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Improving Germination in White Spruce Somatic Embryos with Desiccation and/or Cold Treatments

by

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Requirements for the Degree of

Doctor of Philosophy

in the Department of Biology

We accept this as conforming

to the required standard

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Abstract

Clonal propagation of white spruce (*Picea glauca* (Moench) Voss) through somatic embryogenesis (SE) has important applications in tree improvement programs and will help the forest industry to achieve maximum sustainable yield. The level of induction of embryogenic tissue and the yield of mature embryos through SE has reached acceptable levels using current protocols. However, a large percentage of these embryos produce abnormal seedlings. This problem needs to be assessed and this was done in the work described in this thesis.

Empirically derived, uncontrolled partial desiccation procedures are currently used to improve germinaton. No systematic study has previously been done to correlate the effects of controlled desiccation on germinant quality. My study looked at the effects of controlled partial and complete desiccation of white spruce somatic embryos at four stages of development on subsequent germinant quality. Both slow desiccation at 5°C and flash desiccation at ambient temperature were examined. The effect of temperature treatments as an alternate means of improving germinant quality and its effect on desiccation tolerance were also examined. Dried somatic embryos are likely to suffer imbibitional damage as they (unlike zygotic embryos) have no protective structures surrounding them to regulate water uptake during imbibition. Therefore, the effects of various rehydration methods were also examined.

Large numbers of mature embryos were required for our desiccation experiments. Therefore, a method of squashing the embryogenic tissue into a polypropylene mesh was developed. This method allowed embryogenic tissue to be easily transferred to fresh medium and produced a flat mat of mature embryos that were more accessible for harvesting.

The tolerance of the embryos to desiccation, and the level of desiccation required to improve germinant quality, increased as the embryos matured. The largest improvement in germinant quality was achieved by slowly desiccating 39-d embryos at 5°C for 7 days over a 0.48 M NaCl solution with a water potential of -2 MPa and rehydrating them at 100% RH at a temperature of 5°C. This treatment produced approximately 84% normal germinants. More severe desiccation caused increasing damage.

A temperature treatment of 5 and 10°C also improved germinant quality, producing 70-80% normal germinants. The 5°C treatment can be used as a short-term storage method. Germinant quality from untreated embryos increased with maturity until the embryos became fully mature by 51 d, then quality quickly decreased. Mature 51-d embryos were stored for 8 weeks at 5°C with no loss of germinant quality.

A 5°C temperature treatment for 4-8 weeks significantly improved the tolerance of 39-51 d embryos to flash desiccation (embryos were dried in a laminar flow hood and lost all free cytoplasmic water within 15 minutes). This has important applications in the development of synthetic seed. All of the 8-week cold stored 51-d embryos survived flash desiccation and 58% of them produced normal germinants. The roots developed desiccation tolerance faster than the cotyledons+hypocotyls.

Rehydration experiments showed that slowly and rapidly desiccated embryos responded differently to the method of rehydration. Slowly desiccated embryos suffered less imbibitional damage if they were indirectly rehydrated at 100% RH. Flash desiccated embryos suffered less damage if they were rehydrated directly on germination medium. This suggests that there is no one simple explanation for damage as a result of desiccation and imbibition.

Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) was an effective test for delineating damaged areas in rehydrated embryos, but actual germination tests were the only way of accurately determining germinant quality.

The above treatments have significantly improved germinant quality.

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Chapter 1

Introduction

One objective of modern forestry is to achieve maximum sustainable yield of harvestable trees in the face of increasing demands of an expanding industrial world population. Time-tested silvicultural techniques can meet this objective. However, yield can only be increased further through tree improvement. Tree breeding has traditionally been the main method of improving planting stock. This improved stock may then be mass propagated by cloning, using rooted cuttings. The use of a combination of breeding and cloning strategies speeds up tree improvement programs by capturing both additive and non-additive genetic variances (Park et al. 1998).

One method in particular, clonal propagation by tissue culture, i.e. somatic embryogenesis (SE), first reported in Norway spruce by Hakman et al. (1985) and in white spruce by Hakman and Thorpe (1987) and Lu and Thorpe (1987), has immediate advantages over other clonal propagation methods. A major advantage is that the embryogenic tissue can be frozen in liquid nitrogen (cryopreserved) while the clone is being field tested. In some conifers, it is easy to get rooted cuttings, but they can only be produced by juvenile material. By the time a clone has been field tested, the material is often too mature to produce rooted cuttings. Cryopreservation allows SE tissue to be stored indefinitely in a juvenile state. Part of the tissue from each clone can be cryopreserved while the remainder of the tissue is used to produce trees for field testing to identify the clones that produce trees with desirable traits. Once field testing is completed, these selected clones can then be thawed, quickly multiplied and used to produce seedlings for mass plantings (Park et al. 1998).

The future of SE is even more striking. SE has opened the doors for the use of biotechnology in tree improvement programs. Marker-assisted selection would circumvent lengthy and costly field trials by allowing the early detection of desirable clones based on the presence of molecular markers known to be associated with commercially important genes (Haines 1994). The long-term goal of genetic engineering is the insertion of novel genes or the modification of existing genes (Haines 1994). Some progress has already been made in this area. A gene for glyphosate (herbicide) resistance has been inserted into poplar (Ostry and Michler 1993). Insect resistance has been achieved in agricultural crops by introducing genes that encode the toxin produced by *Bacillus thuringiensis* (Shields 1987). Genetic engineering could produce trees that are incapable of forming reproductive structures, which according to Teasdale (1996) would increase vegetative growth by 15%. Work is ongoing on genetic engineering for male sterility to prevent transfer of genetically altered traits to the wild population.

A reliable SE system is required to produce the tissue to be used for genetic engineering and less importantly to produce the transformed seedlings. As long as a few are produced, these could then be mass propagated by rooting of cuttings. An ideal species to start with is white spruce (*Picea glauca* (Moench) Voss)). This tree is one of the most commonly planted tree species in Canada and is used mainly for wood pulp and general-purpose lumber (Mullin 1997). Genetically improved stock is readily available for this species as active tree breeding and orchard programs for white spruce are present in many Canadian provinces (Mullin 1997). The SE system for white spruce has been used to scale-up for commercial production (Attree et al. 1994) and it is one with which we have great familiarity and experimental expertise.

There are three main stages in the production of seedlings through SE: induction and proliferation of embryogenic tissue masses, maturation of the somatic embryos, and germination of the mature somatic embryos. Park et al. (1993) showed that the induction of white spruce embryogenic tissue is under strong additive genetic control. Therefore, it is possible to introduce the capacity for SE into genetically improved populations by breeding. SE induction rates can further be improved by optimizing tissue culture protocols. In an experiment by Park et al. (1994), all clones produced mature embryos but productivity was variable. They also showed that maturation and, to a lesser extent, germination is still under genetic control, but that the effect is relatively small.

Maturation and germination could benefit more from changes in tissue culture procedures than from changes in breeding strategies. I therefore concentrated my work on maturation and germination in the SE system.

Current induction levels in white spruce SE are acceptable for commercial production. However, maturation and germination protocols need to be optimized and one of the areas to be addressed is the poor germination response. Park et al. (1994) found that, on average, only 22.2% of all white spruce somatic embryos germinated into normal, usable germinants.

Partial or total desiccation of maturing somatic embryos is a likely key for improving germination response. Zygotic embryos undergo desiccation within the seed during the later stages of maturation and thus desiccation is thought to shut down the genes specific for maturation and, after rehydration, to activate those genes required for germination. To obtain proper maturation, somatic embryos are often subjected to empirically derived partial desiccation protocols to synchronize and improve germination (reviewed in Attree and Fowke 1993). These methods meet with limited success.

To be of use for long-term storage protocols or for synthetic seed, embryos must be able to be further desiccated to or below the moisture content of mature zygotic embryos (32%) (Attree et al. 1991). However, Roberts et al. (1990) and Attree et al. (1995) showed that white spruce somatic embryos, matured with the commonly used combination of abscisic acid (ABA) and sucrose as the osmoticant, could not survive desiccation to such low levels. The embryos did survive slow drying to low moisture contents (8%) only if they had been matured with ABA and 5-10% PEG 4000 (polyethylene glycol) as the osmoticant (Attree et al. 1991). Desiccation tolerance has also been improved by applying stress pretreatments to the mature embryos. Nutrient deprivation, short-term cold stress and heat stress improved desiccation tolerance in alfalfa, for example, but the rate of drying was still important (Senaratna et al. 1989). Cold shock and additional ABA improved tolerance to slow desiccation in black spruce somatic embryos (Beardmore and Charest 1995). Obviously, better methods of inducing desiccation tolerance should allow easier and quicker desiccation production methods to be designed.

Longer-term exposure of trees of temperate species to cold temperatures results in cold acclimation and prepares trees to withstand freezing stress in the winter. Cold exposes cells to dehydrative stress similar to that imposed by water stress (Palta 1990) and may also better prepare somatic embryos to withstand desiccation.

The use of cold to improve germination in SE is a neglected area of research. White spruce seed require exposure to low temperature under moist conditions (cold stratification) to break dormancy (Wang 1974) and one effect of cold stratification is thought to be a reduction in endogenous ABA levels as also occurs in response to mild desiccation (Dronne et al. 1997). Consequently, a cold treatment may be useful in improving germination in somatic embryos and is operationally easier to apply than a desiccation treatment.

The purpose of this study was twofold: 1.) to develop protocols that would improve germination in white spruce somatic embryos; and, 2.) to find protocols for desiccation of somatic embryos to low water contents. Developing these protocols required that the effects of various desiccation and rehydration methods on embryo viability and subsequent germination performance be evaluated and explained. Similarly, the role of a cold treatment on subsequent germination and the effect of cold on desiccation were also examined. Finally, the requirement for a large supply of embryos of consistent quality for experiments led to a method for increasing embryo production and dramatically simplifying the handling of the embryos.

Chapter 2

Literature Review

2.1 Introduction

Germination is the transformation of a dry, metabolically quiescent propagule (e.g. a seed, a spore) into a vigorously metabolizing organism ready for growth. Germination starts with the uptake of water by the dried propagule and ends with the first visible signs of growth. However, any discussion of germination would not be complete without a discussion of how the propagule achieves this desiccated state prior to germination.

This section is a review of current knowledge of the effects of desiccation and stratification on germination in seed plants. The role of water, the effect of its loss and re-introduction on cells, and current hypotheses on how organisms cope with water loss will be discussed. The effect of cold-induced changes on embryos as well as a brief description of zygotic and somatic embryo development and germination will round out this section. Much of this literature review deals with angiosperms as little research has been done on conifers.

2.2 Plant-Water Relations

2.2.1 Water

Water is an essential and substantial component of plants comprising 80-90% of the fresh weight of herbaceous plants and over 50% of the fresh weight of woody plants. Water acts as a solvent providing a medium for diffusion of substrates to active enzyme sites or allowing enzymes to undergo conformational changes necessary for catalytic activity. It is not only a substrate for many physiological processes but may also stabilize macromolecules (Kramer 1983, Vertucci 1989). Water maintains cellular turgidity required for plant growth and support (Niklas 1992, Kutschera and Kohler 1994) and a majority of the physiology of the cell relies on it (Webb 1965). Five to 40% of the cell water in non-meristematic cells is in the cell wall, 5-10% in the cytoplasm, and 50-80% in the vacuoles where it acts as a solvent for large quantities of sugars, salts, and organic acids. In meristematic cells, which have a relatively small proportion of vacuole and thin cell walls, the majority of the water is in the cytoplasm (Kramer 1983).

Within the plant cell differentially permeable membranes form compartments with respect to solutes, but only water forms a continuous system, diffusing into and within the cells to maintain an equilibrium in water potential according to the Boyle-Van't Hoff relation (Nobel 1991). Water potential (Ψ), which governs the movement of water within the cell, is determined by the sum of the positive hydrostatic potential, itself a consequence of the elasticity of the cell walls, plus the negative osmotic potentials of the solutes and the matric potential (Nobel 1991). Two methods currently used to determine these cellular water potential components are pressure-volume curves (Anderson et al. 1991) and water-release curves (Livingston and De Jong 1991). They can be used to estimate the osmotic potential of plant tissue at full turgor, the relative water content at its turgor loss point, the volumetric elastic modulus, and apoplastic and symplastic water content.

2.2.2 Thermodynamics

Van't Hoff and D'Arcy/Watt (Vertucci and Leopold 1987b, Rascio et al. 1992) and Guggenheim-Anderson-de Boer (Bruni and Leopold 1991b) analysis of water sorption isotherms can be used to define water in thermodynamic terms. At least five types of water (distinguished by their calorimetric and motional properties) may exist in seeds (Vertucci and Farrant 1995). **Type 1** water is tightly bound to ionic groups on the molecules and does not behave as a solvent. It amounts to less than 0.08 gram H_2O/gram dry mass (g/gdm) water content (or at water potentials greater than -150 MPa) of the water present. **Type 2** water is weakly bound to polar non-charged sites coating the surface of the macromolecule with a thin film of water and solutes. This water has glassy characteristics, and is present at concentrations between 0.08 and 0.25 g/gdm water (or at water potentials between -12 and -150 MPa). **Type 3** water is loosely arrayed over hydrophobic sites, possibly forming bridges. Water that is bound to membrane lipids is in this category at concentrations of 0.25 to 0.45 g/gdm water content (or at water potentials between -4 and -11 MPa). **Type 4** water is concentrated solution or capillary water at concentrations between 0.7 to 0.45 g/gdm (or at water potentials between -2 and -4 MPa). **Type 5** water is dilute solution water, probably required for turgor in seeds. This water is present at water potentials greater than -2MPa (or at water contents greater than 0.6 to 0.9 g/gdm depending on the tissue) (Vertucci and Leopold 1984, Vertucci 1989). More recently, Losch (1993) has generalized that 8% of all water molecules are tightly bound to a macromolecular surface (e.g. proteins, ribosomes, and membranes) as constitutive water (type 1), while the water at 8-11% water content is absorption water (type 2). Water in excess of that he considers as free water.

Extensive studies have been done to determine the amount of bound water in seeds of angiosperms as there was thought to be a positive correlation between the amount of tightly bound water and the level of desiccation tolerance (Vertucci and Leopold 1987a, Rascio et al. 1992). The amount of bound water in plant tissue was found to vary with cell type but water release curve determinations of bound water in mature somatic embryos of three coniferous species were similar, ranging from 0.14 to 0.18 (RWC)(Dumont-BeBoux *et al.* 1996). In *Larix* the bound water increased from 0.023 to 0.106 (RWC) from immature to mature developmental stages (Livingston et al. 1992). However, current research suggests that the amount of bound water present does not determine desiccation tolerance. It is the response of the cells to the removal of the water that determines desiccation tolerance (Pammenter et al. 1991).

2.2.3 Regulating Water Content

Plant cells can lower their osmotic potential and therefore increase the flow of water into the cell by increasing the concentration of solutes (osmotic adjustment) (Niklas 1992). Osmotic adjustment may also be an important mechanism in the temporary survival of severely dehydrated plants by increasing the solute concentration in critical meristematic tissues. This may help maintain cell volume and cell turgor in these cells (Munns 1988). Osmotic adjustment also increases the number of sites for water binding (Rascio et al. 1994). Studies on chili pepper and potato have shown an increase in osmotic potential of cells grown in the presence of an osmoticum (i.e. water stressed)(Socorro Santos-Diaz and Ochou-Alejo 1994a,b; Leone et al. 1994). However, in other species elastic adjustment is more important than osmotic adjustment in maintaining turgor as was found in drought-stressed black spruce (*Picea mariana*) and flooded gum (*Eucalyptus grandis*) seedlings (Fan et al. 1994).

2.3 The Effect of Desiccation on the Plant Cell

Sheie (1970) suggests that molecular changes can cause death in cells when:

1. "....structure or configuration of a crucial molecule is irreversibly altered;
2. an organizational change takes place in some region, i.e. phase change in the membrane;
3. the balance of rates is effected such that degradation overtakes synthesis."

Desiccation of a non-tolerant cell can cause all of these problems.

2.3.1 Changes in the Cell

In plant cells, water is first removed from the larger pores and capillaries in the cell wall. Next the vacuole and cytoplasm respond with corresponding losses of water keeping their water potentials in equilibrium with the cell wall. The result is a decrease in the volume of the protoplast and with further loss of water, the cell wall collapses. This collapse applies a mechanical stress to the cell (Palta 1990), and may also change the interaction between the plasma membrane and the cell wall. With the reduced volume, membranes fold and become susceptible to fusion or vesiculation (Crowe et al. 1988).

Changes also occur in cell contents. As water is lost, the concentration of solutes (salts, metabolites, and macromolecules) increases, changing the ionic and osmotic strength of the cytoplasm, as well as its dielectric constant, pH and viscosity (Caffrey 1986b, Seneratna and McKersie 1986). The interactions and structure of proteins change (Shewfelt 1992). For example, ultrastructural studies of dried lima bean seeds showed polysome breakdown (Klein and Pollock 1968) and condensation of nuclear chromatin (Lopez-Carbonell et al. 1994a). Physiological reactions will slow or stop but those less sensitive to water loss continue (Leopold and Vertucci (1989) and Vertucci (1989)), with a resulting imbalance in metabolic activity. Bruni and Leopold (1991b) propose that the hydration level and threshold of percolative conductivity correlates with the onset of enzymatic activity, while Caffrey (1986b) states that if enough water is lost, the solubility limits of the solutes will be reached. The cytoplasm will then crystallize, form a glass, and/or denature.

2.3.2 Changes in Membranes

2.3.2.1 Membrane Structure

The maintenance of membrane function and integrity following desiccation and rehydration is critical for cell function and survival. Membranes are composed mainly of phospholipids and proteins but also contain steroids, oligosaccharides and water, the latter which can constitute up to 50% of the membrane (Nobel 1991). The precise composition of the membrane varies not only with organism but also with membrane function, cell physiological conditions and maturity (Shewfelt 1992, Kuiper 1985, Navari-Izzo et al. 1993, Filek et al. 1993, Olsson et al. 1994, Lynch 1990, Palta et al. 1993).

The phospholipids are amphiphiles, having a non-polar and polar section. Their most thermodynamically favorable arrangement is in a double molecular layer (lipid bilayer) 60 to 80 Å thick with the long non-polar hydrophobic fatty acid chains pointing toward the inside and the polar hydrophilic phosphate heads pointing toward the outside aqueous layer (Sybesma 1989). Phospholipids are also polymorphic and they can exist in several different phases as defined by the spatial arrangement of the lipid and solvent molecules (Caffrey 1986b). The two most common phases: 1.) liquid crystalline, a fluid phase in which the fatty acid chains possess motional freedom and 2.) gel, a phase where the fatty acid side chains (tails) completely lose their motional freedom and become frozen (Leshem 1992). Under normal conditions, the phospholipids in a membrane exist in the liquid crystalline state. The proteins consist of two types: 1.) peripheral, those loosely bound to the membrane or 2.) integral, those embedded in the membrane. The structure of an integral protein and the extent to which it is embedded in the membrane is determined by the amino acid sequence and polarity. The hydrophobic portions are buried in the membrane and the hydrophilic portions are exposed to the surrounding aqueous solution.

A fluid mosaic model proposed by Singer and Nicholson (1972) visualized the membrane as a two dimensional solution of proteins in the liquid crystalline phase lipid bilayer. The lipids and proteins undergo translational diffusion in the plane of the membrane at a rate determined by the viscosity of the lipids unless the proteins are tied down by a specific interaction with the membrane. The rate of this translational motility (or fluidity) has been assessed using fluorescent probes (Wilson et al. 1991, Leborgne et al. 1992). Modification of the proteins or lipids may affect their distribution and/or rate of translational movement within the membrane. This would change the interaction between the lipids and proteins and thus alter membrane function. Water molecules link via hydrogen bonds to the phosphate head groups of the various phospholipids and water is bound to the phospholipids in amounts ranging from 8 mol water/mol lipid for phosphatidylcholine (PC) to 34 mol/mol for digalactosyldiacylglycerol (DGDG) (Leshem 1992).

2.3.2.2 Effects of Desiccation

Changes occur within the membranes themselves as water is lost, including changes in permeability, elasticity, and compressibility (Caffrey 1986b). For example, Klein and Pollock (1968) observed changes in the membranous structures; in *Solanum tuberosum* and *Fatsia japonica*, lipid droplets appeared in the cytoplasm due to lipid displacement from various cellular membranes as a result of PEG-induced desiccation (Lopez-Carbonell et al. 1994a,b; Leone et al. 1994), and Leone et al. (1994) observed tonoplast reorganization including the disappearance of the large central vacuole and the formation of several small vesicles. If drastic changes occur, loss of membrane potential and cell death result.

There are currently two hypotheses for membrane damage by desiccation and rehydration (see later section on rehydration): 1.) Mechanical Damage Hypothesis- actual physical disruption of the membrane due to radical changes in volume, in particular the stress of shrinking and swelling (Seneratna and McKersie 1986, Powell and Matthews 1978) 2.) Phase Transition Hypothesis- desiccation causes phase changes of the phospholipids in the membrane (Crowe and Crowe 1986a,b). The Phase Transition hypothesis is based on studies of the behaviour of purified phospholipids. The phase in which a phospholipid exists (i.e. liquid crystalline or gel) is dependent upon temperature, hydration level, proton and salt concentration, and pressure (Caffrey 1986b). Studies of purified, fully hydrated phospholipids have shown that each has a characteristic temperature (transition temperature or T_m) at which signs of a phase change from the gel to the liquid crystalline phase can be seen. For example, fully hydrated dipalmitoylphosphatidylcholine (DPPC) has a transition temperature of 41°C; below which it exists in the gel phase and above in the liquid crystalline phase (Crowe and Crowe 1986b). More than one type of lipid, each with its own T_m, can be present between as well as within the membrane bilayer (Sybesma 1989).

As water is removed from the phosphate head groups of the phospholipids, the distance between the fatty acid side chains decreases, increasing van der Waal's forces and consequently increasing the T_m. For example, in purified fully hydrated DPPC, the T_m increases from 41°C to 105°C for totally desiccated DPPC (Crowe and Crowe 1988). If enough water is removed, the lipids will undergo a phase transition, resulting in a gel state under normal conditions. Hoekstra et al. (1991), using Fourier Transform Infrared Spectroscopy (FTIR), showed that the T_m of isolated pollen membranes rose from -6°C to 58°C when desiccated. This correlation of phase transitions with hydration level is also supported by studies on the water content of various purified lipids in the gel and liquid crystalline phase. For example, purified phosphatidylcholine (PC) in the liquid crystalline phase contains 15 mol of water/mol of lipid while PC in the gel phase contains less than 8 mol of water/mol of lipid (Leshem 1992). As one type of lipid changes to the gel phase, it begins to push out the remaining liquid crystalline lipids and proteins resulting in phase separation in the membrane. This changes the potential interaction of the lipids and proteins, limiting their function in the membrane (Crowe and Crowe 1986b). As more water is removed from the polar heads of the phospholipids, the spacing between the polar head groups decreases and the positively charged N on the amino group forms a strong ionic bond with the negatively charged phosphate of the adjacent lipid. The tails now occupy more room than the heads, forcing the lipids out of the bilayer structure and into a lipid tube structure (hexagonal II - H_{II} structure) (Crowe et al. 1989a). The lipid tubes form holes in the membrane disrupting its integrity. As the organism is rehydrated, valuable solutes will be lost through these holes until the membrane sufficiently rehydrates and restores the bilayer arrangement.

The above lipid phase transitions can be detected by means of wide-angle x-ray diffraction (Caffrey 1986a), ^{31}P NMR (Hauser 1986), freeze fracture electron microscopy (Crowe and Crowe 1988), calorimetry (Crowe and Crowe 1988), and FTIR (Crowe et al. 1989d, Casal and Mantsch 1984, Hoekstra et al. 1992a).

2.3.3 Effects of Active Oxygen

Cell metabolic systems can transform relatively unreactive atmospheric oxygen into more reactive forms such as superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen (Smirnoff 1993). Environmental stress (e.g. water stress, chilling) accelerates this transformation (Leprince et al. 1994) and/or decreases the amount or effectiveness of the antioxidants (Chaitanya and Naithani 1994). Smirnoff (1993) proposes the following hypothesis to explain increased active oxygen production as a result of desiccation. Desiccation below ~40% RWC results in the bulk water being lost. The resulting disruption of cytoplasmic structure changes enzyme and substrate concentrations and associations allowing electrons to be misdirected thus producing superoxide. Superoxide can de-esterify the membrane phospholipids disrupting the lipid-protein distribution causing further phase separation as well as formation of gel-phase regions (Seneratna and McKersie 1986). Although not highly reactive, further successive univalent reduction of superoxide results in the formation of the more damaging hydrogen peroxide and hydroxy radical. Hydrogen peroxide can inactivate a number of the Calvin cycle enzymes and hydroxy radicals can initiate lipid peroxidation. This peroxidation is a self-perpetuating reaction that mainly attacks the polyunsaturated fatty acids, breaking down the lipids and impairing membrane function. A hydroxy radical does this by removing a hydrogen atom from the phospholipid to yield an organic free radical that interacts with O_2 forming a peroxyradical. The peroxyradical interacts with a neighbouring unsaturated fatty acid forming an unstable lipid hydroperoxide. This hydroperoxide then degrades to form new free radicals and aldehydes that combine with proteins, causing them to denature. Also, energy transferred from photosynthesizers (e.g. chlorophyll in the chloroplasts) excites molecular oxygen resulting in spin inversion thus producing singlet oxygen that is highly reactive and electrophilic targeting unsaturated fatty acids, histidine, methionine, tryptophan and guanine. Oxidation of these amino acid residues results in loss of catalytic activity and denaturation.

This raises an interesting point. Can desiccation damage be limited, for example by adding antioxidants or by desiccating the embryos in a reduced oxygen atmosphere? In support of this idea, Leprince et al. (1995) demonstrated that desiccation damage could be reduced by manipulating environmental factors, e.g. temperature or O_2 concentration, to limit metabolism or by stopping metabolism with KCN (a respiration inhibitor), thereby decreasing or stopping free radical production. Their results suggest that free radical damage plays as important a role in desiccation damage as do membrane phase transitions.

2.4 Mechanisms to Cope with Desiccation

Most organisms do not appear to be able to survive desiccation without a period of adjustment. For example, Castor beans are not able to withstand desiccation at 20 days after pollination (DAP), but are desiccation tolerant at 25 days (Kermode and Bewley 1985a). At this early stage of desiccation tolerance, the seeds must be dried slowly, but later in development, they will withstand faster drying. The basis for tolerance lies in a number of mechanisms that are physical as well as physiological such as the production of sugars, proteins, amino acids, antioxidants and the presence of repair-based mechanisms.

2.4.1 Sugars

2.4.1.1 Glasses

Prevention of some of the damage in desiccation intolerant species may be helped by the transformation of the cytoplasm to an aqueous glass which is a supersaturated solution with a high solute concentration and high viscosity (i.e. a liquid that has lost its ability to flow) (see Angell (1995) for a recent review of glass). For instance, glass formation has been observed in desiccation tolerant seeds of soybean at water contents below 0.10 g/gdm water at standard storage temperatures (Bruni and Leopold 1991a). Burke (1986) postulates that the formation of a glass may fill up the space in the cell, preventing further tissue collapse and moderating the effects of increases in solute concentration and pH changes. Glasses prohibit molecular diffusion, causing quiescence and preventing damaging interactions between cell components, and also protect proteins and enzymes from denaturation by keeping them in their folded state (Fox 1995). The formation of a glass may also enable tissue to retain water during desiccation. They are so viscous that water molecules are unable to diffuse allowing considerable water to be held (Burke 1986). However, glass formation alone may not be the sole cause of desiccation tolerance as evidenced by studies of Sun et al. (1994). They found that cotyledonary tissue of desiccation tolerant soybeans had similar glass transition dynamics as did red oak cotyledonary tissue that was desiccation sensitive. Glasses are formed from cell solutes, particularly sugars, combinations of which may be important for desiccation tolerance. Leopold and Vertucci (1986) hypothesize that sucrose plays a role in desiccation tolerance as long as raffinose and/or stachyose are present to prevent the crystallization of the sucrose.

2.4.1.2 Water Replacement Hypothesis

Sugars also play a role in the stabilization of proteins and membranes. Using isolated sarcoplasmic reticulum membranes from lobster, Crowe and Crowe (1988) found that trehalose stabilized the membranes during desiccation and subsequent rehydration. Fusion and lateral phase separations were inhibited. Sugars (particularly trehalose) prevented fusion and leakage of dried unilamellar vesicles of palmitoyloleoyl-phosphatidylcholine:phosphatidylserine (POPC:PS) 9:1 (Crowe et al. 1988). Seeds do not have trehalose, but sucrose performs equivalent functions (Crowe et al. 1987). Sugars may be responsible for the maintenance of membrane integrity by preventing the formation of the H_{II} phase. According to the Water Replacement hypothesis, polar carbohydrates (mainly disaccharides, not mono- or tri-saccharides) appear to replace the water molecules by the -OH groups of the sugar binding to the phosphate head groups of the membrane lipid molecules (Crowe et al. 1989a, Crowe and Crowe 1988). By replacing these water molecules, the sugars may preserve the spacing between the lipid heads preventing formation of the H_{II} phase in the membranes. The H_{II} phase has not been found in membranes of anhydrous organisms (reviewed by Crowe et al. 1989b), although Hoekstra et al. (1992a) postulated that a modified H_{II} phase might exist in extremely desiccated pollen membranes. He did not consider it a true H_{II} phase as the membranes quickly recovered when rehydrated.

According to the Water Replacement Hypothesis, replacement of the water molecules with sugars also keeps the T_m of phospholipids low. The T_m of the fully dried phospholipid would be depressed enough for the phospholipids of the dehydrated membrane to remain in the liquid crystalline phase under normal temperatures. Rehydration would not cause a phase change in the membrane (reviewed in Crowe et al. 1987). In support of this hypothesis, sugars and oligosaccharides (e.g. sucrose, raffinose, and stachyose) are known to be present in much higher concentrations in desiccation tolerant stages than in desiccation intolerant stages of soybean (Blackman et al. 1992). For example, desiccation intolerant mutants of *Arabidopsis* have a 400 times lower oligosaccharide/monosaccharide ratio than their desiccation tolerant wild type counterparts suggesting that the conversion of monosaccharides to sucrose and/or oligosaccharides (or their synthesis) may be an important preparation step for desiccation (Ooms et al. 1993).

2.4.2 Proteins

Late embryogenesis abundant (LEA) proteins, especially a subset called dehydrins (Close et al. 1989) may also play a role in desiccation protection. LEA proteins are hydrophilic and highly stable. They cannot provide protection from severe desiccation by themselves, but may work in association with oligosaccharides (Blackman et al. 1995). Blackman et al. (1992) postulate that LEA proteins are important in protecting the cells during the initial stages of desiccation while the levels of saccharides are increasing. An increase in storage proteins would provide additional binding sites for water, increasing the matric potential by helping the cells to bind water during the initial stages of desiccation. There are two types of LEA proteins, those produced early in maturation when abscisic acid (ABA) is on the increase and those produced later in maturation when ABA is declining and maturation-drying has started. Much research activity is centered on the characterization of the storage proteins involved in desiccation tolerance (Blackman et al. 1991, Bradford and Chandler 1992, Lopez et al. 1994). For example, comparisons of protein accumulation in zygotic and somatic embryos have been made to try to understand the deposition of storage reserves during embryo development (Krocho et al. 1994, Misra et al. 1993, Flinn et al. 1993), and Bewley and Black (1994) have determined that LEA protein production is probably regulated by several mechanisms, some of which may involve ABA.

2.4.3 Amino Acids and Abscisic Acid

Not only do sugars increase in response to desiccation, but the amount of amino acids also increases (Good and Zaplachinski 1994). Proline has been shown to increase tolerance to desiccation (Saranga et al. 1992a,b; Kim and Janick 1991). Proline production may be tied to ABA. Ober and Sharp (1994) showed that increased ABA concentrations are required for an increase in proline production in maize roots. However, ABA may not play a direct role in the acquisition of desiccation tolerance. Recent experiments by Bochicchio et al. (1994) on maize embryos showed that the concentration of ABA increased with the acquisition of desiccation tolerance, but that incubation of the embryos with an ABA inhibitor did not affect the acquisition of desiccation tolerance.

2.4.4 Antioxidant Systems

Oxidation processes that occur in the dry seed trap active oxygen/free radicals and imbibition releases them to react with, and damage cellular components (Reuzeau and Cavalie 1995) as discussed in section 2.4.3. Plant cells possess intricate antioxidant systems to protect them from the effects of active oxygen (Senaratna and McKersie 1986). Under normal circumstances, cells can remove the reactive oxygen molecules and/or repair the resulting damage. For example, compounds such as α-tocopherol, ascorbate, superoxide dimutase, catalase and peroxidases react with the active oxygen to keep them at low levels (Smirnoff 1993). Glutathione, glutathione reductase, ascorbate and mono- and dehydrascorbate reductase regenerates the oxidized antioxidants. In an unstressed state, a balance exists between the oxidation rate and the reduction rate leaving a large pool of regenerated antioxidants. Desiccation tolerant soybean axes contain significantly higher quantities of lipid-soluble antioxidants when compared to desiccation intolerant soybean axes (as summarized in Senaratna and McKersie 1986). Also the sugars, polyols, and proline that increase in response to water stress may play multiple roles by also acting as antioxidants and hydroxy radical scavengers (Smirnoff 1993). Similarly flavenoids and polyamines have also been proposed as radical scavengers.

2.4.5 Repair-Based Mechanisms

Some plants have a repair-based desiccation tolerance mechanism in combination with desiccation protective mechanisms (Oliver 1991, Oliver et al. 1998). Scott and Oliver (1994), working with the moss *Tortula ruralis*, confirmed that desiccation and rehydration alters the control of translation of mRNA's. They found that new proteins were not produced, but that the transcription rates were altered to favour the synthesis of specific proteins on rehydration.

2.5 The Role of Water in Seed Development and Germination

2.5.1 Seed Development

Following fertilization, the development of a seed occurs in three steps:

1.) Histodifferentiation- the developmental process by which the unspecialized unicellular zygote undergoes progressive changes to produce a multicellular embryo with well-differentiated organs. The cessation of cell division signals the end of this phase (Smith 1973).

2.) Maturation phase- during this phase, also known as the seed expansion phase, a rapid gain in dry weight occurs as the cells of the embryo and megagametophyte (gymnosperms) or endosperm (angiosperms) expand to accommodate the storage reserves that are synthesized during this stage. This is concomitant with a decrease in water content (Bradford 1994). In pea, for example, the water content (fresh weight basis- fwb) decreases from 84 to 55% (Le Deunff and Rachidian 1988). The storage products consist principally of carbohydrates, fats and oils, and proteins. The carbohydrates are stored as starch in starch grains, hemicellulose in thickened cell walls, or as free sugars (eg. disaccharides and oligosaccharides). The lipids (triaglycerols) are deposited in oil bodies. The carbohydrates and lipids are used during germination as a source of carbon skeleton precursors and as a source of energy for their assembly. The proteins are deposited in protein bodies ready to supply the nitrogenous compounds needed by the seedling until their roots can absorb nitrogen (Boesevinkel and Bouman 1984). The accumulation of storage reserves has been discussed in detail by Krasowski and Owens (1993) and Owens et al. (1993) for *Picea glauca* and *Pseudotsuga menziesii* during post-fertilization megagametophyte and zygotic embryo development. All metabolic events early in this stage are geared towards the synthesis of reserve materials (Kermode et al. 1986). The embryos become desiccation tolerant during this stage.

3.) Maturation drying- the fresh weight decreases as the water content rapidly decreases. There are two possible mechanisms for water loss at this stage: 1.) passive evaporation from the surface of the seed; 2.) metabolically active pumping of the water back to the mother plant (Kermode and Bewley 1986). For example, in pea, this may drop from 55% to 14% fwb over a 9-day period (Le Deunff and Rachidian 1988). Metabolism stops and the seed becomes quiescent and it is the vascular connection between the pod and the mother plant that is severed (Le Deunff and Rachidian 1988).

2.5.2 The Role of Desiccation in enhancing Germination

ABA is thought to play a role in inhibiting germination (although Galau et al. (1991) do not agree with this conclusion). Desiccation enhances ABA breakdown as well as decreases sensitivity of the embryo to ABA (Kermode et al. 1989, Dronne et al. 1997). Perhaps desiccation helps to release the embryo from ABA inhibition of germination. Certainly desiccation causes changes in the membranes, possibly altering hormone receptors. Increasing or decreasing sensitivity to hormones may cause changes in the expression of genes (Bewley and Black 1994). No definitive mechanism for switching metabolism from the developmental to germination mode has been found. mRNAs for both development and germination are present in the dry seed and Bewley and Black (1994) postulate that the germination mRNAs may be sequestered in the nucleus during drying, awaiting immediate use following imbibition. The maturation-related mRNAs may be hydrolyzed during desiccation while those sequestered in the nucleus are protected. However, Bewley (1994) has another hypothesis to account for untranslated mRNAs in the cytoplasm of developing alfalfa embryos. He suggests that repressor proteins inactivate the mRNAs. Such proteins may be important in switching from maturation to germination-related metabolism, or in protecting the germination activated proteins during desiccation.

2.5.3 Rehydration

Once desiccated, the seed must be rehydrated to resume metabolic activity and growth. It is during this rehydration (or imbibition) that much of the damage to the embryo occurs.

There are three phases of rehydration (Haigh and Barlow 1987, Bewley and Black 1994). Rapid water uptake occurs in Phase 1, and it is in this phase that much of the damage to the embryo takes place. Phase 2 is a lag phase with no water uptake during which time cell repairs and germination preparations are being made (Bray 1995). Phase 3 involves another increase in water uptake concurrent with radicle elongation.

The initial Phase I water uptake is believed to occur in 2 stages (Vertucci and Leopold 1983). The first is a wetting phase in which a front separating the wet and dry area of the seed noticeably advances through the tissue. This is followed by a second stage in which the water content increases in the wetted areas. Imbibitional damage apparently occurs during the initial phase (Vertucci and Leopold 1983). Waggoner and Parlange (1976) suggested that the sharp front between the wet and dry portions causes stress resulting in cracks often seen in imbibed cotyledons. Wolk et al. (1989) postulated that the wetting phase consists of the water molecules replacing the sugars at membrane sites in the dry tissue and that imbibitional damage may occur during this water rebinding. The rate of imbibition during Phase 1 is governed by the degree of attraction of water to the molecular components of the seed (i.e. matric potential) until most binding sites are filled. By this time the seed water potential will have reached -8MPa (Bradford 1986). For example, mature dried seeds of spruce have a relative water content (RWC) of 4% to 8% (Young and Young 1992) and could have a water potential of up to -150 MPa (Vertucci and Farrant 1995). (Note: the water content of the intact dry spruce seeds is lower than the 32% quoted on page 3 for the excised dry zygotic embryos because the mass of the dry seed coat is included in measurements of tissue mass). Osmotic potential and turgor potential then regulate the remainder of the water uptake (Bradford 1986). Metabolism begins within minutes of the start of imbibition.

Conductivity measurements of imbibing seed show that they rapidly lose solutes (e.g. sugars, organic acids, ions, amino acids, proteins) into the surrounding medium (Powell and Matthews 1981). The amounts lost will have an effect on the viability of the embryo. Leakage can be explained by a disruption of the membrane either through physical disruption damage, usually caused by rapid swelling due to the rapid water uptake (Mechanical Damage hypothesis), or a disruption through the formation of the H_{II} phase

(Phase Transition hypothesis), or a temporary change in permeability of the membrane caused by phase transitions or phase separations of membrane components (Phase Transition hypothesis) (Senaratna et al. 1984). According to Crowe et al. (1989b,c), the T_m of the lipids decreases as water is removed resulting in membranes entering the solid gel phase when tissues are fully desiccated under normal temperatures. As water is added during imbibition, the T_m increases and the membranes undergo a phase transition to the liquid crystalline phase. During this phase transition the membranes are leaky (Crowe et al. 1989b) allowing solutes to escape from cells. This idea is supported by two types of experimental evidence. 1.) "Chilling injury" i.e. low temperatures cause more leakage (Herner 1986, Sharom et al. 1994) because membrane phospholipids are more likely to exist in a gel phase at lower temperatures. They therefore undergo a phase transition to the liquid crystalline state as they are rehydrated resulting in leakage. 2.) Rehydration in a saturated atmosphere to a critical water content rather than submergence in liquid appears to stop leakage (Crowe et al. 1989a). If membrane phospholipids are rehydrated, without direct contact with a liquid, to a water content that causes them to exist in the liquid crystalline phase at the temperature of imbibition, then there will be no phase change as the tissue rehydrates, hence no leakage.

Upon imbibition metabolism resumes, but it changes from synthesis of storage products to synthesis of enzymes responsible for the catabolism and mobilization of reserves. Desiccation may also result in a change in gene activity, with those genes required for transcription of mRNA's being switched on. Typically, enzymes involved in protein reserve breakdown are produced (Kermode and Bewley 1985b, 1986, 1989; Kermode et al. 1985, 1986). However, it is possible to alter normal sequential events as shown by Kermode and Bewley (1985a). They found that castor bean embryos, which were dried after they had acquired desiccation tolerance, but before normal maturation drying had begun, did not continue maturing when rehydrated, but germinated directly.

2.5.4 Germination

Immediately upon imbibition, the mitochondrial enzymes of the citric acid cycle and electron transport chain are activated, providing a source of ATP for the embryo. Ribosomes aggregate into polysomes to replace those lost during desiccation and within 10-15 minutes of initial imbibition the polysomes are synthesizing protein. This is too short a period for gene activation and mRNA production, which suggests that the mRNAs are already present in the cell. New mRNAs are produced within a few hours and new rRNAs, for incorporation into new ribosomes, are produced within 1 to 2 hours of imbibition. Transfer RNAs are present in the dry seed and within 20 minutes to 1 hour, new tRNAs are produced. Over the next few days the original mitochondria in Norway spruce embryos, for example, continue differentiating and then dividing, producing new mitochondria (Simola 1974). By day 2, breakdown of storage products begins, starting with proteolysis. By day 5 germination in red pine is over as the radicle elongates and emerges (Bewley and Black 1994).

In most seeds cell division does not occur until the radicle emerges and germination is technically over (Bewley and Black 1994). Radicle growth may be triggered by a number of factors singly or in combination. For example, an increase in turgor potential due to solute accumulation occurs during Phase III that results in a lowering of the osmotic potential and an uptake of water. The radicle cell walls relax allowing the radicle to elongate and the endosperm tissue surrounding the radicle tip degrades or weakens allowing the radicle to push through (Bradford 1986). There is no general consensus on the actual mechanism, but it appears to vary with species.

2.6 Somatic Embryogenesis

2.6.1 Comparison of somatic and zygotic embryogenesis

Trees are propagated by sexual reproduction through seeds. Seeds are zygotic embryos surrounded by an embryo sac and integuments. The structures surrounding the embryo provide nourishment, control gas exchange, and water and osmotic changes within the embryo and also impose mechanical pressure on the embryo (Bonga and von Aderkas 1992).

Trees can also be propagated by vegetative propagation (cloning). There are many methods, e.g. rooting of cuttings, grafting, or micropropagation (i.e. axillary shoot elongation, organogenesis, nodule culture, embryogenesis). (See Bonga and von Aderkas (1992) and George (1993) for a discussion of the advantages, limitations and a description of each method). However, the preferred method is somatic embyrogenesis (i.e. the production of bipolar embryos from somatic tissue) if available for that species. Somatic embryos are developmentally and morphologically quite similar to zygotic embryos, but there are differences. Somatic embryos are not enclosed in nutritive or protective tissue, as is the zygotic embryo. Nutrition, water and osmotic regulation, gas regulation, etc. must be provided by the culture medium and culture environment which is not nearly as well adapted to the needs of the embryos as the environment within the seed. But the potential does exist for these naked somatic embryos to be encapsulated thus producing abundant artificial seed that could be handled like conventional seed.

Zygotic embryo formation begins with the fertilization of the egg nucleus. Somatic embryogenesis begins with dedifferentiation of somatic cells. There are three phases in the production of plants through somatic embryogenesis; induction and proliferation, maturation, and germination.

2.6.2 Induction and Proliferation

Explants (immature or mature zygotic embryos or pieces of other juvenile plant material) are placed on semi-solid initiation medium to induce embryogenesis. In most species, the addition of an auxin to the culture medium (and in some instances in combination with a cytokinin) causes cells of the explant to dedifferentiate, divide and become embryogenic (George 1993). The resulting embryogenic tissue mass consists of immature somatic embryos, which have an embryogenic region of small meristematic cells on top of elongate, highly vacuolate suspensor cells. These embryos are sometimes incorrectly called pro-embryos, although they do not resemble zygotic pro-embryos.

The tissue mass grows through continuous cleavage polyembryogenesis (i.e. "the splitting of the embryos into several identical parts each of which is capable of developing into a mature embryo" (the Penguin Dictionary of Botany)). The tissue is usually maintained or proliferated with bi-weekly transfers to fresh semi-solid maintenance medium (which may be the same as the initiation medium or may contain reduced auxin and cytokinin). Scale-up research is focusing on the use of liquid culture and bioreactors for the production of large numbers of embryos (Attree et al. 1994, Tautorus et al. 1992).

2.6.3 Maturation

Histodifferentiation of the immature embryos is accomplished by transferring the embryogenic tissue to semi-solid maturation medium consisting of the same basal medium as the induction medium but usually with no or different plant growth regulators (PGR's) and with or without activated charcoal (George 1993). (Histodifferentiation in the somatic embryos is similar to that in zygotic embryos (Hakman 1993)). The addition of abscisic acid (ABA) in combination with water stress is required to promote the accumulation of storage reserves in the differentiated embryos (Attree et al. 1992, 1995). Water stress, through an increase in osmotic concentration, can be attained by the addition of extra sucrose (6%), mannitol (2-6%)(Roberts 1991) or PEG (polyethylene glycol)(7.5%) to the medium. Klimaszewska and Smith (1997) applied water stress by increasing the concentration of gellan gum to 1% in the medium. Culture conditions have a significant effect on the deposition of storage reserves (Roberts 1991). Somatic embryos generally do not contain as much storage reserves as the corresponding zygotic embryos (McKersie and Acker 1994, Joy et al. 1993), but this can be improved by changes in cultural conditions (Attree et al. 1992, Roberts et al. 1990, Roberts 1991, Leal et al. 1995).

2.6.4. Germination

The mature somatic embryos are germinated by placing them on germination medium containing ½ the basal ingredient concentration and no PGRs. As with zygotic embryos, a period of mild desiccation appears to be required to improve germination (Roberts et al. 1990).

2.7 Low Temperature Injury and Mechanisms of Cold Acclimation

Exposure of unacclimatized plants to low temperatures results in chilling or freezing injury. Chilling injury, a consequence of exposure to sub-optimal but above freezing temperatures, is proposed to be a result of an alteration in membrane properties due to phase transitions in some of the minor lipid fractions (Hallgren and Oquist 1990). This disrupts membrane permeability and membrane-associated enzyme activities resulting in decreases in ATP levels, ion-solute leakage, loss of compartmentalization, and metabolic imbalances (Lynch 1990). However, this may be a simplistic explanation. Shewfelt (1992), in summarizing other studies, suggests that peroxidation of the fatty acids, changes in sterol to phospholipid ratios, changes in the surface properties of the membranes, and increased molecular ordering of the water (accompanied by all the normal dehyration responses) may also play a role in chilling injury. The lipid composition of the membranes will determine the biophysical response of the membrane, but there may be no one simple explanation of injury for all cases. Shewfelt also cautions that the study of membrane turnover (i.e. the rapid degradation and renewal of cell membranes occuring within a 4-hour period) must be included in cold injury and acclimation studies.

Freezing injury results from exposure to below freezing temperatures, but many of the changes associated with chilling injury can also be applied to freezing injury (Shewfelt 1992). Cells subjected to freezing temperatures undergo changes similar to that imposed by desiccation. Extracellular water freezes first. The formation of ice outside the cells increases the solute concentration and decreases the water potential of the remaining water in the extracellular spaces. This causes the water to diffuse from inside to outside of the cell reestablishing the thermodynamic equilibrium, but essentially dehydrating the cells (Palta 1990). The resulting dehydration results in mechanical stress, chemical stress and membrane damage (Hallgren and Oquist 1990)(See section 2.3). Therefore, the development of freezing tolerance is likely to involve mechanisms similar to those that provide desiccation tolerance.

Exposure of plants of temperate origin to low, non-freezing temperatures for several days to weeks can result in any or all of three types of response: 1.) true acclimation- a change in metabolism or structure to be able to withstand the cold 2.) metabolic adjustment- optimization of metabolism for low temperatures and 3.) shock responses- short-term changes in gene expression (Howarth and Ougham 1993). Low temperatures differentially affect the various developmental stages of plants. However, embryos are able to enter a dormant state and are more tolerant to low temperatures (Hallgren and Oquist 1990).

Acclimation to low temperature involves changes in carbohydrates, amino acids, nucleic acids, proteins and phospholipids (Halgren and Oquist 1990, Gusta et al. 1996). For instance, the soluble sugar content, mainly in the form of sucrose and fructans (fructose polymers), can double with cold acclimation. However, the actual role of this carbohydrate accumulation in cold acclimation is under debate. It may serve a cryoprotective role by depressing the freezing point of the cytoplasm or prevent desiccation by increasing intracellular osmotic potential, or it may simply be a side effect of other metabolic shifts (Howarth and Ougham 1993). In addition, as discussed in section 2.4.1, the sugars may protect membranes and proteins and/or play a role in vitrification (i.e. glass formation).

Soluble proteins accumulate in response to low temperatures and several isozymic substitutions occur to reduce their cold sensitivity and alter their catalytic properties. This makes the proteins more efficient in the cold. They also possibly play a desiccation protection role in freezing acclimation by binding water, increasing osmotic concentration, and acting as antifreeze agents and desiccation damage repair proteins (Howarth and Ougham 1993, Gusta et al. 1996). The activity of many enzymes increases with several enzymes involved in free radical scavenging systems (e.g. peroxidase and cellulase in chilled mango fruits (Zauberman et al. 1988)) increasing in response to cold. Also, the amount of polysomes, ribosomal RNA and RNA polymerase activity increase in response to cold probably to support the observed increased protein synthesis (Howarth and Ougham 1993).

Weiser et al. (1990) found that there was an increase in cell wall extensin synthesis in cold acclimatized pea seedlings. They proposed that this might provide increased structural rigidity, thus preventing physical collapse as a result of freeze-induced dehydration. Calcium ions increase in response to cold temperatures (reviewed in Monroy et al. 1993) and calcium-dependent protein phosphorylation may be a signal transduction pathway to trigger cold acclimation (Monroy et al. 1993). Calcium may also play a role in stabilizing membrane structure by binding to phospholipid head groups (Palta 1996). Similarily, ABA also stabilizes membranes by binding to a saturated molecular species of PC. Proline, glycine betaine and polyamines also accumulate and are suspected of having cryoprotective properties, but also may play roles in desiccation tolerance and as antioxidant scavengers (see section 2.4.4).

There are qualitative and quantitative changes in plant membrane lipid composition as a result of exposure to cold temperatures (reviewed in Shewfelt 1992). For example, the overall level of polyunsaturated fatty acids increases in response to cold temperature and this has been shown to confer chilling tolerance in young tobacco leaves (Kodama et al. 1995). The desaturates act by adding double bonds to the fatty acids. They are selective in the lipid species that they modify and the desaturation may also be membrane specific. In a study of orchard grass cold acclimation, fatty acid unsaturation was found in membranes of the endoplasmic reticulum and Golgi, but not in the plasma membrane (Yoshida and Uemura 1984). Lipid unsaturation may help to increase membrane fluidity in cold temperatures and may also have a role in changing the threshold temperature for lipid phase shifts. Finally, to further preserve membrane fluidity, the relative proportions of the membrane components also change in response to cold. Free sterols increase more than the steryl glucosides and the phospholipids increase more than the sterols (Shewfelt 1992).

As with desiccation tolerance, ABA plays an ambiguous role in inducing tolerance to freezing. Reports stating the effect of low temperatures on endogenous ABA production are conflicting, but the general consensus is that ABA can confer freezing tolerance to plants. However, alone it is not as effective as low temperature (Gusta et al. 1996, Howarth and Ougham 1993) and may be induced through a different signal transduction pathway (Monroy et al. 1993).

Apparently water stress and low temperature stress result in many similar defense mechanisms.

2.8 Conclusion

The approach in somatic embryogenesis is the production of a somatic embryo that is similar in quality to its corresponding zygotic embryo. The goal is to be able to encapsulate the somatic embryos (i.e. produce synthetic seed) and handle them the same way as seed. In zygotic embryos, the mother plant provides all the nutrients, many of the hormonal cues, and regulation of their physical environment. Somatic embryos do not have this luxury. They are not protected by a seed coat and have no connection with a mother plant. All nutrients, hormonal cues and environmental conditions must be provided in the nutrient medium and cultural environment. Attempts have been made to duplicate the conditions the zygotic embryos are grown under, but as this process is not well researched or understood, it is hard to replicate in culture.

Desiccation is an important part of normal zygotic embryo development in conifers. It makes the zygotic embryo metabolically quiescent and therefore more tolerant of environmental changes and amenable to long term storage. It also appears to be a necessary prerequisite for germination. This review showed that successful germination in seeds is dependent on the ability of the cells to withstand the stress of desiccation and subsequent rehydration without undergoing irreversible deleterious changes. Clearly membranes are the primary site of cellular injury and can be extensively and often irreversibly damaged by severe desiccation treatments. Damage is prevented or minimized by protective mechanisms involving metabolic and/or structural changes in the cells (e.g. glass formation, prevention of lipid phase transitions, preservation of membrane integrity, and stabilization of proteins). Repair mechanisms must also be available at the onset of rehydration to repair any reversible damage that has occurred. The presence of a functioning antioxidant system upon rehydration is required to prevent further injury as a result of toxic free radicals.

Severe damage can certainly occur when somatic embryos are desiccated in order to obtain proper germination. Because somatic embryos are desiccated in an artificial environment, considerable damage can be expected with uncontrolled desiccation. The objective of my experiments is to find protocols that provide the least harsh environment for the somatic embryos while they are being desiccated and to determine if there are less harsh ways to improve germination.

Chapter 3

Maximizing Embryo Production

3.1 Introduction

In commercial production, economic success depends on the synchronous development of large numbers of usable embryos. However, in vitro procedures often do not yet meet these industrial requirements. Embryos are commonly matured by transferring embryogenic tissue to a Petri dish filled with a semi-solid maturation medium. The tissue grows in a mound with only the lower surface touching the medium. This can cause problems with gaseous exchange in the cells at the centre and the bottom of the mound. Gradients of nutrients, growth factors and waste products will occur between the tissue and the nutrient medium (George 1993), which may partially explain the non-synchronous development of embryos within the mound. Preliminary observations showed that tissue under the mound or in the central core (areas with limited gas exchange) produced fewer embryos and a higher percentage of abnormal ones.

The shape of the embryo at the start of germination influences the way it subsequently performs as a seedling. Barrett (1997) followed normal embryos and embryos with various abnormalities from germination through to the greenhouse. He found that normal shaped embryos produced seedlings that had a better chance of long term survival following transfer to the greenhouse. We found in preliminary studies that abnormal embryos produced seedlings with abnormalities. This suggests that only normal appearing embryos should be used for germination.

This chapter describes work undertaken to maximize and synchronize the production of normal appearing mature embryos. We hoped to achieve this by developing a method of maturing embryos in which all embryogenic tissue had equal contact with the medium. Embryogenic tissue spread in a thin layer on the surface of the medium would have good gaseous exchange and equal contact with the medium. However, simply spreading the tissue on the medium makes transfer to fresh medium cumbersome. Therefore, we decided to squash the tissue into a screen. This allows the tissue to be spread across a large thin surface while maintaining uniform contact with the medium and the air. It also allows for easy transfer to fresh medium, when required, by simply transferring the whole screen with the attached tissue. We tested this idea by using white spruce (*Picea glauca*) embryogenic tissue, material with which we had a great deal of familiarity.

3.2 Materials and Methods

3.2.1 Plant Material

White spruce (*Picea glauca* (Moench)Voss) somatic embryogenic tissue of known genotypes were used. The embryogenic tissue was initially derived in 1991 from six-parent full diallel crosses using trees DNR-27, GPC-02, MRL-03, GPC-03, GPC-15 and MRL-11 (Park et al. 1993). The tissue was cryopreserved in 1992 (Park et al. 1994). Embryogenic tissue from the following controlled pollinations were used: MRL-11 times GPC-15 (clone 6 x 5), MRL-11 times GPC-02 (clone 6 x 2), MRL-11 times MRL-03 (clone 6 x 3), GPC-02 times MRL-11 (2 x 6), DNR-27 times GPC-15 (clone 1 x 5), DNR-27 times GPC-02 (clone 1 x 2), and DNR-27 times MRL-03 (clone 1 x 3). None of the tissue from the GPC-03 controlled pollinations was used in this experiment. The cones of the first parent were pollinated with pollen from the second. Tissue was removed from the liquid nitrogen storage when required, thawed and proliferated according to the methods described in Park et al. (1994). The vials of frozen tissue were immersed in a 37-40°C water bath until completely thawed (approximately 2 min). The vials' contents, suspensions of embryogenic cells in DMSO and sorbitol, were then poured onto sterile filter paper discs placed on top of a plate of initiation medium. Initiation medium consisted of modified half strength Litvay's medium (1/2 LM) (Tremblay 1990) containing 1% (w/v) sucrose, 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 5µM Benzylamineopurine (BA) and 0.9 % agar (w/v). Prolonged exposure to residual DMSO (the cryoprotectant used in the freezing procedure) can be damaging to the tissue. Therefore, after 24 h, the filter paper was transferred to a fresh plate of initiation medium, to remove the last traces of DMSO, and incubated in the dark at 24°C. The embryogenic tissue was transferred to fresh plates of initiation medium as new growth appeared. The tissue was proliferated with bi-weekly subcultures to fresh initiation medium.

3.2.2 Mesh Type and Squash Method

A preliminary experiment, using different types of screen material and different sizes of mesh, was undertaken to determine if the type of screening or the size of the mesh used influence embryo productivity.

3.2.2.1 Mesh Type

Polypropylene screening- 710 µm mesh, polypropylene screening- 980 µm mesh and window screening- 1025 µm mesh, were used. The screening was cut into ~3 cm^2 squares. To sterilize the screens, 10 screens were wrapped in tinfoil and placed between glass Petri dishes for sterilization. This kept the screens flat during autoclaving at 121°C for 15 min.

3.2.2.2 Squash Method

One half gram tissue masses from clones 6 x 5, 6 x 2 and 6 x 3 were squashed into the screens. It was not known how much handling embryogenic tissue could withstand. Therefore, two methods of spreading the tissue into a flat mat were tried, one gentle and one harsher. 1.) Slide method- to spread the tissue gently into a thin mat on the mesh, the screen was placed in the middle of a sterile microscope slide. The tissue was placed on one end of the screen. A sterile microscope slide was gently wiped across the top of the screen to spread the tissue. 2.) Spoon method- the screen was placed in a sterile Petri dish. The tissue was placed in the middle of the screen. The back of a sterilized spoon was used to press the tissue into the screen in as thin a layer as possible. The tissue was actually pressed into the mesh so it was flat with the top of the screen. The tissue exuded liquid when squashed. For later experiments, to soak up this excess liquid, a sterile filter paper disc was placed under the screen prior to squashing. The control consisted of non-squashed tissue placed directly on a screen on the medium. There were five replications for the control and the spoon method and three replications for the slide method.

3.2.2.3 Maturation

To mature the embryos, the screens with tissue were transferred to solid maturation medium 1 (1/2 LM (modified according to Tremblay 1990) with 6% sucrose (w/v), 40 µM abscisic acid (ABA) and 0.4% Gelrite) and grown at 24°C under fluorescent light (24h 55 µmol $m^{-2}s^{-1}$). After 2 weeks, they were transferred to maturation medium 2 (1/2 LM with 3.4% sucrose (w/v), 40 µM ABA and 0.4% Gelrite) for 4 weeks. Two screens were placed on each plate of maturation medium so 1 g of tissue was present on each plate. After 6 weeks, all easily visible embryos were picked and the total number of embryos was counted.

3.2.2.4 Statistical Models and Analysis

The total number of embryos was expressed as the number of embryos g^{-1} of starting tissue (fresh weight). The data was subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + M_i + S_j + C_k + MS_{ij} + MC_{ik} + SC_{jk} + MSC_{ijk} + e_{ijkl} \qquad [3.1]$$

where Y_{ijkl} is the number of embryos produced by the *k*th clone being squashed into the *i*th mesh by the *j*th method; μ is the experimental mean; M_i is the effect of the *i*th mesh (719 µm, 890 µm, 1025 µm); S_j is the effect of the *j*th squashing method (slide, spoon, control(unsquashed)); C_k is the effect of the *k*th clone (6 x 5, 6 x 2, 6 x 3); MS_{ij} is the interaction effect of the *i*th mesh and the *j*th squashing method; MC_{ik} is the interaction effect of the *i*th mesh and the *k*th clone: SC_{jk} is the interaction effect of the *j*th squash method and the *k*th clone; MSC_{ijk} is the interaction effect of the *i*th mesh, the *j*th squashing method and the *k*th clone; e_{ijkl} is the random error component. All main terms were considered to be fixed effects. The non-zero data was tested for normality and transformed using the formula log(x+1). Computations of analysis of variance were performed on the transformed data using the GLM procedure (SAS Institute Inc., Cary NC, USA). The means data are based on actual numbers. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

3.2.3 Recovery Period

Squashing the tissue is damaging. A second preliminary experiment was done to determine if a recovery period (or period of regrowth) was necessary before the tissue was transferred to maturation medium.

3.2.3.1 Method

One half gram tissue masses from clones 2 x 6, 6 x 5, and 6 x 2 were squashed into squares of mesh using the spoon method in section 3.2.2.2, but using 1000 µm polypropylene mesh. One half of the screens were returned to initiation medium for 1 week in the dark at 24°C (a 1-week recovery period) then transferred to maturation medium. The other half of the screens was matured directly with no recovery period (see section 3.2.2.3). Control 1 consisted of unsquashed tissue placed directly on maturation medium (no recovery period). Control 2 consisted of unsquashed tissue placed on a screen on maturation medium (no recovery period). There were 12 replications. After 6 weeks, all easily visible embryos were picked and counted. Synchrony of development was determined by counting the number of mature (normal and abnormal), immature and precociously germinating embryos.

3.2.3.2 Statistical Models and Analysis

The total number of embryos was expressed as the number of embryos g^{-1} of starting tissue (fresh weight). The number of normal, abnormal, immature and precociously germinating embryos was expressed as a percentage of the total number of embryos. The data for each type of embryo was individually subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + T_i + C_j + TC_{ij} + e_{ijkl} \qquad [3.2]$$

where Y_{ijkl} is the number (or percentage) of embryos produced by the *j*th clone subjected to the *i*th treatment; μ is the experimental mean; T_i is the effect of the *i*th treatment (control (unsquashed, no screen, no recovery), control (unsquashed, screen, no recovery), squashed (recovery), squashed (no recovery)); C_j is the effect of the *j*th clone (2 x 6, 6 x 5, 6 x 2); TC_{ij} is the interaction effect of the *i*th treatment and the *j*th clone; e_{ijkl} is the random error component. All main terms were considered to be fixed effects. The data was tested for normality. The data for the total number of embryos was transformed using the formula log(x+1). The non-zero data for the percentage of normal, abnormal, and immature embryos was transformed using the formula sqrt(x). The non-zero data for the percentage of precociously germinating embryos was transformed using the formula log(x+1). Computations of analysis of variance were performed on the transformed data using the GLM procedure. The means data are based on actual numbers (percentages). The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

3.2.4 Clonal Effect

A larger experiment was done to confirm the clonal effect on the response of embryo production to squashing.

3.2.4.1 Method

One half gram tissue masses of clones 6 x 5, 6 x 2, 6 x 3, 1 x 5, 1 x 2, and 1 x 3 were squashed into squares of mesh using the spoon method in section 3.2.2.2, but using 1000 µm polypropylene mesh. The previous experiment showed that there was no statistical difference between the two controls. Therefore, due to the size of the experiment, only one control was used. The control was unsquashed tissue placed on a screen. The screens were placed on maturation medium and the embryos were matured using the method in section 3.2.2.3. There were 10 replications. After 6 weeks, all easily visible embryos were picked and counted. Synchrony of development was determined by counting the number of mature (normal and abnormal), immature and precociously germinating embryos.

3.2.4.2 Statistical Models and Analysis

The total number of embryos was expressed as the number of embryos g^{-1} of starting tissue (fresh weight). The number of normal, abnormal, immature and precociously germinating embryos was expressed as a percentage of the total number of embryos. The data for each type of embryo was individually subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + T_i + C_j + TC_{ij} + e_{ijkl} \qquad [3.2]$$

where Y_{ijkl} is the number (or percentage) of embryos produced by the *j*th clone subjected to the *i*th treatment; μ is the experimental mean; T_i is the effect of the *i*th treatment (control(unsquashed), squashed(no recovery)); C_j is the effect of the *j*th clone (2 x 6, 6 x 5, 6 x 2); TC_{ij} is the interaction effect of the *i*th treatment and the *j*th clone; e_{ijkl} is the random error component. All main terms were considered to be fixed effects. The data was tested for normality. The data for the total number of embryos was transformed using the formula sqrt(x). The data for the percentage of normal embryos was not transformed. The data for the percentage of abnormal and immature embryos was transformed using the formula sqrt(x+0.5) and arcsin(sqrt(x/100)). Computations of analysis of variance were performed on the transformed data using the GLM procedure. The means data are based on actual numbers (percentages). The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

3.2.5 New vs. Old Tissue

Embryogenic tissue changes as it ages and embryo production is thought to decrease with increasing age of the tissue (Dunstan et al. 1993). New tissue (4 months out of cryopreservation) and old tissue (20 months out of cryopreservation) of the same clones were compared to determine if squashing improved embryo production in old tissue.

3.2.5.1 Method

One half gram tissue masses were squashed into squares of mesh using the spoon method in section 3.2.2.2, but using 1000 µm polypropylene mesh. The control consisted of unsquashed tissue placed on the screen. The tissue was matured for 6 weeks (see section 3.2.2.3). There were 20 replications. After 6 weeks, all easily visible embryos were picked and counted. Synchrony of development was determined by counting the number of mature (normal and abnormal), immature and precociously germinating embryos. The experiment was initially done using only clone 6 x 5 (Run 1). It was repeated again using clones 6 x 5 and 6 x 3 (Run 2).

3.2.5.2 Statistical Models and Analysis

Run 1 and Run 2 varied in the number of clones used. Therefore, the results were analyzed separately.

3.2.5.2.1 Run 1

The total number of embryos was expressed as the number of embryos g^{-1} of starting tissue (fresh weight) from clone 6 x 5. The number of normal, abnormal, immature and precociously germinating embryos was expressed as a percentage of the total number of embryos. The data for each type of embryo was individually subjected to analysis of variance using the model:

$$Y_{ijk} = \mu + T_i + A_j + TA_{ij} + e_{ijk} \qquad [3.3]$$

where Y_{ijk} is the number (or percentage) of embryos produced by the *i*th age of tissue subjected to the *j*th treatment; μ is the experimental mean; T_i is the effect of the *i*th treatment (control, squash(no recovery)); A_j is the effect of the *j*th age of the tissue (new, old); TA_{jk} is the interaction effect of the *i*th treatment and the *j*th age; e_{ijk} is the random error component. All main terms were considered to be fixed effects. The data was tested for normality. The data for the total number of embryos was transformed using the formula sqrt(x). The data for the percentage of abnormal and immature embryos was transformed using the formulas log(x+1) and sqrt(x+1) respectively. The data for the percentage of precociously germinating embryos was transformed by the formula 1/(x+1). The data for the percentage of normal embryos showed normal distribution. Therefore, transformation was not required. Computation of analysis of variance was performed on the transformed data separately for each type of embryo data using the GLM procedure. The means data are based on actual numbers (percentages). The Duncan's multiple-range test at the significance level $\alpha \leq 0.05$ was performed on all main-effect means.

3.2.5.2.2 Run 2

The total number of embryos was expressed as the number of embryos g^{-1} of starting tissue (fresh weight). The number of normal, abnormal, immature and precociously germinating embryos was expressed as a percentage of the total number of embryos. The data for each type of embryo was individually subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + C_i + T_j + A_k + CT_{ij} + CA_{ik} + TA_{jk} + CTA_{ijk} + e_{ijkl} \qquad [3.4]$$

where Y_{ijkl} is the number (or percentage) of embryos produced by the *i*th clone of the *k*th age subjected to the *j*th treatment; μ is the experimental mean; C_i is the effect of the *i*th clone (6 x 5, 6 x 3); T_j is the effect of the *j*th treatment (control (unsquashed), squashed(no recovery)); A_k is the effect of the *k*th age of the tissue (new, old); CT_{ij} is the interaction effect of the *i*th clone and the *j*th treatment; CA_{ik} is the interaction effect of the *i*th clone and the *k*th age; TA_{jk} is the interaction effect of the *j*th treatment and the *k*th age; CTA_{ijk} is the interaction effect of the *i*th clone, the *j*th treatment and the *k*th age; e_{ijkl} is the random error component. All main terms were considered to be fixed effects. The data was tested for normality. The data for the total number of embryos and the percentage of abnormal embryos data was transformed using the formula sqrt(x). The data for the percentage of immature embryos was transformed using the formula sqrt(x+0.5). The data for the percentage of normal embryos showed normal distribution. Therefore, transformation was not required. Computation of analysis of variance was performed on the transformed data separately for each type of embryo data using the GLM procedure. The means data are based on actual numbers (percentages). The Duncan's multiple-range test at the significance level $\alpha \leq 0.05$ was performed on all main-effect means.

3.3 Results

3.3.1 Mesh Type and Squash Method

There was a significant clonal influence on embryo production (Table 3.1.1). Clone 6 x 2 produced significantly fewer embryos g^{-1} of tissue, an overall mean of 5.1 embryos g^{-1} as compared to 17.7 from clone 6 x 5 and 11.2 from clone 6 x 3 (Table 3.1.2). Embryo production in clones 6 x 5 and 6 x 3 was generally improved by spoon squashing, but not

Table 3.1.1. Mesh Type and Squash Method Experiment. Analysis of variance summary table for the effect of mesh type and squash method on the number of embryos produced per gram of starting tissue (fresh weight). Treatments were arranged in a 3 x 3 x 3 factorial and the data were analyzed using the GLM procedure (SAS). F-values are significant at the 0.05(*), 0.01(**), 0.001(***) level.

Table 3.1.2. Mesh Type and Squash Method Experiment. Duncan's Multiple Range Test results for the effects of the mesh type and squash method on the number of embryos produced per gram of starting tissue (fresh weight). Data are means averaged over all treatment combinations. Means with different letters are significantly different ($p = 0.05$) from each other.

Table 3.1.3. Mesh Type and Squash Method Experiment. The effects of mesh type, squash method and clone on the number of embryos (±standard error) produced per gram of starting tissue (fresh weight). The numbers of embryos g^{-1} from the spoon squashed and the unsquashed control tissue are the averages of five replicates. The number of embryos g^{-1} from the slide squashed tissue are the average of three replicates.

Table 3.1.1.

Source	DF	F-value
Mesh (M)	2	0.70
Squash Method (S)	2	0.36
Clone (C)	2	9.61***
M x S	4	0.59
M x C	4	2.46
S X C	4	1.51
M x S x C	7	1.78
Error	40	2.08
Corrected Total	65	

Table 3.1.2.

	Number of embryos
Mesh	
710 µm	11.5a
980 µm	8.0a
1025 µm	14.6a
Squash Method	
Spoon	16.6a
Slide	8.9a
Control	7.3a
Clone	
6 x 5	17.7a
6 x 2	5.1b
6 x 3	11.2a
Mean	11.3

Table 3.1.3.

	Spoon	Slide	Control
Clone 6 x 5			
710 µm mesh	38.6 (19.0)	26 (15.0)	19 (10.2)
980 µm mesh	10.6 (7.8)	5 (2.6)	13 (8.1)
1025 µm mesh	35.6 (14.2)	0.7 (0.7)	2.2 (1.4)
Clone 6 x 2			
710 µm mesh	0.7 (0.4)	3.0 (1.5)	6.4 (2.6)
980 µm mesh	7.6 (4.4)	1.7 (1.2)	6.4(4.0)
1025 µm mesh	1.4 (0.5)	3.0 (3.0)	12.8 (6.3)
Clone 6 x 3			
710 µm mesh	4.8 (2.8)	1.0 (1.0)	2 (1.3)
980 µm mesh	7.4 (5.2)	17.7 (16.2)	1.8 (1.8)
1025 µm mesh	43 (38.8)	22.3 (20.4)	0

in clone 6 x 2 (Table 3.1.3). Mesh type had no significant influence on embryo production (Table 3.1.1). The overall mean number of embryos produced per gram of tissue from each mesh type was not significantly different (Table 3.1.2).

The spoon method of squashing the tissue produced more embryos than the slide method or the unsquashed control (16.6 vs. 8.9 and 7.3)(Table 3.1.2) but the results were not statistically different (Table 3.1.1). With the slide method, spreading the tissue was problematic. It was very hard to get a uniform mat, as the tissue tended to aggregate on the edges. Also, this method was more cumbersome. The spoon method was easier to use and was therefore used for the remainder of the squash experiments. The spoon method showed that embryogenic tissue is not particularly fragile and that compressing the tissue mass into screens did not harm the tissue.

3.3.2 Recovery Period

The effect of the treatment of the tissue on total embryo production and on the percentage of normal and abnormal embryos was statistically significant (Table 3.2.1). The squashed tissue without a recovery period produced a significantly higher total number of embryos (an overall average of 36.0 g^{-1} of tissue). There was no significant difference in the total number of embryos produced by the unsquashed controls and the spoon squashed sample with a 1-week recovery period (Table 3.2.2). Squashing with no recovery period also produced significantly less normal and abnormal embryos (Table 3.2.2). Squashing with no recovery period produced more immature embryos (an overall mean of 69.5%), but this was not statistically different from the other treatments (Table 3.2.2). Examination of the individual clone/treatment combinations (Table 3.2.3) shows that in all cases, squashing with no recovery period resulted in a larger percentage of the embryos being immature.

For the remainder of the squash experiments, tissue squashed with a spoon was transferred directly to maturation medium for maturation (without a recovery period).

Table 3.2.1. Recovery Period Experiment. Analysis of variance summary table for the effects of tissue treatment and clone on the total number of embryos produced per gram of starting tissue (fresh weight) and the percentage of these that were normal, abnormal, immature or precocious (i.e. precociously germinated). F-values are significant at the 0.05(*), 0.01(**), 0.001(***) level.

Table 3.2.2. Recovery Period Experiment. Duncan's Multiple Range Test results for the effects of tissue treatment and clone on the number of embryos produced per gram of starting tissue (fresh weight). Data are means averaged over all treatment combinations. Means within the same column with different letters are significantly different ($p = 0.05$) from each other.

Table 3.2.3. Recovery Period Experiment. The effects of tissue treatment and clone on the mean total number of embryos (±standard error) produced per gram of starting tissue (fresh weight) and on the mean percentage (±standard error) of these embryos that were normal, abnormal, immature or precocious (i.e precociously germinated). The numbers of embryos g^{-1} are the averages of 12 replicates.

Table 3.2.1.

	Total		% Normal		% Abnormal		% Immature		% Precocious	
Source	DF	F-value	DF	F-value	DF	F-value	DF	F-value	DF	F-value
Treatment (T)	3	3.63*	3	4.63*	3	5.08**	3	3.06*	3	0.92
Clone (C)	2	4.58*	2	0.26	2	5.96**	2	8.59***	2	0.71
T x C	6	1.57	6	0.50	5	1.20	6	0.78	2	0.50
Error	74		37		36		52		22	
Corrected Total	85		48		46		63		29	

Table 3.2.2.

	Total	% Normal	%Abnormal	%Immature	%Precocious
Treatment					
Control (no screen)	13.8b	21.7a	22.4a	45.0a	10.9a
Control (screen)	15.7b	32.7a	25.3a	30.6a	11.4a
Squash (recovery)	12.3b	40.0a	31.7a	25.6a	2.7a
Squash (no recovery)	36.0a	9.5b	9.1b	69.5a	11.9a
Clone					
2 x 6	18.9a	22.7a	38.3a	25.3c	13.6a
6 x 5	26.9a	28.8a	15.3b	43.6b	12.3a
6 x 2	10.1b	26.5a	9.8ab	62.4c	1.4a
Mean	19.3	25.9	22.0	42.3	9.8

Table 3.2.3.

	Total	% Normal	%Abnormal	% Immature	% Precocious
Clone 6 x 5					
Control (no screen)	11.1 (3.3)	11.9 (7.9)	28.1 (9.6)	40.8 (12.7)	19.3 (8.5)
Control (screen)	12.6 (3.3)	45.1 (16.6)	15.2 (9.2)	30.5 (12.2)	9.2 (3.9)
Squash (recovery)	16.2 (4.7)	66.7 (16.2)	15.8 (14.0)	17.5 (9.2)	0.0 (0)
Squash (no recovery)	56.3 (12.0)	7.0 (2.0)	6.1 (3.5)	70.4 (7.1)	16.5 (4.1)
Clone 6 x 2					
Control (no screen)	9.9 (2.6)	24.4 (12.9)	1.8 (1.8)	68.1 (14.1)	5.6 (3.8)
Control (screen)	8.2 (2.3)	33.0 (12.6)	15.6 (11.4)	51.5 (13.5)	0.0 (0)
Squash (recovery)	14.4 (10.6)	35.5 (20.3)	16.6 (7.4)	47.9 (20.3)	0.0 (0)
Squash (no recovery)	9.5 (2.2)	3.6 (3.6)	0.0 (0)	96.4 (3.6)	0.0 (0)
Clone 2 x 6					
Control (no screen)	19.2 (6.3)	28.1 (9.6)	32.9 (12.1)	31.4 (11.6)	7.5 (7.5)
Control (screen)	24.3 (6.5)	22.2 (10.3)	41.6 (8.9)	13.7 (5.6)	22.4 (8.0)
Squash (recovery)	7.3 (1.8)	21.4 (12)	57.5 (14.9)	13.9 (9.0)	7.1 (7.1)
Squash (no recovery)	19.9 (5.4)	17.5 (12.2)	20.2 (9.5)	50.0 (13.2)	12.2 (8.7)

3.3.3 Clonal Effect

Clone and treatment and their interaction had a significant influence on the total number of embryos g^{-1} of tissue produced by the various clones (Table 3.3.1). Clone also had a significant influence on the percentage of normal, abnormal and immature embryos produced. In this experiment, the control non-squashed tissue overall produced more embryos g^{-1} tissue than the squashed tissue (67.7 vs. 34.4) (Table 3.3.2). The percentage of normal, abnormal and immature embryos was significantly different between clones. Squashing did not significantly affect the percentage of normal, abnormal and immature embryos produced by the clones (Table 3.3.2). Examination of the individual clone/treatment combinations (Table 3.3.3) shows that squashing produced only marginally larger total numbers of embryos than the unsquashed controls in clones 1 x 5 and 3 x 6. These two clones also produced the least number of embryos.

3.3.4 New vs. Old Tissue

3.3.4.1 Run 1

Treatment had a significant influence on the total number of embryos produced per gram of starting tissue (fresh weight) (Table 3.4.1). The squashed tissue produced significantly more embryos g^{-1} than the unsquashed controls (181.4 vs. 70.4 and 51.2)(Table 3.4.2). Treatment did not significantly influence the percentage of normal, abnormal, immature or precociously germinating embryos produced (Table 3.4.1). The majority of the embryos were immature (58.4 to 61.5%)(Table 3.4.2). The age of the tissue had a significant influence on the percentages of normal, abnormal and immature embryos produced (Table 3.4.1). The old tissue produced significantly less normal and abnormal embryos than the new tissue (21.6 and 7.3% vs. 40 and 12.8%) (Table 3.4.2). The old tissue produced significantly more immature embryos than the new tissue (69.5 vs. 46.4%). Examination of the individual treatment by age interactions (Table 3.4.3) shows that both new and old tissue produced more embryos g^{-1} when squashed than did either unsquashed control. The old tissue produced more immature embryos than the new tissue 63.3-74.1% vs. 42.3-45.5%). The new tissue produced more normal embryos than the old tissue (35.8-45.2% vs. 17.6-28.8%).

Table 3.3.1. Clonal Effect Experiment. Analysis of variance summary table for the effects of clone and tissue treatment on the total number of embryos produced per gram of starting tissue (fresh weight) and the percentage of these that were normal, abnormal, immature or precocious (i.e. precociously germinated). F-values are significant at the 0.05(*), 0.01(**), 0.001(***) level.

Table 3.3.2. Clonal Effect Experiment. Duncan's Multiple Range Test results for the effects of clone and tissue treatment on the number of embryos produced per gram of starting tissue (fresh weight). Data are means averaged over all treatment combinations. Means within the same column with different letters are significantly different ($p = 0.05$) from each other.

Table 3.3.3. Clonal Effect Experiment. The effects of clone and tissue treatment on the mean total number of embryos (±standard error) produced per gram of starting tissue (fresh weight) and on the mean percentage (±standard error) of these embryos that were normal, abnormal, immature or precocious (i.e precociously germinated). The numbers of embryos g^{-1} are the averages of 10 replicates.

Table 3.3.1.

	Total		% Normal		% Abnormal		% Immature		% Precocious	
Source	DF	F-value	DF	F-value	DF	F-value	DF	F-value	DF	F-value
Clone (C)	5	10.88***	5	3.37**	5	3.07*	5	3.59**	5	0
Treatment(T)	1	22.09***	1	1.04	1	1.50	1	0.88	1	0
C x T	5	6.77***	5	0.66	5	3.20*	5	1.51	5	0
Error	92		92		92		92		92	
Corrected Total	103		103		103		103		103	

Table 3.3.2.

	Total	% Normal	% Abnormal	% Immature	% Precocious
Clone					
6 x 5	48.2b	27.0bc	18.2c	54.8a	0
6 x 2	80.5a	34.2bc	20.7bc	45.1ab	0
6 x 3	47.8b	50.4b	24.0abc	25.6bc	0
1 x 5	33.3bc	37.0ab	25.6abc	37.5abc	0
1 x 2	21.0c	39.1ab	39.8a	21.0c	0
1 x 3	97.1a	20.0c	36.7ab	43.3ab	0
Treatment					
Squashed	34.4b	31.9a	30.2a	37.8a	0
Control	67.7a	36.8a	24.5a	38.7a	0
Mean	52.6	34.6	38.3	27.1	0

Table 3.3.3.

	Total	% Normal	% Abnormal	% Immature	% Precocious
Clone 6 x 5					
Control	68.3 (14.1)	31.5 (8.9)	12.2 (5.3)	56.4 (9.2)	0
Squash	25.8 (9.1)	22.0 (5.1)	24.8 (8.4)	53.2 (11.6)	0
Clone 6 x 2					
Control	133.7 (11.8)	35.6 (3.4)	24.0 (2.4)	40.4 (5.3)	0
Squash	27.3 (5.7)	32.8 (10.5)	17.5 (5.6)	49.7 (12.8)	0
Clone 6 x 3					
Control	55.8 (10.1)	56.3 (4.2)	10.7 (1.9)	33.0 (4.8)	0
Squash	30.0 (18.5)	37.1 (16.8)	54.1 (16.5)	8.9 (8.4)	0
Clone 1 x 5					
Control	29.2 (5.6)	40.5 (7.6)	19.1 (5.1)	40.4 (10.2)	0
Squash	37.4 (9.0)	33.4 (7.0)	32.1 (5.1)	34.6 (9.6)	0
Clone 1 x 2					
Control	19.3 (5.1)	36.3 (5.3)	40.4 (3.5)	23.3 (6.3)	0
Squash	23.0 (3.9)	42.3 (5.3)	39.2 (8.8)	18.5 (6.9)	0
Clone 1 x 3					
Control	106.6 (23.6)	18.7 (6.2)	43.0 (12.7)	38.4 (15.0)	0
Squash	81.8 (14.8)	22.1 (6.4)	26.7 (3.5)	51.2 (6.2)	0

Table 3.4.1. New vs. Old Tissue Experiment (Run 1). Analysis of variance summary table for the effects of age of the embryogenic tissue and tissue treatment on the total number of embryos produced per gram of starting tissue (fresh weight) and the percentage of these that were normal, abnormal, immature or precocious (i.e. precociously germinated). F-values are significant at the 0.05(*), 0.01(**), 0.001(***) level.

Table 3.4.2. New vs. Old Tissue Experiment (Run 1). Duncan's Multiple Range Test results for the effects of age of the embryogenic tissue and tissue treatment on the number of embryos produced per gram of starting tissue (fresh weight). Data are means averaged over all treatment combinations. Means within the same column with different letters are significantly different ($p = 0.05$) from each other.

Table 3.4.3. New vs. Old Tissue Experiment (Run 1). The effects of age of the embryogenic tissue and tissue treatment on the mean total number of embryos (±standard error) produced per gram of starting tissue (fresh weight) and on the mean percentage (±standard error) of these embryos that were normal, abnormal, immature or precocious (i.e precociously germinated). The numbers of embryos g^{-1} are the averages of 20 replicates.

Table 3.4.1.

	Total		% Normal		% Abnormal		% Immature		% Precocious	
Source	DF	F-value	DF	F-value	DF	F-value	DF	F-value	DF	F-value
Treatment (T)	2	35.70***	2	0.33	2	1.04	2	0.29	2	0.90
Age (A)	1	2.39	1	16.79***	1	9.96**	1	14.61***	1	0.23
T x A	2	1.56	2	1.75	2	1.03	2	1.30	1	0.62
Error	79		79		79		79		7	
Corrected Total	84		84		84		84		11	

Table 3.4.2.

	Total	% Normal	%Abnormal	% Immature	% Precocious
Treatment					
Control1	70.4b	27.6a	11.2a	58.8a	2.3a
Control2	51.2b	29.8a	8.6a	61.5a	0.1a
Squashed	181.4a	31.8a	9.0a	58.4a	0.7a
Age					
Old	95.7a	21.6b	7.3b	69.5a	1.5a
New	108.5a	40.0a	12.8a	46.4b	0.7a
Mean	101.3	29.6	9.7	59.5	1.1

Table 3.4.3.

	Total	% Normal	%Abnormal	% Immature	% Precocious
Clone 6 x 5					
New Tissue					
Control1	69.8(10.2)	45.2(6.2)	8.9(2.0)	455(5.2)	0.3(0.3)
Control2	79.0(18.6)	39.6(5.9)	17.6(4.3)	42.3(5.9)	0.5(0.4)
Squash	178.5(22.6)	35.8(5.8)	10.9(2.4)	52.0(6.7)	1.3(0.7)
Old Tissue					
Control1	36.6(8.6)	17.6(4.9)	8.3(4.0)	74.1(6.7)	0.0(0)
Control2	63.7(10.3)	18.4(4.4)	6.3(1.7)	71.6(6.8)	3.7(2.6)
Squash	183.5(20.5)	28.8(5.8)	7.6(1.8)	63.3(6.9)	0.3(0.2)

Table 3.5.1. New vs. Old Tissue Experiment (Run 2). Analysis of variance summary table for the effects of clone, tissue treatment and age of the embryogenic tissue on the total number of embryos produced per gram of starting tissue (fresh weight) and the percentage of these that were normal, abnormal, immature or precocious (i.e. precociously germinated). F-values are significant at the 0.05(*), 0.01(**), 0.001(***) level.

Table 3.5.2. New vs. Old Tissue Experiment (Run 2). Duncan's Multiple Range Test results for the effects of clone, tissue treatment and age of the embryogenic tissue on the number of embryos produced per gram of starting tissue (fresh weight). Data are means averaged over all treatment combinations. Means within the same column with different letters are significantly different (p = 0.05) from each other.

Table 3.5.3. New vs. Old Tissue Experiment (Run 2). The effects of clone, tissue treatment and age of the embryogenic tissue on the mean total number of embryos (±standard error) produced per gram of starting tissue (fresh weight) and on the mean percentage (±standard error) of these embryos that were normal, abnormal, immature or precocious (i.e precociously germinated). The numbers of embryos g^{-1} are the averages of 20 replicates.

Table 3.5.1.

	Total		% Normal		% Abnormal		% Immature		% Precocious	
Source	DF	F-value	DF	F-value	DF	F-value	DF	F-value	DF	F-value
Clone (C)	1	0.85	1	7.00**	1	0.85	1	0.91	1	0
Treatment (T)	1	0.92	1	4.84*	1	5.23*	1	0.17	1	0
Age (A)	1	0.27	1	0.02	1	8.35**	1	3.89	1	0
C x T	1	0.03	1	0.35	1	0.71	1	1.20	1	0
C x A	1	1.03	1	0.10	1	8.50**	1	11.32**	1	0
T x A	1	11.35**	1	0.02	1	0.93	1	3.66	1	0
C x T x A	1	0.32	1	0.22	1	2.63	1	1.81	1	0
Error	51		51		51		51		51	
Corrected Total	58		58		58		58		58	

Table 3.5.2.

	Total	% Normal	%Abnormal	% Immature	% Precocious
Clone					
6 x 5	47.6a	28.5b	31.8a	39.7a	0.0
6 x 3	55.7a	47.6a	20.9a	31.5a	0.0
Treatment					
Control	53.9a	41.7a	21.0b	37.2a	0.0
Squashed	46.36a	28.1b	36.4a	35.5a	0.0
Age					
Old	53.96a	35.3a	35.5a	28.8a	0.0
New	48.03a	36.5a	20.5b	42.9a	0.0
Mean	50.7	36.0	27.5	36.5	0.0

Table 3.5.3.

	Total	% Normal	%Abnormal	% Immature	% Precocious
Clone 6 x 5					
New Tissue					
Control	68.3(14.1)	31.5(8.9)	12.2(5.3)	56.4(9.2)	0(0)
Squash	25.8(9.1)	22.0(5.1)	24.8(8.4)	53.2(11.6)	0(0)
Old Tissue					
Control	36.7(13.7)	34.8(8.5)	41.9(7.9)	23.3(9.6)	0(0)
Squash	61.6(11.5)	23.8(9.2)	54.3(10.1)	21.9(4.0)	0(0)
Clone 6 x 3					
New Tissue					
Control	55.8(10.1)	56.3(4.2)	10.7(1.9)	33.0(4.8)	0(0)
Squash	30.0(18.5)	37.1(16.8)	54.1(1.9)	8.9(8.4)	0(0)
Old Tissue					
Control	56.6(20.0)	49.8(8.3)	15.6(2.4)	34.6(9.6)	0(0)
Squash	75.2(24.2)	38.0(13.7)	17.9(2.2)	44.0(13.1)	0(0)

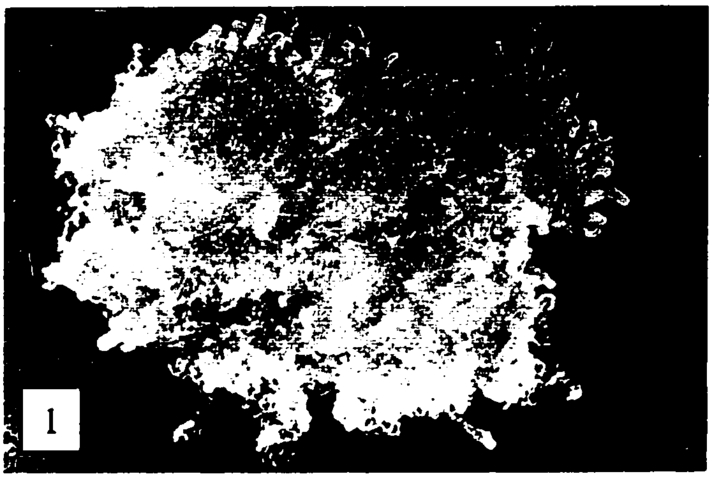
Figure 3.1. Comparison of embryo production in:

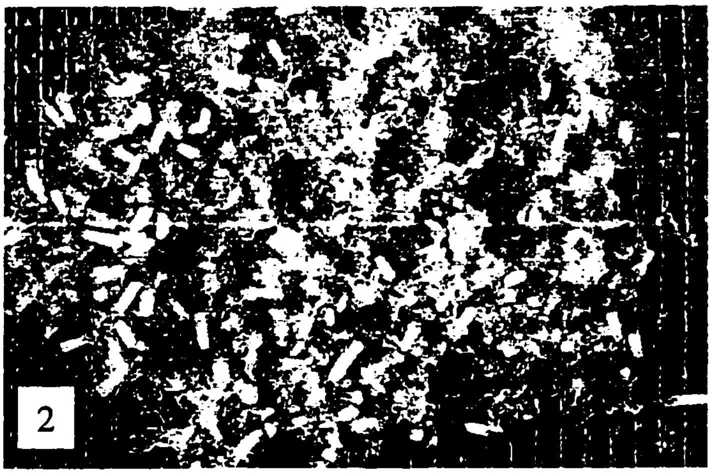
Figure 3.1.1. Unsquashed embryogenic tissue.

Figure 3.1.2. Embryogenic tissue squashed into a screen.

Magnification is [2.4x].

Fig. 3.1





3.3.4.2 Run 2

Treatment times age interaction had a significant influence on the total number of embryos produced (Table 3.5.1). Squashing decreased the total number of embryos produced by the new tissue in both clones, but increased total embryo production in the old tissue (Table 3.5.3). The morphology of the embryos was also affected by clone, treatment, age and the clone times age interaction (Table 3.5.1). Both clone and treatment affected the percentage of normal embryos produced. Clone 6 x 3 produced an overall mean of 47.6 normal embryos g^{-1} as compared to 28.5 from clone 6 x 5 (Table 3.5.2). The unsquashed controls produced more normal embryos g^{-1} than the squashed tissue (41.7 as compared to 28.1) (Table 3.5.2). Treatment and age affected the percentage of abnormal embryos produced (Table 3.5.1) with the squashed tissue producing more abnormal embryos (Table 3.5.2). The old tissue also produced more abnormal embryos g^{-1} as compared to the new tissue (Table 3.5.2). The clone by age interaction affected both the percentages of abnormal and immature embryos produced (Table 3.5.1). New tissue from clone 6 x 5 produced a high percentage of immature embryos and a low percentage of abnormal embryos while the old tissue produced a high percentage of abnormal and a low percentage of immature embryos (Table 3.5.3). Clone 6 x 3 produced a high percentage of immature embryos and a low percentage of abnormal embryos (Table 3.5.3). The ratio of abnormal to immature embryos in the new tissue of clone 6 x 5 was affected by squashing (Table 3.5.3).

3.4 Discussion

Squashing tissue onto screens with a spoon is a fast and simple way of making a flat mat of tissue. Tissue is quickly and easily transferred to fresh medium by moving the whole screen. Squashing also produced a mat of tissue with exposed embryos that were much easier to pick than those found in clumps (Fig. 3.1). This is important in increasing the speed and ease with which embryos can be harvested. Protocols could be designed, using screens, to decrease the amount of labour involved in the current production system. A commercial system could use large screens of tissue (cut to fit in a Petri dish). Harvesting of embryos for germination pre-treatment (e.g. desiccation) is the most time consuming part of the current protocol. Maturation of embryos on a screen would allow the whole screen of embryos and tissue to be transferred to the germination pre-treatment and then transferred to germination medium. This would decrease labour costs substantially by eliminating the task of harvesting individual embryos. Culling of unacceptable germinants would be done as the embryos are transplanted to the greenhouse.

Klimaszewska and Smith (1997) used another method of producing a flat mat of tissue for maturing *Pinus strobus* somatic embryos. They placed embryogenic tissue in a test tube with liquid medium and vigorously shook it creating a slurry. Aliquots of the suspension were poured onto a filter paper and the supernatant removed. The filter paper was then transferred to maturation medium. This method is more labour intensive and there is an increased chance of contamination.

In all our experiments using multiple clones, the largest influence on embryo production was due to clonal effect. The total number of embryos g^{-1} was clone dependent. This correlates well with the results of Park et al. (1994) using the same embryogenic material. They found that during maturation, variance due to clones was large (34.3% to 43.2% of the total variance). Barrett et al. (1997) investigated the effects of various nitrogen sources on tissue growth and embryo production in some of the same clones. They again found that the number of mature embryos produced was clone dependent. This suggests that the number of embryos produced by a clone may be genetically determined and that there may be limitations to the improvement in embryo production that can be attained by changes in cultural methods.

The rate of maturation and the morphology of the embryos is also clone dependent. All experiments showed some clonal influence on the percentage of normal, abnormal immature and/or precociously germinating embryos. Treatment and age also affected morphology and synchrony, but the results were variable and no clear trend was found.

The age of the tissue affected embryo production. The unsquashed older tissue produced fewer embryos. This phenomenon was also cited by Dunstan et al. (1993) and Fourre et al. (1997) who studied the effect of long-term culture on embryo production in white and Norway spruce respectively. Fourre cites a decrease in embryo production in one clone from 1000 embryos g^{-1} down to almost zero. He postulates that this may be due to "genetic deviations, or a progressive change in the embryo organization, or a physiological evolution resulting in a diminished capacity in the synthesis of endogenous compounds required for embryo production." Meins (1989) says that older tissue may become habituated to growth regulators used to proliferate the tissue.

In our experiments, squashing increased embryo production in older tissue. Perhaps there is decreased plant growth regulator (PGR) transport in older mound tissue or sensitivity to the hormones may have decreased which may require more of the PGRs to be transported to the embryos to induce proper maturation. Squashing of the tissue ensures that the embryos will have closer contact with the medium and more access to the PGRs. It is also possible that mound formation in older tissue results in a substantial build up of toxic waste products. Squashing of the tissue into a flat mat allows more contact with the medium and therefore more of the waste products could diffuse into the medium.

Variability in embryo production was prominent in this study. Interclonal variability is common in conifer tissue culture and is often reported, but intraclonal variation, while known to be present, has only been reported by Ruaud (1993), Dunstan et al. (1993) and Fourre et al. (1997). In our experiments, intraclonal variability was frequently observed between plates, between the 2 screens on each plate and also in different areas on the screen. Although the tissue came from the same clone, not all parts of the tissue were equally productive. This could be an effect of the growth of the tissue in clumps during the proliferation phase. The centre of the clump becomes necrotic and may have very few immature embryos present to mature. The majority of the embryos are produced on the outside of the clump. The size and the age of the clump probably have an effect on productivity. Larger clumps have a larger non-productive central core, hence fewer embryos to mature. Fourre et al. (1997) also found high weekly variation in embryo production within the same clone. This variation was also seen in our experiments with embryo production and response to squashing changing between the different experiments. For example, squashing improved embryo production in clone 6 x 5 in the Recovery Period experiment, but decreased embryo production in the Clonal Effect Experiment. The reasons for this cyclical variability and other intraclonal variations are not known. Fourre et al. (1997) could not detect any RAPD intraclonal variation. George (1993) postulates that temporary variations *in vitro* may be a result of changes in gene expression (epigenetic variation) in response to temporary environmental changes or plant growth regulators.

3.5 Conclusion

Squashing of tissue into a polypropylene mesh with the back of a spoon is a quick and easy method to create a mat of exposed embryos that are easy to pick. Squashing significantly improved embryo production in older tissue. This is important as most tissue culture programs use older tissue, as this is easier than continually replenishing the tissue supply with newly thawed tissue. Squashing of new embryogenic tissue had variable results on the total number of embryos produced confirming that intraclonal variability is a problem in tissue culture, especially in maturation. The total number of embryos produced per gram of tissue appears to be genetically influenced. Contrary to what was expected, squashing had no effect on the morphology of the embryos. Squashing did not increase the number of normal embryos, but it also usually did not increase the number of abnormal embryos. Embryo morphology appears to be genetically influenced as shown by the strong clonal influence. Squashing did not appear to change the synchrony of the maturing embryos. The rate of maturation was most strongly influenced by clone. The improvement in total embryo production with old tissue, the ease with which the tissue can be handled and the embryos harvested, make this a very useful method of maturing embryos and one worth further development for commercial applications.

Chapter 4

Improving Germinant Quality with a Desiccation Treatment

4.1 Introduction

The form of the mature somatic embryo influences the development of the resulting seedling. Abnormal embryos germinate to form abnormal seedlings. However, normal appearing embryos, germinated directly after removal from maturation medium, do not always germinate into normal seedlings, many being vitreous and/or without roots. Vitreous (or hyperhydric) germinants have a watery, translucent and often swollen appearance, are easily damaged by desiccation, and usually will not survive transfer to the greenhouse (George 1993, Bonga and von Aderkas 1992). Good quality germinants with normal elongating cotyledons, hypocotyls and roots stand a much better chance of surviving in the greenhouse and growing into usable planting stock. A pre-treatment may be necessary to improve germinant quality by increasing the produce these normal seedlings.

Drying of mature somatic embryos has been shown to improve germination (as discussed in Attree and Fowke 1993). It has been achieved by suspending mature embryos over a saturated salt solution in a desiccator at room temperature (Attree et al. 1991). Similar methods using 6 or 12 well petri dishes involve putting embryos in one half of the wells and filling the other half with water (Roberts et al. 1990) or with saturated salt solutions at 25°C (Beardmore and Charest 1995) or at 4°C (Dronne et al. 1997). Most desiccation procedures were empirically derived, not strictly controlled and therefore are not easy to duplicate.

To optimize desiccation protocols, several aspects of desiccation and its effect on germination will have to be studied. There is no published systematic study that correlates increasing levels of desiccation with changes in germinant quality. It is important that this be done.

Tolerance to desiccation increases as embryos mature (Attree et al. 1995), but at the same time, plantlet conversion rates of undesiccated embryos (defined as the number of normal germinants produced) decrease with increased maturity (Lelu et al. 1995). The effect of embryo maturity on desiccation response should also be examined to determine the optimum stage of development for the application of a desiccation treatment.

In addition, dried embryos respond differently to imbibition with germinant quality depending on the method of rehydration. Most of the currently used protocols rehydrate the embryos by placing them directly on germination medium (Compton et al. 1995, Attree et al. 1995). Tetteroo et al. (1995) showed that desiccated embryos of *Daucus carota* suffer imbibitional shock when they are placed directly into liquid. However, this shock can be lessened by partially rehydrating the embryos in moist air before they come into contact with actual liquid (Crowe et al. 1989a). Therefore, the response of the desiccated embryos to different rehydration methods should be examined.

This chapter describes work conducted to optimize desiccation as a pre-treatment for improving germinant quality. By using the unsaturated salt solution technique, we dried embryos of four stages of development to well defined and previously characterized relative water contents. Next, the dried embryos were rehydrated directly on germination medium or indirectly in a saturated atmosphere, and then the rehydrated embryos were germinated. Germinant quality was correlated with the maturity of the embryo, the level of desiccation, and the method of rehydration, to determine the most effective desiccation protocol for improving germination in white spruce somatic embryos.

4.2 Materials and Methods

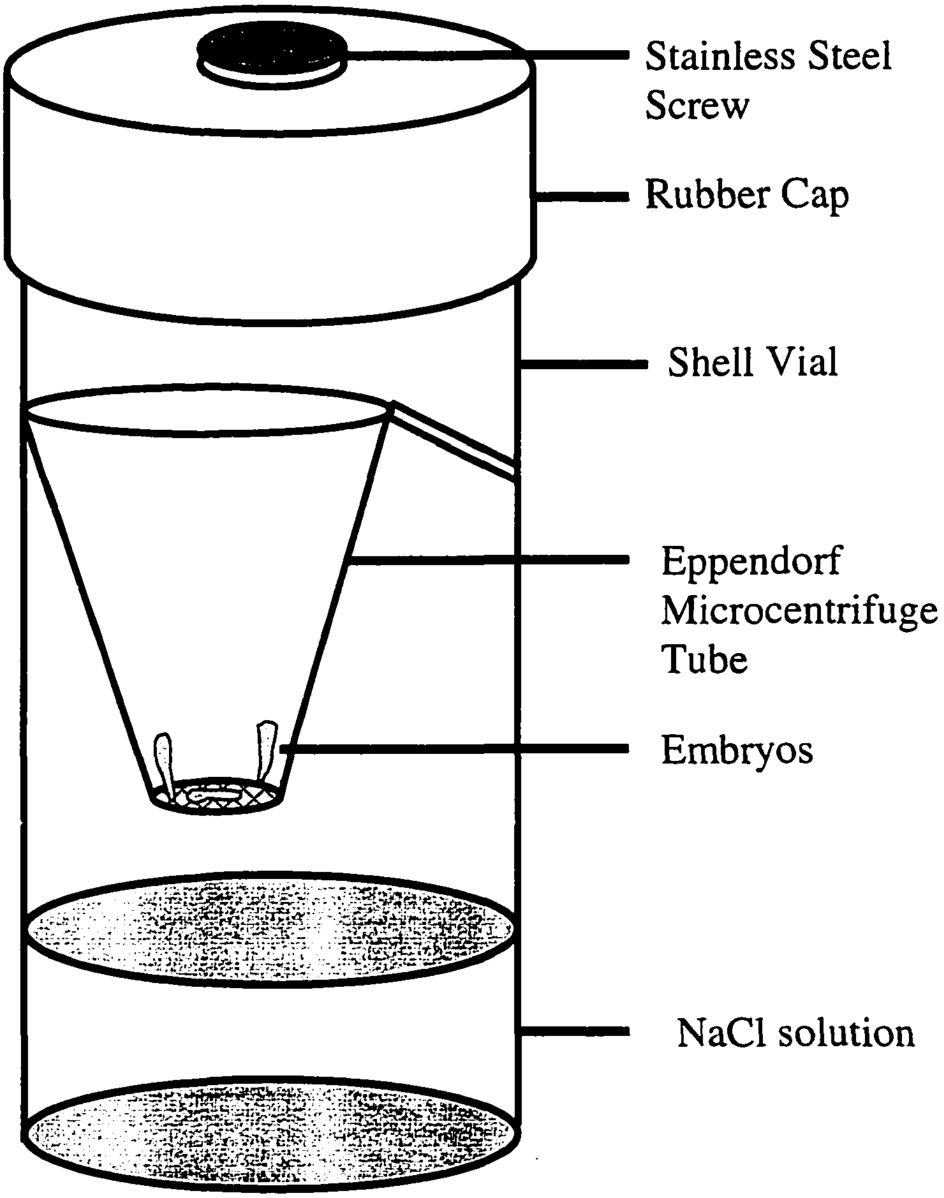
4.2.1 Culture Conditions

Embryogenic tissue from cryopreserved white spruce cross MRL-11 times GPC-15 (clone 6x5) was proliferated on initiation medium (1/2 LM with 1% (w/v) sucrose, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 μM Benzylaminopurine (BA) and 0.9% (w/v) Difco-Bacto agar) (Park et al. 1993). Plates of tissue were grown in the dark at 24°C with bi-weekly subcultures. Two transfers were made to maintenance medium (initiation medium with the 5 µM BA replaced with 5 µM kinetin)(Park et al. 1994) before the beginning of the maturation treatment. The tissue was transferred to maturation medium 1 (1/2 LM (modified according to Tremblay (1990)) with 6% sucrose (w/v), 40 µM abscisic acid (ABA) and 0.4% Gelrite) and grown at 24°C under fluorescent light (24 h 55 µmol $m^{-2}s^{-1}$). After 2 weeks, the tissue was subcultured monthly to fresh maturation medium 2 (1/2 LM with 3.4% sucrose, 40 µM ABA, and 0.4% Gelrite) (Park et al. 1994). The tissue was either transferred as a mound to the maturation medium or squashed into a mesh screen (see Chapter 3). Embryos at four stages of development were harvested for the desiccation treatments. The stages were based on the number of days the embryogenic tissue had been on maturation medium (15, 21 39 and 51 d).

4.2.2 Desiccation

Individual embryos at the four stages of development were equilibrated to precise moisture end points. The desiccation procedure was similar to the method initially described by Livingston et al. (1992) and Dumont-Beboux et al. (1996) for generating water release curves and subsequently used by Percy (1997) for controlled desiccation of embryos and somatic tissue. Embryos were placed in modified 0.5mL Eppendorf tubes (the tip cut off and replaced with a nylon mesh (335 µm openings) which had the cap removed but the arm left attached) (Fig. 4.1). Three embryos were placed in each Eppendorf. A 10 mL shell vial lined with filter paper was sterilized and the vial filled with 2 mL of either sterilized deionized-distilled water or filter-sterilized NaCl solution at a specific concentration. The Eppendorfs were wedged in the shell vial and topped with a rubber cap, creating a closed system. The mesh of the Eppendorfs was thus positioned above the salt solution. The caps had previously been sterilized in 70% ETOH for 15 minutes and air-dried in the laminar flow hood. The cap was weighted down with a 5/16" x 1/2" stainless steel screw and the whole unit was placed in a water bath at 5.0° ± 0.1°C. The water and salt solutions used resulted in water potentials at 5°C of 0, -1, -2, -3, -4, -5, -8, -10, -15, -20 MPa (Appendix 1). Embryos had equilibrated to their respective salt solution water potentials within 7 d, at which time they were removed from the

Figure 4.1. Schematic diagram of the desiccation apparatus. Embryos are suspended in a modified eppendorf tube over a salt solution with a known water potential. The inside of the shell vial is lined with filter paper. A rubber cap tightly seals the vial. The whole unit is weighted down with a screw and immersed in a water bath at 5°C


Stainless Steel Screw
Rubber Cap
Shell Vial
Eppendorf Microcentrifuge Tube
Embryos
NaCl solution

Eppendorfs and rehydrated. For each of the four embryo developmental stages there were 48 embryos per solution (3 embryos per Eppendorf, 16 Eppendorfs per NaCl solution).

4.2.3 Rehydration

The embryos were directly rehydrated by being placed on germination medium or indirectly rehydrated in a cuvette which had an atmosphere approaching 100% RH at 5°C for 16-24 h. They were then germinated. The control embryos were not desiccated. They were placed directly on germination medium after being picked off the maturation medium.

4.2.4 Germination

The embryos were placed on germination medium (modified 1/4 strength LM containing 1/2 strength $Fe_2(SO_4)_3$, EDTA, KNO_3, $MnSO_4.H_20$, 2% (w/v) sucrose and 0.4% (w/v) Gelrite) and placed in the growth room at 24°C under continuous light (24h 55 μmol $m^{-2}s^{-1}$). After 4 weeks, they were assessed for the presence of normal, elongating cotyledons, hypocotyls and roots and were graded for quality (quality categories 1-8) (Appendix 2). A dead category was also added as embryos were killed by some of the desiccation treatments. Swollen germinants with a bumpy or shiny surface were classified as vitreous in a separate scoring system (quality categories 1v-7v; Appendix 3).

From each desiccation level/rehydration method combination, 24 embryos were put on one plate of germination medium. Each plate was considered a sample unit and the number of embryos in each quality category was calculated as a percentage of total embryos for each plate. There were 24 embryos x 10 NaCl solutions x 2 rehydration methods x 4 embryo development stages for a total of 1920 embryos used for the experiment. The experiment was repeated three times. The results from the three repeats were averaged.

The change in germinant quality as a result of the desiccation treatment was assessed by examining the change in the percentage of the germinants within the various quality categories. Category 1 germinants are seedlings with normal, elongating cotyledons, hypocotyls and roots. They are the desired end product of any germination method. Therefore, an improvement in germinant quality was considered to be an increase in the percentage of category 1 germinants produced.

4.2.5 Statistical models and analysis

The germination responses, expressed as the percentage of germinants in each quality category, were subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + M_i + P_j + H_k + MP_{ij} + MH_{ik} + PH_{jk} + MPH_{ijk} + e_{ijkl}$$

where Y_{ijkl} is the percentage of germinated embryos of the *i*th maturity (developmental stage) desiccated over a NaCl solution with the *j*th water potential and rehydrated by the *k*th rehydration method. μ is the experimental mean; M_i is the effect of the *i*th maturity state of the embryo (15, 27, 39 or 51 d); P_j is the effect of desiccation over NaCl solution with a water potential of (0, -1, -2, -3, -4, -5, -8, -10, -15, -20 MPa); H_k is the effect of the rehydration method (direct; indirect); MP_{ij} is the interaction effect of the *i*th maturity and the *j*th water potential; MH_{ik} is the interaction effect of the *i*th maturity and the *k*th rehydration method; PH_{jk} is the interaction effect of the *j*th water potential and the *k*th rehydration method; MPH_{ijk} is the interaction effect of the *i*th maturity, the *j*th water potential and the *k*th rehydration method; e_{ijkl} is the random error component. All main effect terms were considered to be fixed effects. The data for each quality category was tested for normality. Computations of analysis of variance were performed on the transformed data (Thoni 1967) for each quality category separately using the GLM procedure (SAS Institute Inc., Cary NC, USA). The non-zero data for the percentage of germinants in categories 1v, 3v, 5, 6v, 7, 7v, 8 and dead were transformed by using the formula log(x+1), where x is the percentage of germinated embryos in that quality category. The non-zero data for the percentage of germinants in category 1 was transformed using the formula arcsin(sqrt(x/100)). The non-zero data for the percentage of germinants in categories 2, 2v, 3, and 4 were transformed using the formula 1/(x+1). The non-zero percentage data for the percentage of germinants in category 5v was transformed by using the formula sqrt(x). The means data are based on actual percentages. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

The percentage of germinants in the individual vitreous categories was relatively small. By summing the percentage of germinants in all vitreous categories (1v- 3v and 5v-7v) the change in the total percentage of germinants that were scored as vitreous was examined. Prior to statistical analysis, the vitreous data was transformed using the formula arcsin(sqrt(x/100)), where x was the total percentage of vitreous germinants.

Preliminary experiments indicated that the cotyledons and hypocotyls were more sensitive to damage by desiccation than the roots (further discussed in Chapter 6). Therefore, the combined response of the cotyledons and hypocotyls to desiccation was examined. Summing categories 1 and 5 gave the total percentage of germinants with undamaged cotyledons+hypocotyls (C+H) and the resulting non-zero data was normalized prior to analysis using the formula arcsin(sqrt(x/100)), where x was the total percentage of germinants with undamaged cotyledons+hypocotyls.

By summing the percentage of all germinants in those categories that involve roots (1, 1v, 2, 2v, 3, 3v, 4), the effect of desiccation on the overall percentage of germinants with elongating roots (Roots) was made apparent. The data was normalized prior to analysis using the formula arcsin(sqrt(x/100)), where x was the total percentage of germinants with elongating roots.

The data was graphed using Microsoft Excel.

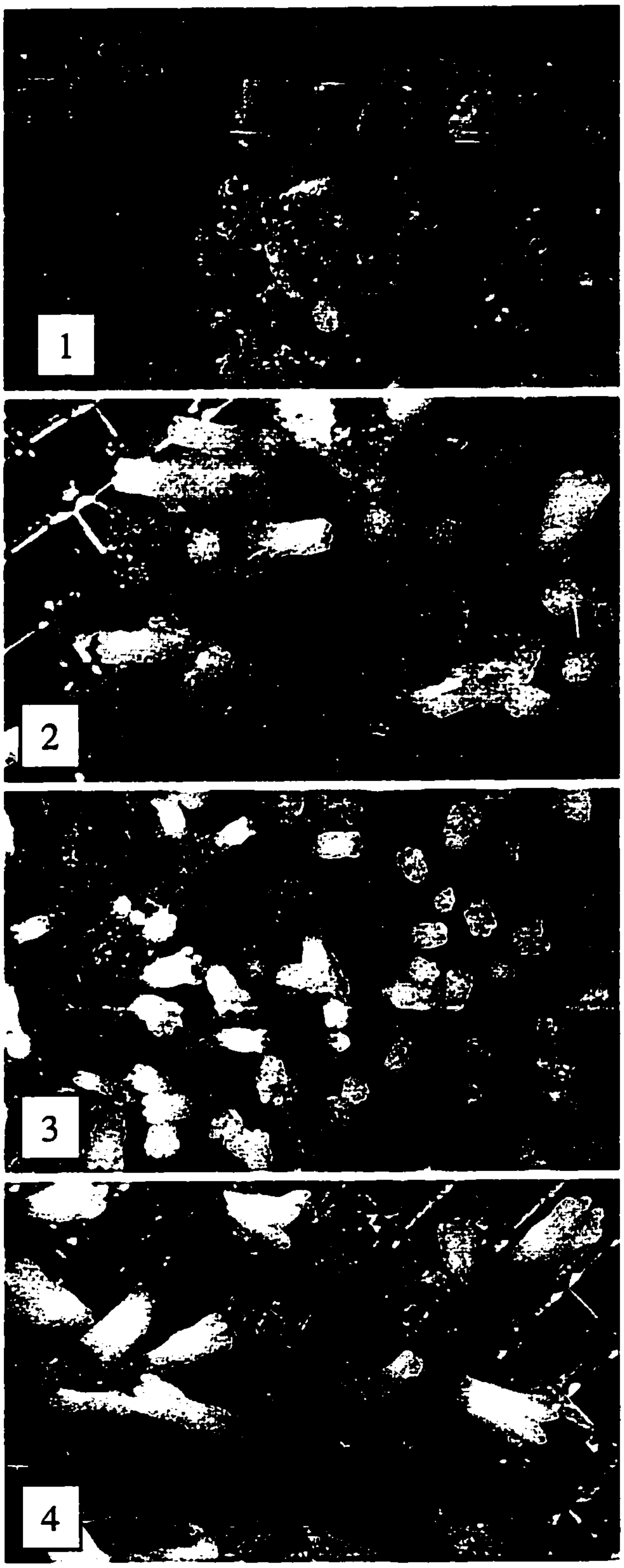
4.3 Results

Embryo development was asynchronous resulting in various stages of development being present at each of the chosen sampling times. The 15-d embryos (Fig 4.2.1) were translucent and ranged in stage of development from globular to early cotyledonary with the cotyledonary buttresses just visible. The majority of the embryos had a distinct shoot

Figure 4.2. Developmental stages of white spruce somatic embryos after culture on maturation medium on polypropylene 1000 μm mesh screen for various lengths of time. Embryos at these four developmental stages were used for the desiccation experiment:

Fig. 4.2.1. - 15 d. Magnification is [8.5x]
Fig. 4.2.2 - 27 d. Magnification is [6.8x]
Fig. 4.2.3 - 39 d. Magnification is [4.3x]
Fig. 4.2.4 - 51 d. Magnification is [8.2x]

Figure 4.2



apex and no cotyledons or only cotyledonary buttresses. These were selected for the desiccation treatments. The developmental stage of the 27-d embryos (Fig. 4.2.2) ranged from having a distinct shoot apex with no cotyledons, to cotyledons topping the shoot apex. The more mature cotyledonary embryos were used. The 39-d embryos (Fig 4.2.3) were opaque and well-formed with fully developed cotyledons, hypocotyl and root apex when selected for desiccation. The 51-d embryos (Fig 4.2.4) were fully mature and in some cases had already germinated precociously but only the mature non-germinated embryos were used.

After 4 weeks on germination medium, the germinants from the desiccated embryos could be divided into non-vitreous and vitreous germinants and dead embryos. Based on the presence of normal elongating cotyledons, hypocotyls and roots, the germinants were further divided into eight quality categories (Appendix 2). The vitreous germinants were divided into six categories (Appendix 3).

4.3.1 General Analysis

The analysis of variance showed that the maturity of the embryos, the water potential and the rehydration method all had a significant effect on the percentage of germinants in the different quality categories (Table 4.1).

The maturity (i.e. stage of development) of the embryo had a significant effect on the percentage of germinants in seven of the 15 individual germination categories. The effect was significant at the $p \leq 0.001$ significance level for quality categories 1, 1v, 4, 8, and dead and for the combined categories of Roots, Vitreous and Cotyledons+Hypocotyls (C+H).

The method of rehydration had a significant effect on the percentage of germinants in three of the 15 individual quality categories (Table 4.1). The effect was significant at the 0.001 level for category 1, at the 0.01 level for the dead embryos and for the combined categories of Roots and Cotyledons+Hypocotyls.

Table 4.1. Analysis of variance summary table for the effect of embryo maturity, level of desiccation (water potential) and method of rehydration on the subsequent percentage of germinants in each germination category. For each quality category the change in the percentage of germinants was analyzed separately. F-values are significant at the 0.05(*), 0.01(**) and 0.001(***) level.

Table 4.1

	Germination Category													
	Category 1		Category 1v		Category 2		Category 2v		Category 3		Category 3v		Category 4	
Source	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value
Maturity (M)	3	9.07***	3	8.64***	3	9.23	2	1.77	3	5.73**	3	2.70	3	6.26***
Rehydration (R)	1	22.39***	1	0.09	1	0.00	1	0.00	1	6.27*	1	0.02	1	1.60
Water Potential (P)	10	6.80***	10	1.25	10	5.36	8	1.27	10	2.28	10	6.52**	10	1.27
M x R	3	0.04	3	1.19	3	2.70	1	0.12	3	1.25	2	0.56	3	0.83
M x P	30	0.62	27	0.91	15	2.65	2	9.99	24	1.06	10	1.88	24	1.80
R x P	9	0.74	9	1.13	4	2.90	0		9	0.69	1	0.46	9	1.20
M x R x P	21	0.47	10	1.36	1	5.96	0		7	0.73	0		8	1.43
Error	84		38		2		1		17		10		29	
Corrected total	161		101		39		15		74		37		87	

	Germination Category													
	Category 5		Category 5v		Category 6v		Category 7		Category 7v		Category 8		Dead	
Source	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value	DF	F-Value
Maturity (M)	3	3.91*	3	0.49	3	2.85	3	0.73	3	1.20	3	27.26***	3	8.58***
Rehydration (R)	1	1.34	1	2.16	1	1.10	1	0.03	1	0.00	1	3.43	1	8.01**
Water Potential (P)	10	2.63**	10	1.70	10	1.86	10	2.78	10	1.06	10	2.60**	10	10.34***
M x R	3	0.31	3	0.41	3	2.13	3	1.79	3	0.29	3	1.80	3	0.97
M x P	28	0.72	28	1.10	15	2.61	15	1.56	17	0.30	30	1.05	20	0.76
R x P	9	0.63	9	0.88	5	1.31	4	2.02	5	0.65	9	1.01	7	0.69
M x R x P	20	0.79	20	0.61	0		2	2.32	0		20	0.55	8	0.38
Error	62		56		7		7		14		89		46	
Corrected total	136		130		44		45		53		165		98	

	Combined Germination Categories					
	Roots		Vitreous		C+H	
Source	DF	F-Value	DF	F-Value	DF	F-Value
Maturity (M)	3	32.62***	3	26.20***	3	18.33***
Rehydration (R)	1	43.56***	1	3.39	1	26.90***
Water Potential (P)	10	10.81***	10	2.86**	10	8.72***
M x R	3	1.29	3	3.73	3	0.35
M x P	30	0.64	30	1.45	30	0.57
R x P	9	0.94	9	1.68	9	1.02
M x R x P	27	0.33	27	0.74	24	0.67
Error	123		123		94	
Corrected total	206		206		174	

The extent to which the embryos were desiccated, (i.e., the water potential of the NaCl solutions used to desiccate the embryos) also had a significant effect on the percentage of germinants in four of the germination categories (Table 4.1). The effect was significant at the 0.001 level for categories 1 and dead and for the combined categories of Roots and Cotyledons+Hypocotyls.

The interaction of these three main factors had no significant influence on the germination response (Table 4.1).

4.3.1.1 Maturity

The more mature embryos (i.e. at later stages of development) were better able to withstand desiccation as shown by the Duncan's test results in Table 4.2.1. The mean percentage (averaged over all treatment combinations) of normal (category 1) germinants increased with maturity from 11.6% for 15-d embryos to a high of 42.1% for 51-d ones. The mean percentage of category 8 germinants decreased with maturity with the largest drop from 42.8% at 15 d to 18.2% at 27 d. The mean percentage of dead embryos decreased with maturity from 25.7% at 15 d to 3.0% at 51 d. The percentage of germinants with elongating roots (Roots) increased with maturity from 22.2% at 15 d to 57.6% at 51 d as did the percentage of vitreous germinants from 8.1% at 15 d to 29.9% at 51 d. The percentage of germinants with undamaged Cotyledons+Hypocotyls (C+H) increased with maturity from 14.2% at 15 d to 51.1% at 51 d maturity.

4.3.1.2 Rehydration Method

Indirect rehydration improved the quality of the germinants by decreasing the damage suffered as a result of the desiccation treatments as shown by the Duncan's test results in Table 4.2.2. The mean percentage of normal (category 1) germinants increased from 23.2% to 39.9% and that for dead embryos decreased from 19.8% to 7.3%. As well, the mean percentage of vitreous germinants decreased from 21.1% to 15.5% and lastly, indirect rehydration increased the percentage of germinants with elongated roots or

Table 4.2.1. Duncan's Multiple Range Test results for the effect of embryo maturity on the subsequent percentage of germinants in each germination category. The percentage data for each germination category were analyzed separately. Data are means averaged over all levels of desiccation (water potentials) and both rehydration methods. Means with different letters are significantly different (p=0.05) from each other.

Table 4.2.2. Duncan's Multiple Range Test results for the effects of rehydration method on the subsequent percentage of germinants in each germination category. The percentage data for each germination category were analyzed separately. Data are means averaged over the four stages of embryo maturity and all levels of desiccation (water potentials). Means with different letters are significantly different (p=0.05) from each other.

Table 4.2.1

	Maturity			
Germination Category	15-D	27-D	39-D	51-D
1	11.6c	24.6b	39.9a	42.1a
1v	1.7b	3.8b	4.1b	7.9a
2	1.2b	1.2ab	2.2ab	0.8a
2v	0.4a	0.0a	0.3a	1.1a
3	1.0a	2.2b	3.1b	2.3b
3v	0.8a	0.8ab	0.5b	2.3ab
4	5.5b	2.2a	2.0a	1.9a
5	2.5b	7.8a	9.9a	9.0a
5v	4.2a	9.8a	10.6a	11.4a
6	0.5a	0.1a	0.1a	0.7a
6v	0.8ab	1.6b	2.4ab	1.4a
7	1.0a	1.8a	1.8a	2.7a
7v	0.2a	2.5a	3.0a	5.9a
8	42.8a	18.2b	12.3c	8.4c
Dead	25.7a	23.5a	7.4b	3.0b
Combined Categories				
Roots	22.2c	34.7b	52.1a	57.6a
Vitreous	8.1c	18.5b	20.9b	29.9a
C+H	14.2c	32.3b	49.8a	51.1a

Table 4.2.2

	Rehydration Method	
Germination Category	Direct	Indirect
1	23.2b	39.9a
1v	4.3a	4.2a
2	1.3a	1.5a
2v	0.5a	0.2a
3	1.6a	3.1b
3v	1.5a	0.3a
4	2.4a	3.3a
5	6.8a	8.1a
5v	9.7a	7.5a
6	0.4a	0.2a
6v	1.6a	1.5a
7	1.8a	1.9a
7v	3.5a	1.7a
8	21.5a	19.3a
Dead	19.8a	7.3b
Combined Categories		
Roots	34.9b	52.6a
Vitreous	21.1a	15.5b
C+H	30.0b	47.9a

undamaged cotyledons+hypocotyls from 34.9% or 30.0% to 52.6% or 47.9% respectively.

4.3.1.3 Increasing Levels of Desiccation

Mild desiccation improved the quality of the embryos but additional desiccation resulted in increasing damage and eventually death as shown in the Duncan's test results in Table 4.3. The mean percentage (averaged over all treatment combinations) of category 1 germinants increased slightly up to a maximum of 51% at -1MPa, and then progressively decreased to a low of 8.4% at -20 MPa. The percentage of category 8 germinants progressively increased with increasing water potential from 9.5% for the control to 30.3% at -5 MPa. Desiccation past -5 MPa resulted in more dead embryos up to a maximum of 45.2% at -20 MPa. The mean percentage of vitreous germinants decreased with mild desiccation from 27.4% for the non-treated control to 16.7% with desiccation at -1 MPa, the same water potential that produced the maximum percentage of category 1 germinants. Mild desiccation increased the percentage of germinants with elongating roots to a maximum of 66.3% at -1 MPa. However, increased desiccation resulted in progressively more damage to the cotyledons +hypocotyls.

4.3.2 Maturity x Desiccation x Rehydration Interaction

The effect of various desiccation levels and rehydration methods on embryos at each developmental stage was examined to determine the treatment combination that produced the largest percentage of normal germinants. The percentage of normal (category 1) germinants produced by the undesiccated (control) embryos increased with the level of development of the embryo from 16% for the 15-d embryos to 56% for the 51-d ones (Graphs 4.1.1-4).

4.3.2.1 Desiccation with Direct Rehydration

Desiccation followed by direct rehydration on germination medium improved the percentage of category 1 germinants, for example, to 26% for the 15-d embryos and 66% for the 39-d ones (Graphs 4.1.1-4). More advanced developmental stages (more mature)

Table 4.3. Duncan's Multiple Range Test results for the effect of level of desiccation (water potential) on the subsequent percentage of germinants in each germination category. The percentage data for each quality category were analyzed separately. Data are means averaged over the four stages of embryo maturity and both methods of rehydration. Means with different letters are significantly different (p=0.05) from each other.

Table 4.3

	Water Potential (-MPa)										
Germination Category	Control	0	1	2	3	4	5	8	10	15	20
1	41.3ab	42.2ab	51.0a	44.3ab	36.2bc	29.8abc	21.5bcd	17.5bcd	14.9cd	15.7cd	8.4d
1v	5.4b	4.5ab	6.2ab	4.1ab	6.2ab	4.5ab	5.8ab	3.3ab	2.8a	2.3ab	2.2ab
2	0.5a	1.7abc	1.6abc	1.8abc	1.8abc	1.0ab	1.4bc	0.2ab	0.4ab	2.4c	1.5abc
2v	0.0a	0.2a	0.5a	0.0a	1.0a	0.2a	1.1a	0.2a	0.2a	0.4a	0.6a
3	0.6a	1.5bc	3.5bc	1.7bc	1.5bc	2.4bc	1.9bc	2.0c	3.5bc	3.1bc	1.4b
3v	0.3d	1.4bc	0.2bc	1.8bc	1.2bc	0.5bc	2.4bc	0.8bc	0.1cd	0.9a	1.7ab
4	2.2a	2.7b	3.3ab	3.6b	2.0ab	2.4ab	3.7b	1.8ab	3.4ab	1.4ab	3.7b
5	17.5a	9.2abc	4.2c	4.1bc	10.0abc	7.5abc	7.2abc	7.8a	8.9ab	3.6bc	4.0abc
5v	16.8a	12.4ab	6.1b	9.0ab	9.2ab	10.9a	10.3ab	7.9ab	6.5b	5.8b	6.0ab
6	0.2a	0.3a	0.2a	0.2a	0.4a	0.6a	0.4a	0.2a	0.4a	0.6a	0.0a
6v	3.7ab	1.0b	1.2ab	1.2ab	1.0a	1.1ab	0.6ab	3.2ab	1.1ab	2.0ab	1.8ab
7	0.1b	0.3a	1.6a	2.3a	1.1a	0.6a	0.7a	2.5a	2.4a	5.4a	2.8a
7v	1.2b	5.9ab	2.6ab	3.1ab	2.1ab	5.3a	3.2ab	2.3ab	1.0ab	2.2ab	1.5ab
8	9.5c	13.6bc	16.0ab	20.3ab	22.1ab	27.6a	30.3a	22.8ab	21.8ab	20.9ab	19.3ab
Dead	0.6c	3.1c	2.0bc	2.6c	4.3c	5.6c	9.3abc	27.4ab	32.5ab	33.3a	45.2a
Combined Categories											
Roots	50.4bcd	54.6abc	66.3a	57.3ab	49.9bcd	40.8cd	37.9de	26.2f	26.0ef	25.4ef	19.5f
Vitreous	27.4a	25.5ab	16.7bcd	19.2abcd	20.8abc	22.6abcd	23.4ab	17.8bcd	11.8d	13.5cd	13.8cd
C+H	58.8a	51.6ab	55.2ab	48.3abc	46.2abc	37.3bcd	28.8de	25.3cde	23.8de	19.3de	12.3e

Graph 4.1. The percentage of directly rehydrated embryos that were either killed by the desiccation treatments or germinated and scored as Category 1 (normal), vitreous or category 8. Embryos at four stages of development were desiccated over water or NaCl solutions with water potentials ranging from 0 to –20 MPa. The control embryos (C) were not desiccated. Each data point is the average of 3 replications.

Four developmental stages of embryos were used:

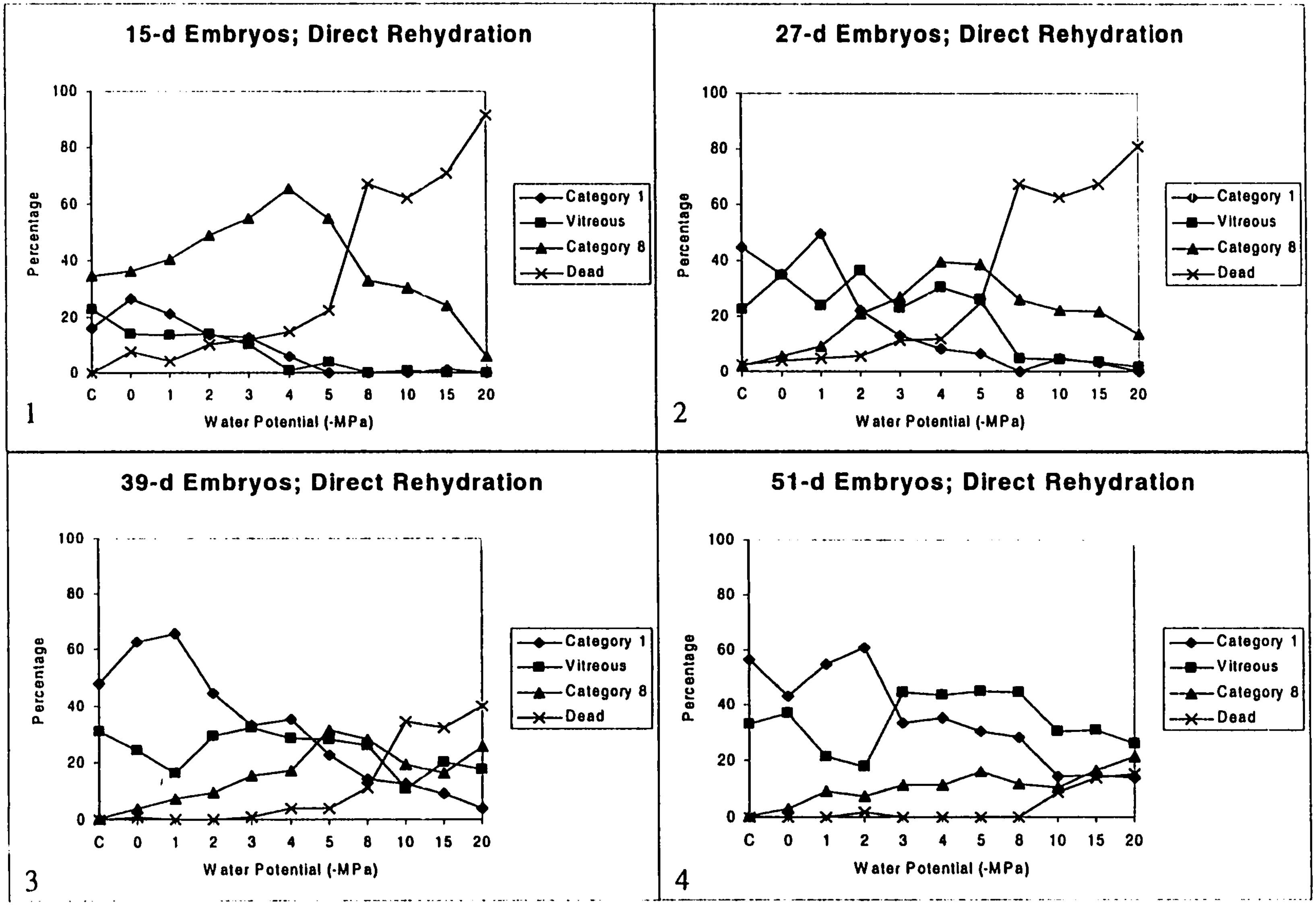
Graph 4.1.1. 15-d embryos

Graph 4.1.2. 27-d embryos

Graph 4.1.3. 39-d embryos

Graph 4.1.4. 51-d embryos

Graph 4.1



15-d Embryos; Direct Rehydration

27-d Embryos; Direct Rehydration

39-d Embryos; Direct Rehydration

51-d Embryos; Direct Rehydration

required stronger desiccation to achieve maximum improvement in the percentage of category 1 germinants produced. Equilibration over water at 5°C was all that was required to improve the percentage of category 1 germinants for the 15-d embryos (Graph 4.1.1) while 27, 39 and 51-d embryos required desiccation at -1, -1, and -2 MPa respectively (Graphs 4.1.2, 4.1.3, 4.1.4). For the older 39 and 51-d embryos, this improvement correlated with a decrease in the percentage of vitreous germinants. Tolerance to desiccation improved as the embryos matured. Damage was shown by an increase in category 8 and dead embryos. Desiccation of the immature 15-d embryos increased the percentage of category 8 germinants (Graph 4.1.1). Desiccation past -5 MPa resulted in a significant increase in the percentage of embryos killed by desiccation up to a maximum of 92%. The 27-d embryos produced fewer category 8 germinants, but the percentage also increased with increasing desiccation (Graph 4.1.2). The percentage of embryos killed by desiccation increased significantly with desiccation past -5 MPa. The percentage of category 8 and dead embryos produced by the 39 and 51-d embryos increased with increasing desiccation, but at significantly lower levels. The percentage of dead embryos increased with desiccation past -6 MPa.

4.3.2.2 Desiccation with Indirect Rehydration

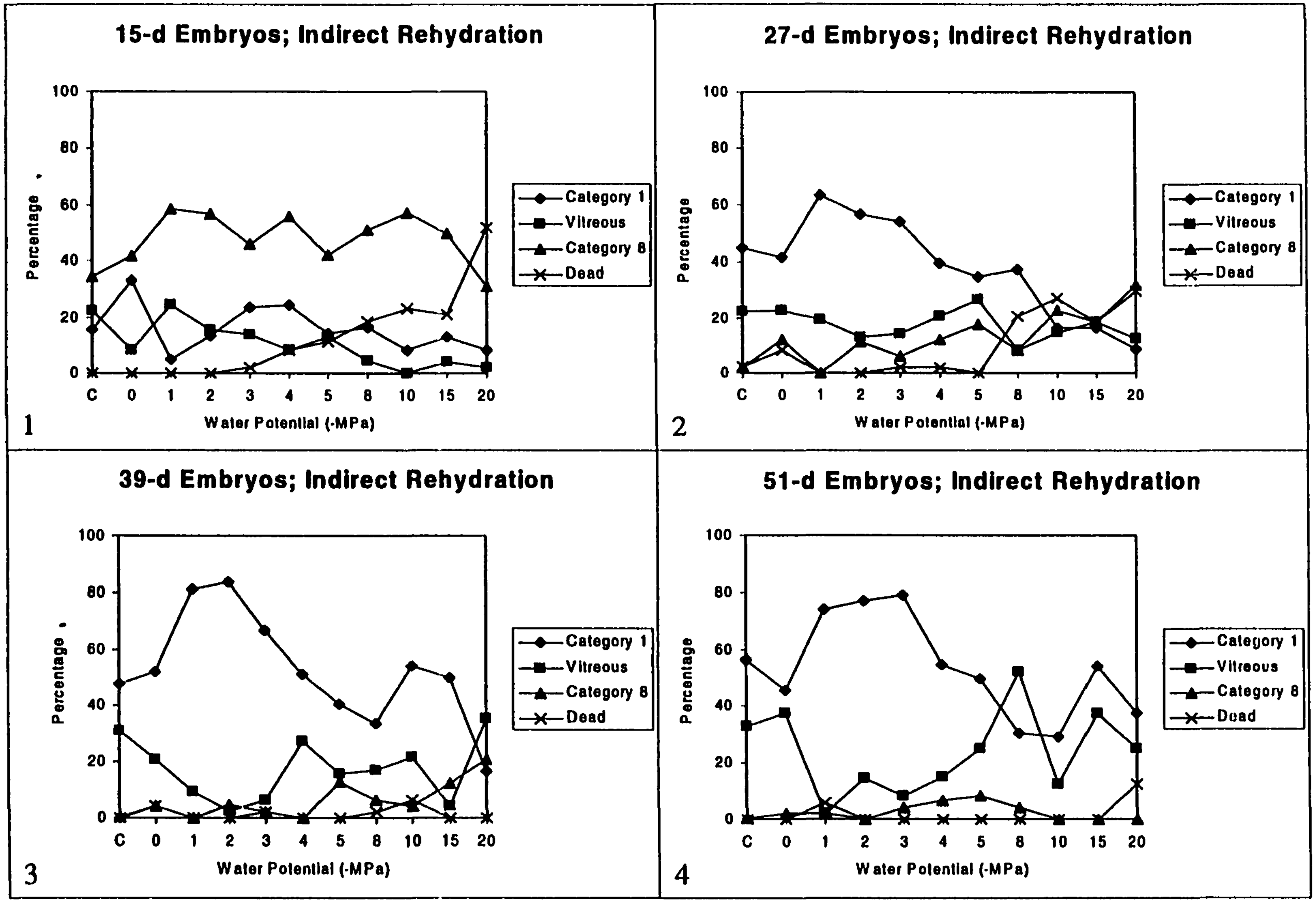
Indirect rehydration had a significant effect on the germination results (Graphs 4.2.1-4). Overall, a higher percentage of normal (category 1) germinants were produced when the desiccation treatments were followed by indirect rehydration as compared to the direct rehydration values. The level of desiccation required to increase the percentage of category 1 germinants produced by the more mature embryos also changed with direct rehydration. Desiccation at -1 and -2 MPa (vs. -1 MPa only) increased the percentage of category 1 germinants for the 39-d embryos. Desiccation at -1, -2 and -3 MPa (vs. -1 and -2 MPa) increased the percentage of category 1 germinants for the 51-d embryos. The most noticeable difference between the two methods of rehydration was the change in the percentage of category 8 and dead embryos. Fewer embryos are killed by the desiccation treatments if they are rehydrated indirectly after desiccation.

Graph 4.2. The percentage of indirectly rehydrated embryos that were either killed by the desiccation treatments or germinated and scored as Category 1 (normal), vitreous or category 8. Embryos at four stages of development were desiccated over water or NaCl solutions with water potentials ranging from 0 to –20 MPa. The control embryos (C) were not desiccated. Each data point is the average of 3 replications.

Four developmental stages of embryos were used:

- **Graph 4.2.1.** 15-d embryos
- **Graph 4.2.2.** 27-d embryos
- **Graph 4.2.3.** 39-d embryos
- **Graph 4.2.4.** 51-d embryos

Graph 4.2.


15-d Embryos; Indirect Rehydration

27-d Embryos; Indirect Rehydration

39-d Embryos; Indirect Rehydration

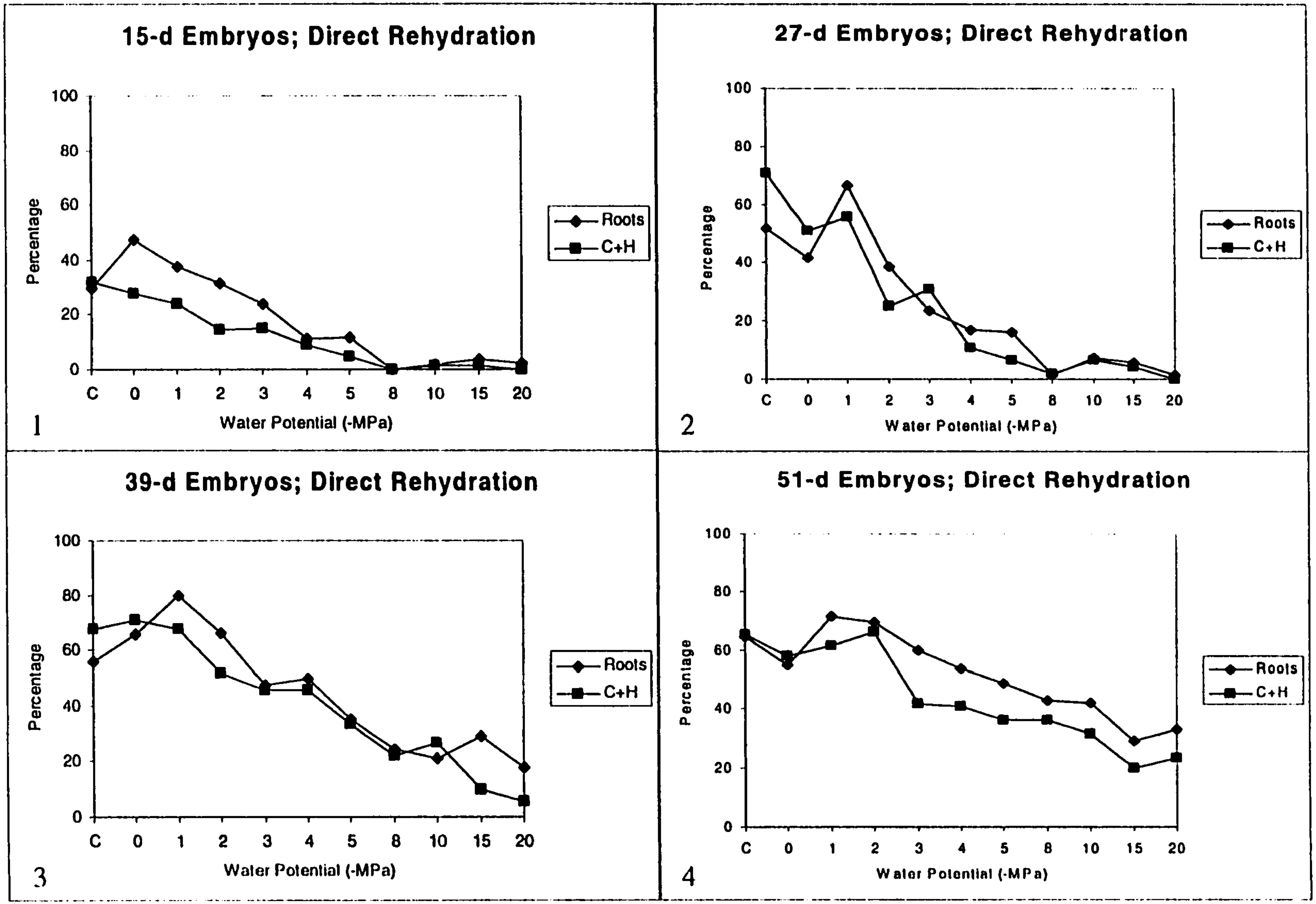
51-d Embryos; Indirect Rehydration

Graph 4.3. The percentage of directly rehydrated embryos that produced germinants with undamaged roots (Roots) or undamaged cotyledons+hypocotyls (C+H). Embryos at four stages of development were desiccated over water or NaCl solutions with water potentials ranging from 0 to –20 MPa. The control embryos (C) were not desiccated. Each data point is the average of 3 replications.

Four developmental stages of embryos were used:

Graph 4.3.1. 15-d embryos
Graph 4.3.2. 27-d embryos
Graph 4.3.3. 39-d embryos
Graph 4.3.4. 51-d embryos

Graph 4.3


15-d Embryos; Direct Rehydration

27-d Embryos; Direct Rehydration

39-d Embryos; Direct Rehydration

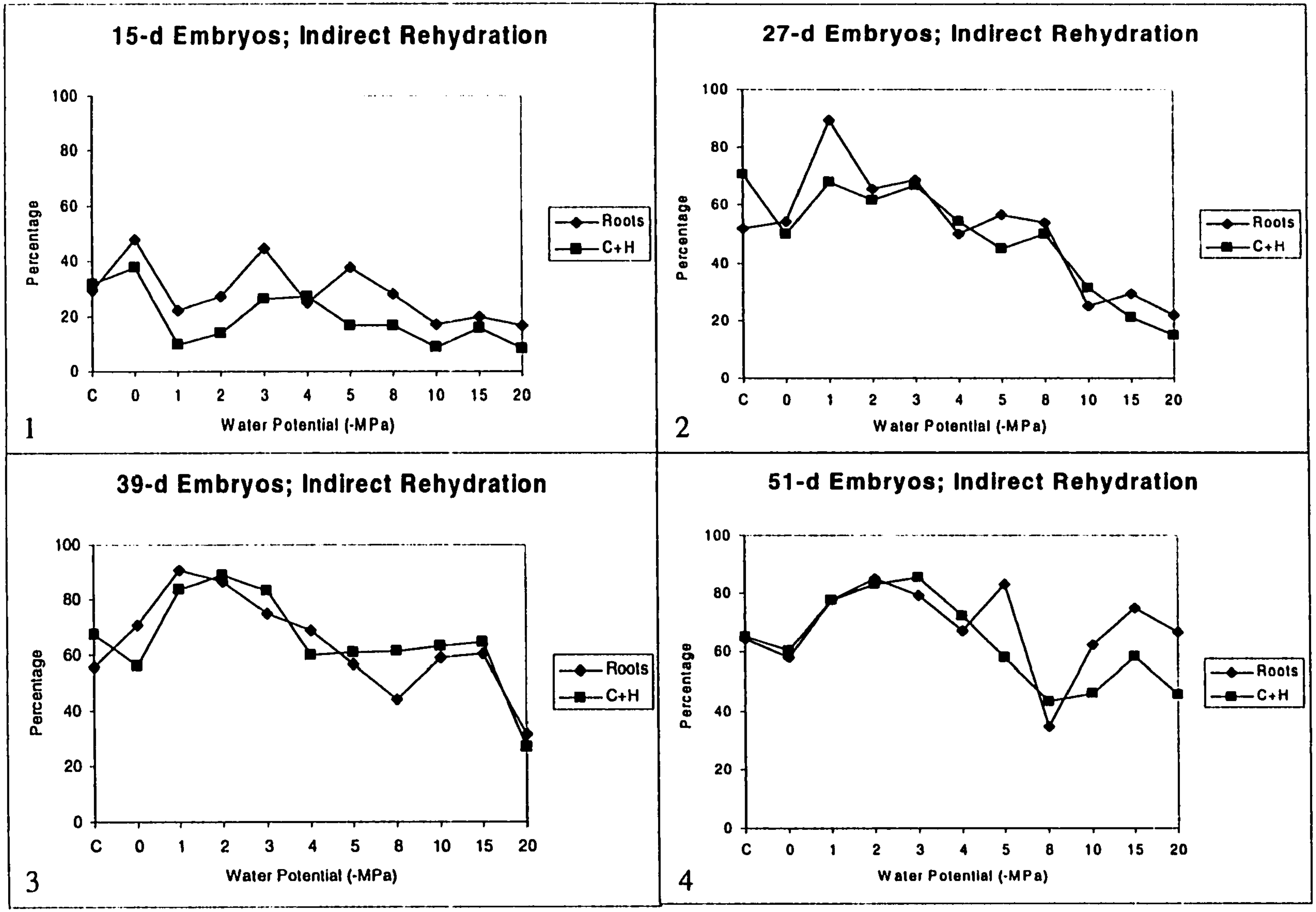
51-d Embryos; Direct Rehydration

Graph 4.4. The percentage of indirectly rehydrated embryos that produced germinants with undamaged roots (Roots) or undamaged cotyledons+hypocotyls (C+H). Embryos at four stages of development were desiccated over water or NaCl solutions with water potentials ranging from 0 to –20 MPa. The control embryos (C) were not desiccated. Each data point is the average of 3 replications.

Four developmental stages of embryos were used:

Graph 4.4.1. 15-d embryos
Graph 4.4.2. 27-d embryos
Graph 4.4.3. 39-d embryos
Graph 4.4.4. 51-d embryos

Graph 4.4


15-d Embryos; Indirect Rehydration
27-d Embryos; Indirect Rehydration
39-d Embryos; Indirect Rehydration
51-d Embryos; Indirect Rehydration
Percentage
0
20
40
60
80
100
C
0
1
2
3
4
5
8
10
15
20
Water Potential (-MPa)
Roots
C+H
1
2
3
4

4.3.2.3 The effect of desiccation on roots and cotyledons+hypocotyls

Undesiccated embryos developed the potential to produce normal roots and cotyledons+hypocotyls at different times during their development. About 30% of the undesiccated (control) 15-d embryos produced germinants with elongating roots and normal cotyledons and hypocotyls (Graph 4.3.1). Seventy percent of the undesiccated 27-d embryos produced normal cotyledons+hypocotyls while only 52% produced elongating roots (Graph 4.3.2). Sixty-seven percent of the 39-d embryos produced normal cotyledons + hypocotyls compared to 56% with elongating roots (Graph 4.3.3). About 65% of the undesiccated embryos produced normal cotyledons+hypocotyls and elongating roots (Graph 4.3.4). In all cases, mild desiccation stimulated root production but had little effect on the cotyledons and hypocotyls (Graphs 4.3.1-4). More severe desiccation damaged both the cotyledons+hypocotyls and roots.

Indirect rehydration also changed the effect of desiccation on the roots and cotyledons+hypocotyls (Graph 4.4.1-4). Mild desiccation followed by indirect rehydration increased the percentage of germinants with both normal cotyledons+hypocotyls and elongating roots.

4.4 Discussion

The percentage of embryos that germinated and became normal seedlings with elongating cotyledons, hypocotyls and roots (i.e. category 1 germinants) was significantly improved with the use of strictly controlled desiccation procedures. The largest improvement occurred as result of the desiccation of the 39-d embryos at -2 MPa followed by indirect rehydration. This increase in the percentage of category 1 germinants produced is important because the production of unusable germinants is time consuming and expensive as these embryos must subsequently be culled.

Researchers have reported improvements in germination in conifers and other species in response to desiccation treatments (Kermode and Bewley 1985a (castor bean zygotic embryos), Tremblay and Tremblay 1991(black spruce)). Roberts et al. (1990) reported that a moisture loss of approximately 3-5% (described as a high humidity treatment) improved germination of interior spruce. Their high humidity treatment was done in a 12-well petri dish at 27°C. Six of the wells were filled with water. The embryos were placed in the remaining wells and the dish was sealed with parafilm. If the seal was tight, the embryos should have taken up water to come to equilibrium with an environment approaching 100% RH and not lost water as claimed by the authors. However, because parafilm is not impermeable to water vapour and does not create a closed system, the embryos are partially desiccated during the process. Moreover, there is no control of exactly how much desiccation occurs because the ambient atmosphere will influence the amount of desiccation. This method is used as the basis for most desiccation procedures, making exact duplication of desiccation treatments impossible (as discussed in Percy 1997). In our method embryos were dried in a closed system to precise, repeatable levels of desiccation that were then correlated to the germination response.

These experiments not only showed that tolerance to desiccation progressively increases as the embryos mature but that the level of desiccation required to improve germination also increases as the embryos mature. The change in desiccation tolerance with maturity was reported by Attree et al. (1995) with white spruce somatic embryos matured with PEG as the osmoticum. They showed that the embryos became tolerant to slow desiccation after 3 weeks of culture on maturation medium, but required 7-8 weeks growth to survive rapid desiccation. Lelu et al. (1995) found a similar increase in desiccation tolerance in hybrid larch somatic embryos in which a minimum of 5 weeks growth on maturation medium was required for the embryos to withstand severe desiccation.

The four developmental stages used in our experiments showed varying degrees of desiccation tolerance. The 15-d embryos were not fully developed. They had just finished a period of rapid cell division (Hakman et al. 1987), had started tissue differentiation, and were very sensitive to desiccation and the method of rehydration. The results can be related to studies of Kermode and Bewley (1985a) who found that immature castor bean embryos were stimulated to germinate with a mild desiccation treatment as long as the seeds had passed from the desiccation-intolerant state to the desiccation-tolerant state (between 20 and 25 d after fertilization). Previous to this transition, the embryos appear to be insensitive to desiccation as a germination cue. Our 15-d embryos were at the desiccation-intolerant state and therefore, as expected, desiccation did not improve germination. The most likely reason for the low percentage of normal germinants from these 15-d embryos is that the embryos had not finished their development phase and still required exogenous growth regulators to continue development.

As the 15-d embryos were not desiccation-tolerant, desiccation over increasingly stronger NaCl solutions followed by direct rehydration on germination medium resulted in increasing damage. The damage initially manifested itself as a disruption of organized growth as shown by an increase in category 8 germinants. When the embryos were desiccated at water potentials lower than -4 MPa, the percentage of dead embryos increased substantially. However, indirect rehydration lessened the damage with the majority of the germinants being classed as category 8 or vitreous but with only a small percentage of dead embryos. The large percentage of dead embryos as a result of direct rehydration must therefore be a result of injury during rehydration (imbibition) and not a result of desiccation.

Attree et al. (1995) correlated the appearance of early cotyledonary stages of embryos with tolerance to slow drying. Our results confirmed his results. The 27-d embryos were at the early cotyledonary stage. The rapid cell division and differentiation phase had finished and the cells were rapidly expanding. The embryos showed a rapid gain in dry weight as the vacuoles were beginning to fill with storage reserves. As expected, the 27-d embryos tolerated mild desiccation. They were starting to become desiccation tolerant and therefore mild desiccation (-1 MPa) followed by direct rehydration slightly increased the percentage of category 1 germinants. Desiccation beyond -1 MPa damaged the embryos, resulted in an increase in the percentage of vitreous and category 8 germinants. More severe desiccation (higher than -5 MPa) significantly increased the percentage of dead embryos. As was seen in the 15-d embryos, desiccation followed by indirect rehydration lessened the damaging effects of desiccation enabling the embryos to withstand more severe desiccation. Desiccation at water potentials of -1, -2 and -3 MPa increased the percentage of category 1 germinants. Some embryos were still killed by desiccation, but only when they had been desiccated below -6 MPa, and in much lower numbers than the directly rehydrated embryos. Again most of the lethal damage is apparently a result of imbibitional shock. Indirect rehydration of the embryos in moist air before placing them on germination medium lessens this damage.

As embryos age, they become even more desiccation tolerant (Attree et al. 1995). The 39-d embryos were in the maturation phase of development during which time dry weight increases and cellular water content decreases as the water is displaced with storage reserves (i.e. carbohydrates, lipids and proteins- See section 2.5.1). Hoekstra et al. (1991) showed that increased endogenous sucrose concentrations protected *Typha latifolia* L. pollen membranes from undergoing phase transitions during desiccation. This may partially explain the increased desiccation tolerance in these direct rehydrated embryos as compared to the more immature embryos. Mild desiccation (-1MPa) followed by direct rehydration significantly increased the percentage of category 1 germinants. This increase in category 1 germinants was mirrored by a decrease in the percentage of vitreous germinants. One of the problems with germinating untreated mature embryos is that the percentage of vitreous germinants increases with embryo maturity. Mild desiccation appears to counteract this anomaly. Desiccation below -1 MPa resulted in a sharp decline in the percentage of category 1 germinants and an increase in the percentage of vitreous, category 8 and dead germinants. The two rehydration methods again made a difference in the response to desiccation, but not to the same extent as with the more immature embryos. Indirect rehydration resulted in a substantial improvement in category 1 germinants when the embryos were desiccated at -1 or -2 MPa (81.2% and 83.9%). Desiccation below -2 MPa resulted in a sharp decline in category 1 germinants. As with direct rehydration, the improvement in germinant quality was mirrored by a substantial decrease in the percentage of vitreous germinants. Damage started to show up as an increase in the percentage of vitreous and category 8 germinants as the embryos were desiccated below -2MPa. There were very few dead embryos.

The 51-d embryos were fully mature and filled with storage reserves (Attree et al. 1995). Again the method of rehydration made a difference in the response of the embryos to desiccation. Desiccation at -1 or -2 MPa followed by direct rehydration slightly improved the percentage of category 1 germinants. This was mirrored by a decrease in the percentage of vitreous embryos. Desiccation past -2 MPa resulted in damage, with the percentage of category 1 germinants decreasing, and the percentage of vitreous and category 8 germinants increasing. Desiccation at -1, -2, or -3 MPa followed by indirect rehydration resulted in a significant improvement in germinant quality (category 1 germinants) mirrored by a decrease in vitreous embryos.

The cotyledons+hypocotyls of directly rehydrated embryos were more sensitive to damage than the roots. This may be a consequence of the positioning of the physical structures in the embryo. The root meristem is within the embryonic root cap (Hakman 1993). The root meristem does not have direct exposure to the rehydration medium. This may help to protect these tissues during direct rehydration on germination medium (further discussed in chapter 7). The cotyledons and hypocotyl are in direct contact with the medium and therefore may suffer more imbibitional damage.

Indirect rehydration lessened the damage suffered by the cotyledons+hypocotyls. The decrease in damage may be explained by the membrane phase transition theory and/or the presence of repair mechanisms. According to the theory, phospholipids in the membranes undergo a phase change when dried, from the liquid crystalline phase to the solid gel phase, and then back again when rehydrated. The membranes are leaky during this transition and leakage of soluble cell components occurs during imbibition (Crowe et al. 1989 a,b,c). Prehydration in moist air before imbibition returns the membranes to the liquid crystalline phase before they come into contact with liquid water in the medium, thus preventing damaging or lethal leakage of cell components and thereby decreasing irreparable damage suffered by the cells. This could explain the decrease in dead embryos as a result of indirect rehydration.

Another possible explanation is that slow rehydration allows proteins, damaged during the desiccation and rehydration process, to be repaired as soon as they are denatured thus preventing an accumulation of degradation products that can be detrimental to cellular metabolism. L-isoaspartyl methyltransferase (MTs) can repair damaged proteins by converting the abnormal L-aspartyl residues to normal l-aspartyl residues. For example, Mudgett and Clarke (1994) found that the enzymatic activity of the MT increased in wheat in response to ABA, desiccation and salt stress and remained active for 24 h post-imbibition and Mudgett et al. (1997) found that MTs were present in 45 species from 23 plant families including the two species of pine that they studied.

Desiccation also had an effect on the embryos. Severe desiccation results in mechanical stress in the cells and causes plasmolysis which may be responsible for the disruption in organized growth seen in a majority of the embryos.

Livingston et al. (1992) showed that larch embryogenic tissue lost a significant amount of weight when suspended over water at a temperature of 20°C, and that little weight loss occurred at a temperature of 5°C. They attributed the large weight loss to respiration. Therefore, for our experiment, to eliminate the confounding effects of weight loss due to respiration, desiccation was carried out at a temperature of 5°C. However, tissues can acclimate to prolonged exposure to cold. Therefore, desiccation of the embryos for 1 week at 5°C may have changed the germination response of the embryos as evidenced by the improvement in germination response in some of the maturity categories of the embryos that had been desiccated over water for 1 week. The effect of cold on germination response will be further examined in Chapter 5.

The exact method by which desiccation improves germination is not known, but there are many theories. For example, Kermode and Bewley (1985a) found that castor bean required desiccation to improve germination. They propose that desiccation may result in a change in genomic activity, switching on those genes required for transcription of mRNA's required for germination (Kermode et al. 1986). Bewley and Black (1994) postulate that desiccation affects the membranes, possibly altering hormone receptors, and increasing or decreasing sensitivity to hormones thereby causing changes in the expression of the genes. ABA levels increase as somatic embryos mature (Label and Lelu 1994). ABA is required for maturation of the embryos and one of its effects is to inhibit germination (Garciarrubio et al. 1997). Kermode et al. (1989) found that desiccation decreases the sensitivity of the embryo to ABA, but Dronne et al. (1997) showed that desiccation actually reduces ABA content in hybrid larch. Dunstan et al. (1998) postulate that the transition in metabolism from the developmental to the germination mode is probably influenced by this decrease in ABA.

Dumont-BeBoux et al. (1996) examined a variety of water relation parameters of embryogenic tissue and mature embryos of western larch, loblolly pine, and white spruce through the generation of water release curves. The SE parameters, including bound water, elastic modulus, turgor loss point and osmotic potential of the three conifer species varied with the developmental stage of the tissue, but were conservative across species. This suggests that a desiccation protocol optimized for one species of conifer should be applicable to other conifer species. Therefore, these desiccation methods, optimized for white spruce, should be applicable to other species.

4.5 Conclusion

A controlled mild desiccation improves germination in white spruce somatic embryos by increasing the percentage of embryos that produce normal seedlings. The level of desiccation required to improve germinant quality increases as the embryos mature. The majority of the damage occurs during rehydration. Therefore, rehydrating the embryos in moist air before putting them on germination medium significantly minimizes the damage. The 15, 27, 39 and 51-d embryos require desiccation at 0, -1, -2, and -3MPa, respectively, to improve germination. The most significant improvement in germinant quality occurs when the mature (39-51 d) embryos are desiccated at -1 to -2 MPa and rehydrated in moist air. This results in approximately 80-84% of the embryos germinating into normal germinants, a significant improvement over the untreated control embryos. Further improvements in maturation protocols and more restrictive selection criteria for choosing embryos to use for germination will probably improve the success rate even more.

Chapter 5

Improving Germinant Quality without a Desiccation Treatment

5.1 Introduction

Chapter 4 confirmed that controlled, mild desiccation significantly improved the quality of the germinants. However, controlled desiccation requires specialized equipment, is labour intensive and takes 1 week of drying time. The number of embryos that can be dried at one time is limited to the capacity of the drying apparatus.

This chapter describes work undertaken to develop an alternative method for improving germinant quality. The desiccation experiments in Chapter 4 were carried out at a temperature of 5°C, a lower temperature than normally used in SE procedures. Exposure of the embryos to this temperature without any desiccation (in an atmosphere approaching 100% RH) resulted in a slight improvement in germinant quality over the untreated control embryos. This suggests that the application of a cold treatment might be worth exploring as an alternative method of improving germination in white spruce somatic embryos. Such a treatment would be operationally easier to apply and would not require the specialized equipment used to desiccate embryos. Plates of embryos could simply be left in an incubator at the appropriate temperature for the required length of time.

Subjecting plants to water stress or cold temperatures may result in similar stress responses (Palta 1990). Therefore, cold stress might be expected to elicit the same improvement in germination response as mild desiccation does. Also, the positive effect of a cold treatment can be seen in seeds of temperate species. Stratification (chilling of seeds in moist conditions for several weeks to months) improves germination in a majority of these species by releasing the seeds from dormancy (Bewley and Black 1994). Stratification is usually efficacious in the range of 1-10°C, although higher temperatures, 12-15°C are used for wheat and *Rumex*. The process requires up to 60 d. Although somatic embryos do not have seed coat imposed dormancy, some of the metabolic changes associated with stratification may also result in improved germination for these somatic embryos.

To test the hypothesis that cold stress can be used to improve germination, white spruce somatic embryos at four stages of development were germinated after exposure to various temperatures, and the quality of the germinants assessed.

5.2 Materials and Methods

5.2.1 Culture Conditions

Embryogenic tissue from cryopreserved white spruce cross MRL-11 times GPC-15 (clone 6x5) was used to produce the somatic embryos. This clone was one of many obtained from explants from seeds of 30 full-sib families from a 6-parent diallel cross (Park et al. 1993). See section 3.2.1 for details. The embryogenic tissue was proliferated on initiation medium followed by two transfers on to maintenance medium before the beginning of the maturation treatment. See section 4.2.1 for details. Approximately 250 plates of embryogenic tissue (eight tissue masses per plate) were required to produce sufficient numbers of embryos. The embryogenic tissue was transferred to maturation medium 1 and grown at 24°C under fluorescent light (24 h 55 μmol $m^{-2}s^{-1}$). Four masses of tissue were placed on each of 500 plates. After 2 weeks, the tissue was subcultured monthly to fresh maturation medium 2. The tissue was either transferred as a mound to the maturation medium or squashed into a mesh screen as described previously in section 3.2. The temperature treatments were then applied to embryos at the four stages of development (15, 21 39 and 51 d) designated as the maturity level.

5.2.2 Treatments

Plates of maturing embryos (on maturation medium 2) were put in darkened incubators at the desired temperatures (1, 5, 10, 20, or 30°C) for 0, 1, 2, 4, or 8 weeks. Tissue was transferred to fresh maturation medium 2 every 4 weeks.

5.2.2.1 Second Temperature Treatment Experiment

An additional 12 plates of 15-d embryos were treated at 30°C for 8 weeks and then treated at a temperature of 1, 5, or 10°C for a period of 0, 1 or 2 weeks.

5.2.3 Germination

For each stage of embryo development (maturity), 100 embryos per temperature and duration combination were placed on germination medium and placed in the growth room at 24°C under continuous light. Germinant quality (non-vitreous quality categories 1-8 (Appendix 2) and vitreous quality categories 1v-7v (Appendix 3) was assessed after 4 weeks on germination medium and the resulting change in germinant quality was measured by the change in the percentage of germinants within the various quality categories.

A maximum of 25 embryos was put on each plate of germination medium with each plate being considered a sample unit. The distribution of embryos in each quality category was calculated as the percentage of total embryos for a plate. There were four replications of 25 embryos for each temperature and duration combination, which were averaged to determine the overall results for each treatment. The experiment was repeated three times, once in August 1995-January 1996, May 1996-October 1996, and in January 1997-July 1997. The results from the three experiments were averaged. The 30°C temperature treatment and the second temperature treatment experiment were added in January 1997-July 1997 only.

5.2.4 Dry Mass/Fresh Mass Ratio

The dry mass/fresh mass ratio of a subset of embryos was determined at the beginning and end of each temperature treatment. Forty embryos were used for each weight measurement. The dry mass was obtained after drying the embryos in an oven at 65°C for 48 h.

5.2.5 Statistical Analysis and Models

The germination responses, expressed as a percentage of germinated embryos in each quality category, were subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + M_i + T_j + D_k + MT_{ij} + MD_{ik} + TD_{jk} + MTD_{ijk} + e_{ijkl}$$

where Y_{ijkl} is the percentage of germinated embryos of the *i*th maturity (developmental stage) subjected to the *j*th temperature for the *k*th length of time; μ is the experimental mean; M_i is the effect of the *i*th maturity of the embryo (15, 27, 39 or 51-d); T_j is the effect of the *j*th temperature (1, 5, 10, 20, 30°C); D_k is the effect of the *k*th duration of the treatment (0, 1, 2, 4 or 8 weeks); MT_{ij} is the interaction effect of the *i*th maturity and the *j*th temperature; MD_{ik} is the interaction effect of the *i*th maturity and the *k*th duration; TD_{jk} is the interaction effect of the *j*th temperature and the *k*th duration; MTD_{ijk} is the interaction effect of the *i*th maturity, the *j*th temperature and the *k*th duration; e_{ijkl} is the random error component. All main effect terms were considered to be fixed effects. The data for each quality category was tested for normality. Computations of analysis of variance were performed for each quality category separately on the transformed data using the SAS GLM procedure. The data for the percentage of germinants in category 1 showed a normal distribution and required no transformation. The percentage data in all remaining categories had the zero values removed to help normalize the data. Data for categories 3, 4, 8, 1v, 3v, 6v, 7v were transformed using the formula log (x+1), where x is the percentage of germinated embryos in the quality category. The percentage data for categories 2 and 2v were transformed using the formula arcsin (sqrt (x/100)). Data for categories 5 and 5v were transformed using the formula sqrt(x). Data for categories 6 and 7 were transformed using the formula 1/(x+1). The means data are based on the actual percentages. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

By summing the percentage of germinants in all vitreous categories (1v-3v and 5v-7v) the change in the total percentage of vitreous germinants was examined. Prior to statistical analysis using the SAS GLM procedure, the non-zero vitreous data was normalized using the formula sqrt(x).

The effect of the temperature treatments on the overall percentage of germinants with elongating roots was examined by summing the percentage of germinants in those categories that involve roots (1, 1v, 2, 2v, 3, 3v, 4). The data showed a normal distribution and required no transformation. The data was analyzed using the SAS GLM procedure.

The data was graphed using Microsoft Excel.

5.3 Results

5.3.1 Embryo Development

The five temperature treatments had a significant effect on embryo development as shown in Figs. 5.1- 5 4. Temperature treatments of 1 and 5°C halted any further morphological development. The embryos were arrested at the developmental stage at which the temperature treatment was applied (15, 27, 39 and 51-d). The temperature treatments of 10, 20 and 30°C allowed embryo development to continue. At 10°C some of the immature 15-d embryos matured into embryos that had cotyledon deficiencies ranging from one to all cotyledons being absent (Fig. 5.1.4), but those that did mature properly were well-formed and closely resembled zygotic embryos. The more mature 27, 39 and 51-d embryos developed normally when subjected to a temperature of 10°C (Figs. 5.2.4, 5.3.4, 5.4.4). All embryos developed normally when grown at a temperature of 20 or 30°C. However, after 4-8 weeks at a temperature of 10, 20 or 30°C, the majority of the embryos (especially the 39 and 51-d embryos) became swollen and vitreous or germinated precociously (Figs. 5.3.4-6 and 5.4.4-6).

5.3.2 Dry Mass/Fresh Mass Ratio

The change in the average fresh mass as a result of the embryo maturity and the temperature treatments (Table 5.1) correlates with the visual assessments of embryo development discussed in section 5.3.1. The embryos exposed to a temperature of 1 and 5°C showed little increase in average fresh mass/embryo while those exposed to a temperature of 10 and 20°C showed a substantial mass gain with increasing length of

Figure 5.1. Morphological development of the 15-d embryos

Figure 5.1.1. Before the temperature treatments. Magnification is [21x]

After 8 weeks of exposure to temperatures of:

Figure 5.1.2. 1°C. Magnification is [8.5x]

Figure 5.1.3. 5°C. Magnification is [10x]

Figure 5.1.4. 10°C. Magnification is [11x]

Figure 5.1.5. 20°C. Magnification is [12x]

Figure 5.1.6. 30°C. Magnification is [12x]

Figure 5.1

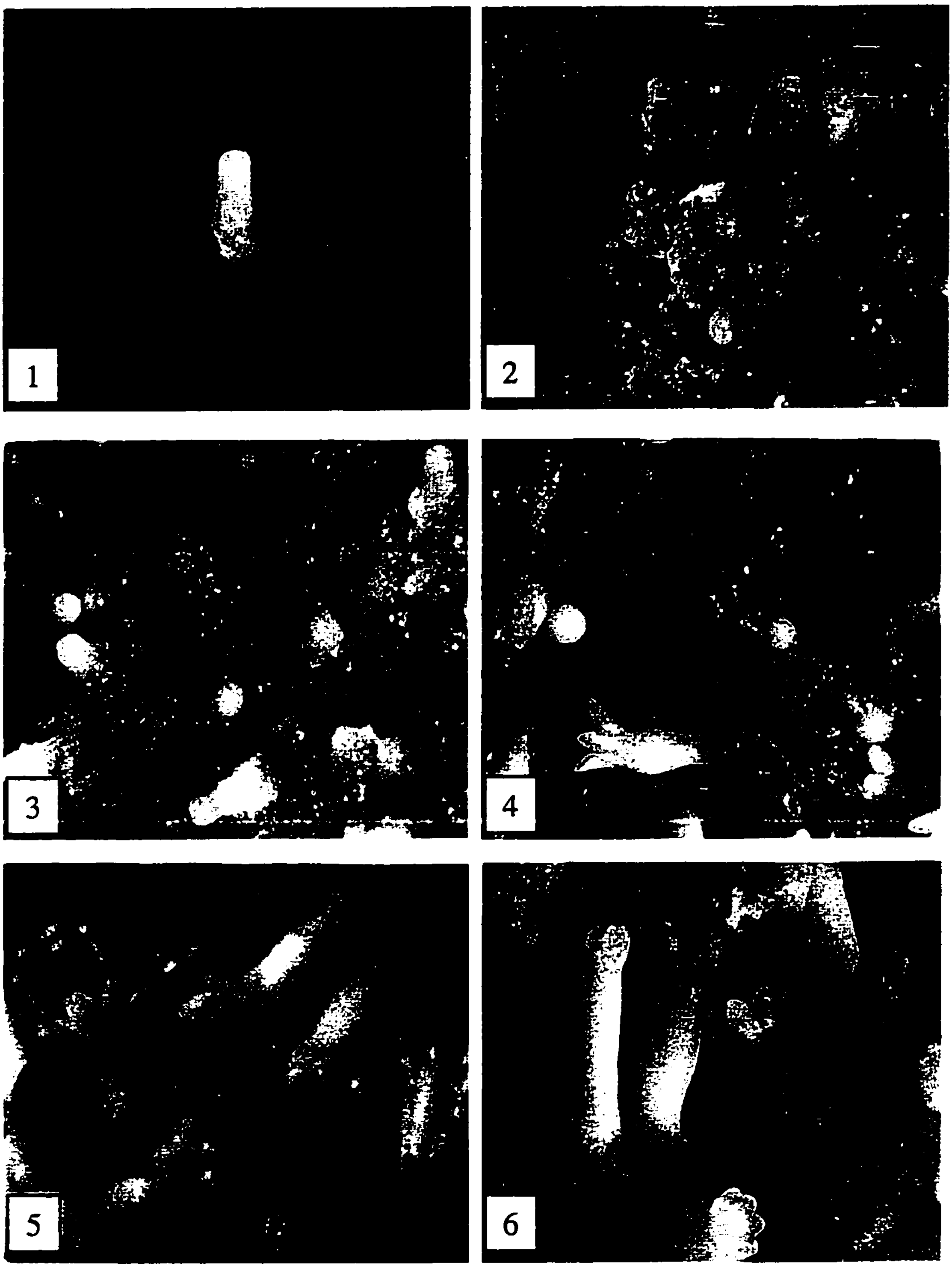


Figure 5.2. Morphological development of the 27-d embryos

Figure 5.2.1. Before the temperature treatments. Magnification is [8x]

After 8 weeks of exposure to temperatures of:

Figure 5.2.2. 1°C. Magnification is [7x]

Figure 5.2.3. 5°C. Magnification is [8x]

Figure 5.2.4. 10°C. Magnification is [8x]

Figure 5.2.5. 20°C. Magnification is [8x]

Figure 5.2.6. 30°C. Magnification is [9x]

Figure 5.2.

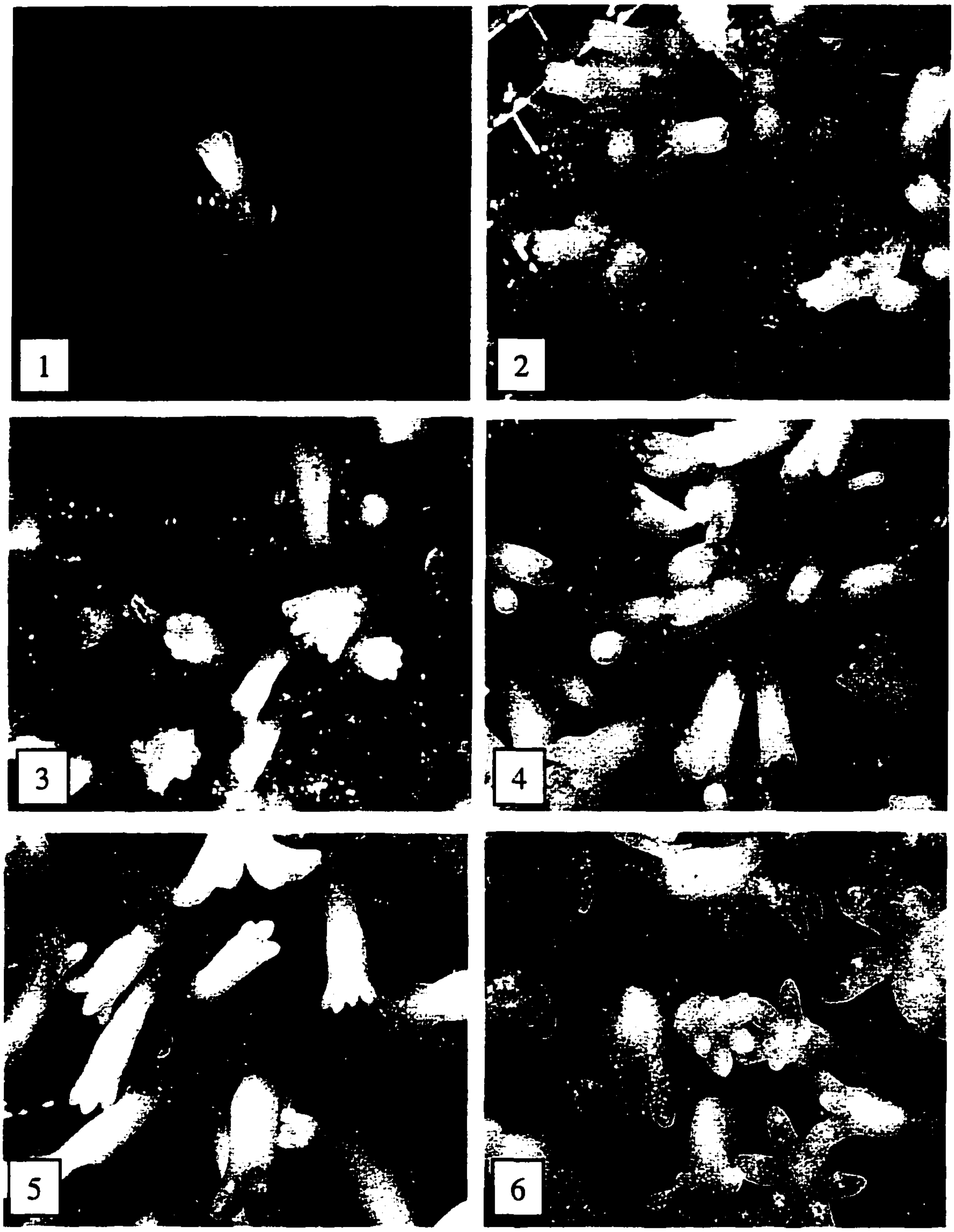


Figure 5.3. Morphological development of the 39-d embryos

Figure 5.3.1. Before the temperature treatments. Magnification is [15x]

After 8 weeks of exposure to temperatures of:

Figure 5.3.2. 1°C. Magnification is [4x]

Figure 5.3.3. 5°C. Magnification is [5.5x]

Figure 5.3.4. 10°C. Magnification is [5.5x]

Figure 5.3.5. 20°C. Magnification is [5.5x]

Figure 5.3.6. 30°C. Magnification is [5.5x]

Figure 5.3

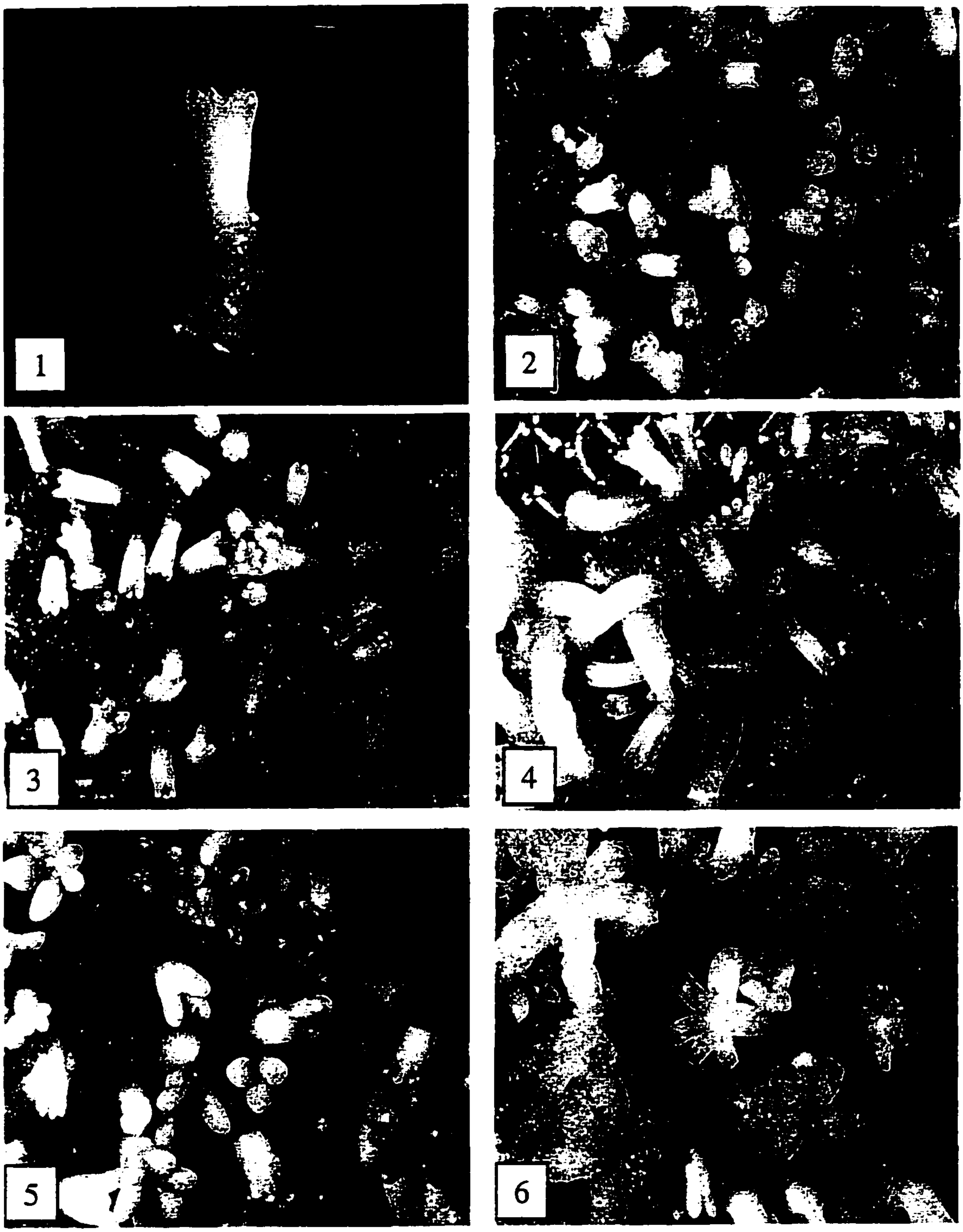


Figure 5.4. Morphological development of the 51-d embryos

Figure 5.4.1. Before the temperature treatments. Magnification is [12x]

After 8 weeks of exposure to temperatures of:

Figure 5.4.2. 1°C. Magnification is [8x]

Figure 5.4.3. 5°C. Magnification is [9x]

Figure 5.4.4. 10°C. Magnification is [10x]

Figure 5.4.5. 20°C. Magnification is [9x]

Figure 5.4.6. 30°C. Magnification is [8x]

Figure 5.4.

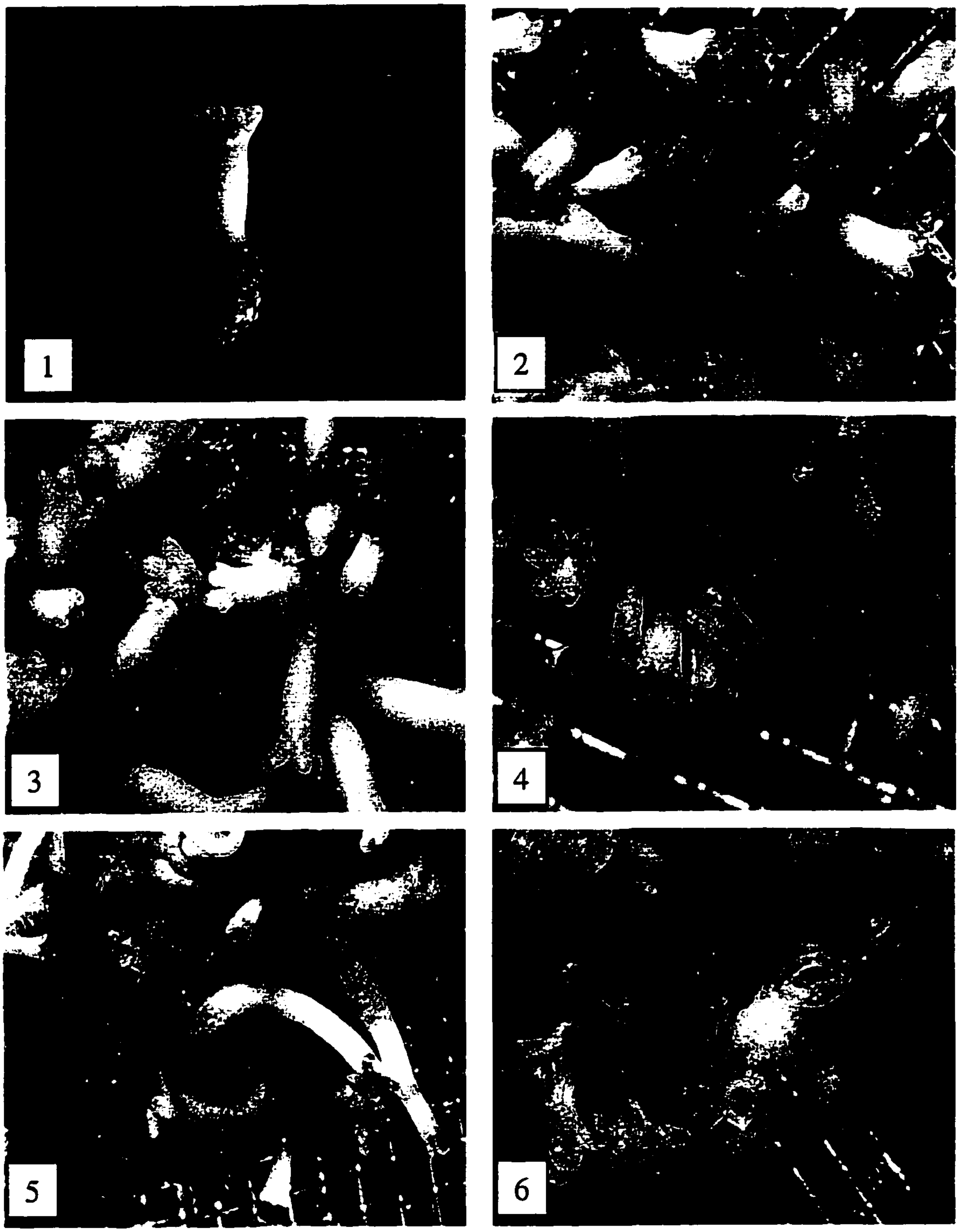


Table 5.1. The fresh mass (in mg) and dry mass/fresh mass ratio (dm/fm) of the four developmental stages of the embryos after 0, 1, 2, 4, and 8 weeks of storage at a temperature of 1, 5, 10 and 20°C. Each weight is the average of 40 embryos when available.

Table 5.1

	1°C		5°C		10°C		20°C	
15-d embryos	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm
0 Weeks	1.6	0.11	1.6	0.11	1.6	0.11	1.6	0.11
1 Week	1.1	0.23			1.3	0.23	1.7	0.24
2 Weeks	1.1	0.16	1.9	0.14	1.6	0.16	3.3	0.22
4 Weeks	2.1	0.15	1.9	0.19	4.6	0.22	5.8	0.19
8 Weeks	2.7	0.07	3.6	0.11	21.5	0.13	22.4	0.27
27-d embryos	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm
0 Weeks	5.5	0.17	5.5	0.17	5.5	0.17	5.5	0.17
1 Week	4.1	0.20	4.1	0.19	4.1	0.18	5.0	0.20
2 Weeks	6.4	0.20	4.0	0.20	6.2	0.18	12.9	0.14
4 Weeks	6.7	0.20	1.9	0.26	13.1	0.25	17.7	0.24
8 Weeks	2.8	0.23	4.2	0.19	5.8	0.30	20.8	0.24
39-d embryos	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm
0 Weeks	8.6	0.12	8.6	0.12	8.6	0.12	8.6	0.12
1 Week	8.1	0.13	12.7	0.18	14.4	0.17	10.7	0.14
2 Weeks	6.4	0.22	10.9	0.23	15.3	0.22	14.8	0.25
4 Weeks	13.3	0.23	12.5	0.26	16.9	0.22	22.8	0.22
8 Weeks	14.8	0.27	13.1	0.30	25.6	0.29	26.5	0.33
51-d embryos	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm	Fresh	dm/fm
0 Weeks	10.4	0.30	10.4	0.30	10.4	0.30	10.4	0.30
1 Week	12.5	0.21	10.4	0.21	15.5	0.19	20.0	0.16
2 Weeks	13.3	0.24	10.5	0.24	12.6	0.25	18.0	0.24
4 Weeks	15.6	0.28	18.9	0.27	14.8	0.34	10.5	0.31
8 Weeks	18.2	0.26	9.8	0.35	13.9	0.22	24.3	0.23

exposure to these temperatures. Dry mass/fresh mass ratio (dm/fm) increased with the maturity of the embryo and also as a result of all temperature treatments. There was no clear pattern to changes in the dm/fm for the temperature treated 15-d embryos, but the largest increase in dry weight occurred at 20°C. The dm/fm increased during all temperature treatments in the 27-d embryos, with the largest gain in dry matter accumulation occurring when the embryos were treated for 8 weeks at a temperature of 10°C. The dm/fm ratio increased with increasing length of exposure to all temperatures for the 39-d embryos. Exposure to a temperature of 1°C resulted in the smallest dry matter accumulation. With the 51-d embryos, there was an increase in dry matter accumulation with increasing length of exposure to a temperature of 5°C. The dm/fm also increased with 1-4 weeks of exposure to a temperature of 10 or 20°C, but there was a large decrease with 8 weeks of exposure.

5.3.3 Germination

The analysis of variance showed that the distribution of embryos in the different quality categories changed with the maturity of the embryos and also with the temperature and duration of the treatments.

The maturity (i.e. developmental stage) of the embryo had a significant effect on the percentage of germinants in five of the 14 individual quality categories. The effect was significant at the $p \leq 0.001\%$ level for quality categories 1, 5 and 8 and for the combined category of roots (Table 5.2).

The temperature also had a significant effect on the percentage of germinants in six of the 14 individual quality categories. The effect was significant at the $p \leq 0.001\%$ level on categories 1, 5v, 7v, and 8 and the combined categories of roots and vitreous (Table 5.2).

Table 5.2. Analysis of variance summary table for the effect of embryo maturity, temperature treatment and duration of the temperature treatment on the subsequent percentage of germinants in each germinant quality category. The change in the percentage of germinants in each quality category was analyzed separately. F-values are significant at the 0.05(*), 0.01(**) and 0.001(***) level.

Table 5.2

Germinant Quality Category

	Category 1		Category 1v		Category 2		Category 2v		Category 3		Category 3v		Category 4	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Maturity (M)	3	53.94***	3	4.82**	3	1.75	2	0.43	3	0.95	3	5.01**	3	4.20**
Temperature (T)	4	18.73***	4	4.00**	4	0.99	4	0.46	4	1.17	4	2.00	4	1.50
Duration (D)	4	7.18***	4	5.52***	4	0.96	3	0.11	4	5.38**	4	1.20	4	1.84
M x T	12	6.88***	12	2.60**	6	0.42	2	1.00	11	1.39	10	1.12	5	0.90
M x D	12	9.84***	12	2.09*	5	3.14	1	0.14	11	2.59*	7	0.92	4	0.49
T x D	16	8.53***	16	2.49**	9	0.48	1	0.11	14	1.53	11	0.80	9	0.72
M x T x D	48	3.62***	46	0.88	3	0.27	0		6	0.91	9	0.77	4	1.22
Error	739		285		8		9		26		30		47	
Corrected total	993		488		49		23		96		103		101	

Germinant Quality Category

	Category 5		Category 5v		Category 6		Category 6v		Category 7		Category 7v		Category 8	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Maturity (M)	3	41.93***	3	1.47	3	3.29	3	0.81	3	3.32	3	0.84	3	147.26***
Temperature (T)	4	2.90*	4	12.97***	4	2.21	4	0.55	4	1.34	4	7.15***	4	6.12***
Duration (D)	4	26.84***	4	10.00***	4	9.22	4	3.55	4	0.38	4	1.95	4	1.08
M x T	12	4.83***	12	1.35	2	5.52	6	1.14	11	0.27	12	1.51	10	1.27
M x D	12	5.11***	12	3.33***	0		2	0.82	11	0.67	10	0.66	11	2.14*
T x D	16	2.54***	16	5.03***	0		6	0.75	10	0.71	15	2.15*	11	2.53**
M x T x D	48	2.2***	48	2.87***	0		0		6	0.42	20	0.78	17	2.21**
Error	608		500		1		7		19		113		113	
Corrected total	856		723		16		35		71		232		217	

Combined Categories

	Roots		Vitreous			Roots		Vitreous	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**Source**			**DF**	**F-Value**
Maturity (M)	3	31.11***	3	1.46	T x D	16	9.50***	16	6.68***
Temperature (T)	4	23.81***	4	19.50***	M x T x D	48	3.55***	48	2.66***
Duration (D)	4	12.26***	4	21.21***	Error	736		586	
M x T	12	4.06***	12	2.23*	Corrected total	989		817	
M x D	3	5.72***	12	4.56***					

The duration of the treatment significantly influenced five of the 14 individual quality categories. The effect was significant at the $p \leq 0.001\%$ level on the individual categories 1, 1v and 5 and on the combined categories of roots and vitreous (Table 5.2).

The interaction effect of the three main factors also had a significant influence on the distribution of germinants in the various quality categories, especially on the individual categories 1, 1v, 5, 5v and 8 and on the combined categories of roots and vitreous.

5.3.3.1 Maturity

The percentage of category 1 germinants (i.e. normal germinants with elongating cotyledons, hypocotyls and roots), germinants with elongating roots, and vitreous germinants increased with maturity as shown by the Duncan's Test results in Table 5.3.1. The percentage of category 8 germinants (unorganized growth) decreased with maturity. The percentage of category 5 germinants (normal cotyledons and hypocotyls but no roots) increased from 13.4% for the 15-d embryos to 25.4% for the 27-d embryos and then decreased with maturity.

5.3.3.2 Temperature

A temperature treatment of 10 and 5°C had a positive effect on germination, improving the percentage of category 1 (i.e. normal) and rooted germinants and decreasing the percentage of vitreous germinants (Table 5.3.2). The percentage of vitreous germinants produced increased when the embryos were exposed to temperature of 1, 20 or 30°C.

5.3.3.3 Duration

For the production of the maximum percentage of category 1 germinants, the temperature treatment had to be applied for a minimum of 2 weeks (Table 5.4). Increasing duration of the temperature treatments had a positive influence on the percentage of germinants with elongating roots. The percentage of vitreous germinants decreased with 1 and 2 weeks exposure to the temperature treatments, but then increased with longer-term exposure.

Table 5.3.1. Duncan's Multiple Range Test for the effect of embryo maturity on the subsequent percentage of germinants in each germinant quality category. The percentage data for each quality category were analyzed separately. Data are means averaged over all treatment temperatures and durations. Means within the same quality category with different letters are significantly different ($p=0.05$) from each other.

Table 5.3.2. Duncan's Multiple Range Test for the effect of the treatment temperature on the subsequent percentage of germinants in each germinant quality category. The percentage data for each quality category were analyzed separately. Data are means averaged over the four stages of embryo maturity and all treatment durations. Means within the same quality category with different letters are significantly different ($p=0.05$) from each other.

Table 5.3.1

	Maturity			
Quality Category	15 Day	27 Day	39 Day	51 Day
1	31.1c	46.3b	50.3a	51.5a
1v	5.0a	5.2a	4.4b	6.0ab
2	0.4a	0.5a	0.1a	0.0a
2v	0.6a	0.2a	0.1a	0.0a
3	0.6a	0.3a	0.4a	0.9a
3v	0.6b	0.5b	1.3b	0.8a
4	3.6a	0.7a	0.0a	0.0a
5	13.4d	25.4a	22.7b	16.7c
5v	15.0a	14.8a	16.8a	17.0a
6	0.2a	0.1a	0.0a	0.1a
6v	0.6a	0.4a	0.0a	0.0a
7	0.8a	0.9ab	0.7ab	0.4b
7v	1.7b	1.4a	3.0a	6.0a
8	21.2a	3.3b	0.3c	0.7c
Combined Categories				
Roots	47.1c	53.9b	56.4b	59.2a
Vitreous	23.6b	22.4ab	25.5b	29.7a

Table 5.3.2

	Temperature				
Quality Category	1°C	5°C	10°C	20°C	30°C
1	45.0b	46.0b	52.2a	43.2b	46.2b
1v	5.9a	5.3a	5.0a	5.0a	3.7b
2	0.3a	0.4a	0.3a	0.2a	0.1a
2v	0.1a	0.3a	0.3a	0.1a	0.2a
3	0.4a	0.5a	0.6a	0.6a	0.7a
3v	0.6ab	0.6ab	0.8b	1.5b	0.1a
4	1.4a	1.6a	0.9a	0.4a	0.1a
5	19.5ab	19.7ab	19.2b	21.1a	20.4b
5v	16.1b	13.6b	12.7b	17.8a	24.7a
6	0.0a	0.2a	0.1a	0.2a	0.0a
6v	0.2a	0.4a	0.2a	0.2a	0.1a
7	0.7a	0.6a	0.7a	0.8a	0.4a
7v	3.0b	2.3b	1.7b	5.5a	2.8b
8	6.8ab	8.5a	5.3ab	3.8bc	0.5c
Combined Categories					
Roots	53.8bc	54.8b	60.1a	50.8cd	51.4d
Vitreous	25.8bc	22.5c	20.7d	30.0a	31.6ab

Table 5.4. Duncan's Multiple Range Test for the effect of treatment duration on the subsequent percentage of germinants in each germinant quality category. The percentage data for each quality category were analyzed separately. Data are means averaged over the four stages of embryo maturity and all treatment temperatures. Means within the same quality category with different letters are significantly different (p=0.05) from each other.

Table 5.4.

	Duration				
Quality Category	0 Weeks	1 Week	2 Weeks	4 Weeks	8 Weeks
1	45.2bc	46.2bc	50.1a	48.1ab	43.2c
1v	3.2b	3.3b	3.5b	7.6a	9.0a
2	0.3a	0.1a	0.2a	0.3a	0.6a
2v	0.4a	0.1a	0.0a	0.2a	0.4a
3	0.6c	0.7bc	0.5a	0.5ab	0.4ab
3v	0.4a	0.2a	0.9a	0.9a	1.8a
4	1.0b	0.7ab	1.0ab	0.6ab	1.5a
5	24.2b	25.7a	20.0c	17.1c	10.8d
5v	17.0a	12.7b	12.5b	16.3b	20.9a
6	0.1b	0.2b	0.1b	0.1a	0.1b
6v	0.1a	0.4a	0.3a	0.4a	0.7a
7	1.1a	1.1a	0.4a	0.4a	0.4a
7v	1.3b	2.5ab	3.3a	2.9ab	6.2a
8	5.1c	6.4b	7.3a	4.8b	4.8a
Combined Categories					
Roots	51.4b	51.3b	53.2a	58.1a	56.8a
Vitreous	22.4b	19.0c	20.4c	28.4b	38.3a

5.3.3.4 Maturity x Temperature x Duration Interactions

5.3.3.4.1 Effect on the Percentage of Normal Germinants

The temperature treatments had variable effects on the percentage of normal germinants produced by the different developmental stages of the embryos (Graphs 5.1.1-4). The largest improvement in the production of normal germinants was seen with the application of temperature treatments to the 15-d embryos.

A temperature treatment of 10, 20 and 30°C increased the percentage of normal (category 1) germinants produced by the 15-d embryos to a maximum of 80.5, 56.8 and 73.0%. The improvement was seen after 4 weeks of exposure to a temperature of 10°C, after 2 weeks of exposure to a temperature of 20°C, and after 1 week of exposure to a temperature of 30°C. However, 8 weeks of exposure to any of these three temperatures decreased the percentage of normal germinants. Temperature treatments of 1 or 5°C did not affect the percentage of category 1 germinants produced.

The percentage of normal germinants increased after 2 weeks of exposure of the 27-d embryos to a temperature of 5 °C and after 4 weeks at a temperature of 10°C. Longer-term exposure to any of the temperatures decreased the percentage of normal germinants.

Four to eight weeks of exposure of the 39-d embryos to temperatures of 5 and 10°C improved the percentage of normal germinants.

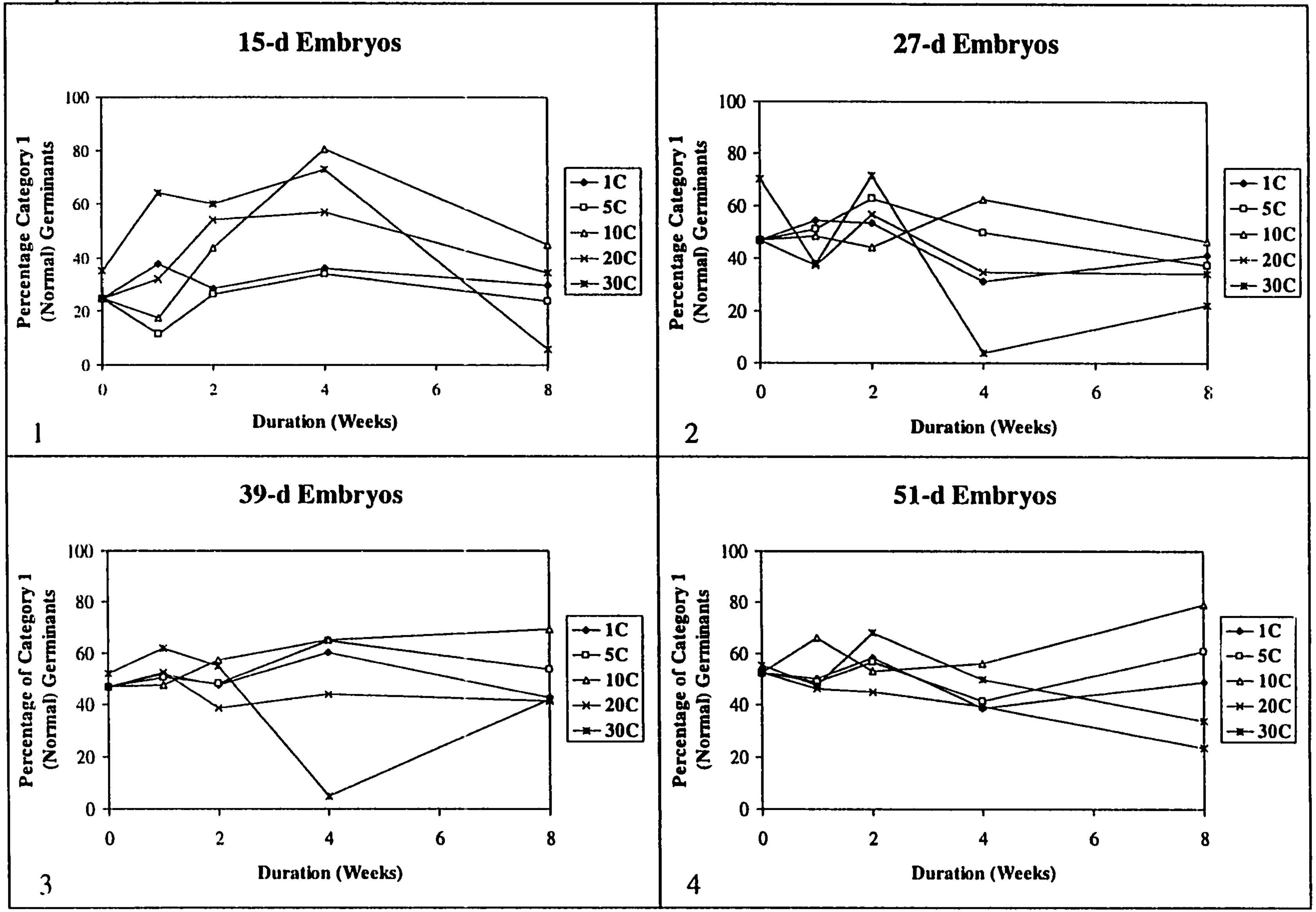
The 51-d embryos required a full 8 weeks at 10°C to increase the percentage of normal germinants produced to a maximum of 79.0% while exposure to 5°C maintained or slightly improved the percentage of category 1 germinants produced. As with all developmental stages of embryos, long-term exposure to a temperature of 1, 20 or 30°C decreased the percentage of normal germinants produced.

Graph 5.1. The percentage of temperature treated embryos that produced category 1 (normal) germinants. Embryos at four stages of development were subjected to five temperatures for varying lengths of time. Each data point is the average of 4 replicates. The experiment was repeated 3 times and the results averaged.

Four developmental stages of embryos were used:

Graph 5.1.1. 15-d embryos
Graph 5.1.2. 27-d embryos
Graph 5.1.3. 39-d embryos
Graph 5.1.4. 51-d embryos

Graph 5.1.


15-d Embryos
Percentage Category 1 (Normal) Germinants
Duration (Weeks)
0
2
4
6
8
0
20
40
60
80
100
1C
5C
10C
20C
30C
1
27-d Embryos
Percentage Category 1 (Normal) Germinants
Duration (Weeks)
2
39-d Embryos
Percentage of Category 1 (Normal) Germinants
Duration (Weeks)
3
51-d Embryos
Percentage of Category 1 (Normal) Germinants
Duration (Weeks)
4

5.3.3.4.2 Effect on the Percentage of Germinants in Other Quality Categories

The developmental stage of the embryo also influenced the effect the temperature treatments had on the percentage of germinants in the other quality categories (Graphs 5.2 - 5.5).

A temperature of 10 and 30°C significantly increased the percentage of germinants with elongating roots produced by the 15-d embryos (Graphs 5.2.3 and 5.2.5). The percentage of vitreous germinants significantly increased with 8 weeks of exposure to a temperature of 20 and 30°C (Graphs 5.2.4 and 5.2.5).

A temperature treatment of 10°C increased the percentage of germinants with elongating roots produced by the 27-d embryos (Graph 5.3.3). The percentage of vitreous germinants increased with length of exposure to the temperature treatments (Graphs 5.3.1-5.3.5) with a temperature of 20 and 30°C producing the most vitreous germinants.

Exposure of the 39-d embryos to a temperature of 1, 5, or 10°C (Graphs 5.4.1-3) increased the percentage of germinants with elongating roots with the 10°C treated embryos yielding the best response. The percentage of vitreous germinants increased with long-term exposure to a temperature of 1, 20 and 30°C (Graphs 5.4.1, 5.4.4, 5.4.5). A temperature treatment of 5 and 10°C maintained, or decreased the level of vitreousness.

For the mature 51-d embryos, a temperature treatment of 1°C (Graph 5.5.1) maintained the level of rooted embryos while temperatures of 5 and 10°C (Graphs 5.5.2, 5.5.3) increased the percentage of rooted germinants produced. Long-term exposure to a temperature of 20 or 30°C (Graphs 5.5.4, 5.5.5) decreased the percentage of rooted germinants but increased the percentage of vitreous germinants.

5.3.4 Second Temperature Treatment Experiment

Exposure of 15-d embryos, matured for an additional 8 weeks at 30°C, to a second temperature treatment of 1, 5, or 10°C improved germinant quality. The embryos without

Graph 5.2. The percentage of temperature treated 15-d embryos that produced category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots). The embryos were subjected to five temperatures for varying lengths of time. Each data point is the average of 4 replicates. The experiment was repeated 3 times and the results averaged. The 30°C treatment was not repeated.

The following temperatures were used:

Graph 5.2.1. 1°C

Graph 5.2.2. 5°C

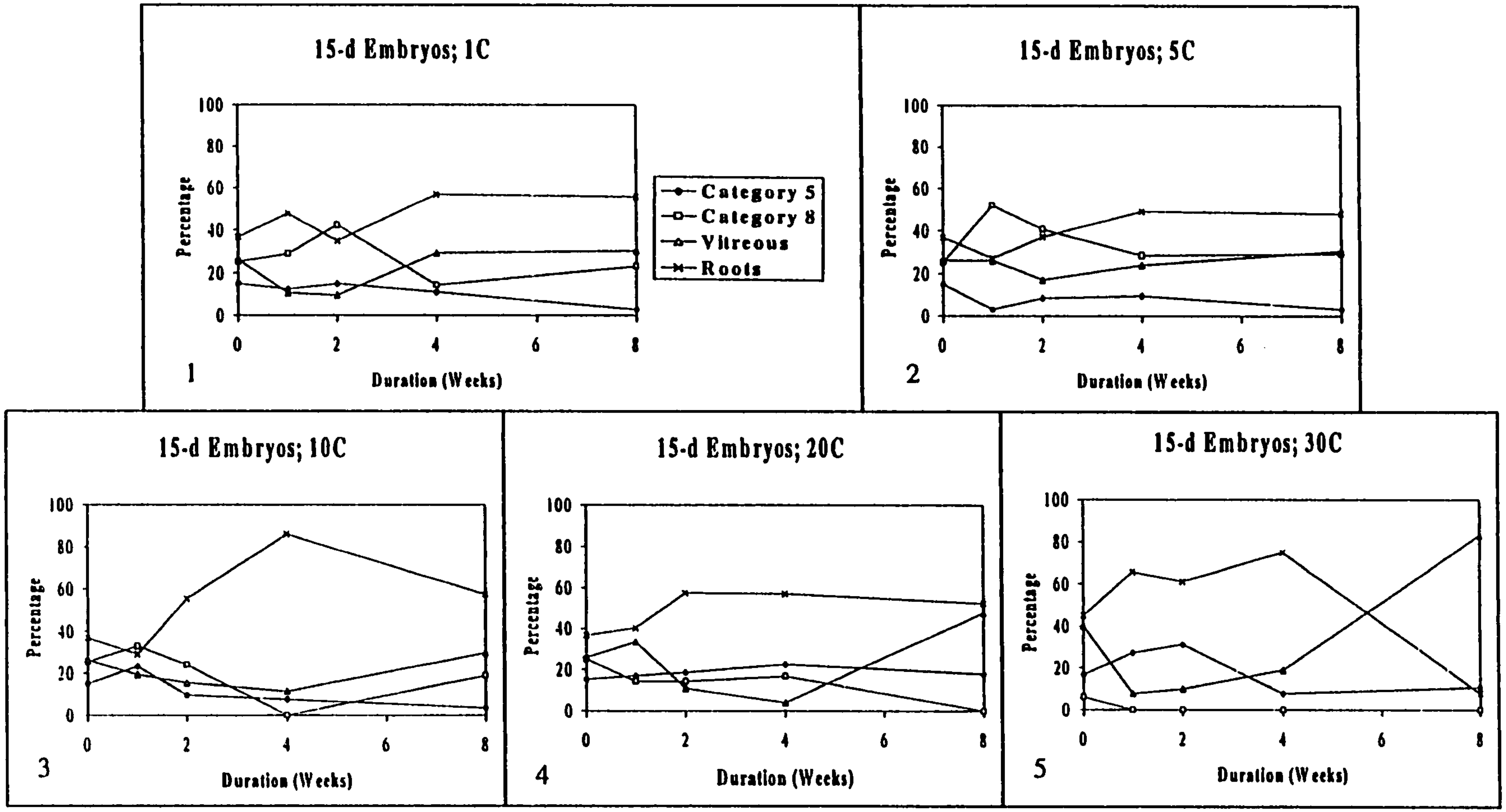
Graph 5.2.3. 10°C

Graph 5.2.4. 20°C

Graph 5.2.5. 30°C

See Graph 5.2.1 for a description of the symbols.

Graph 5.2


15-d Embryos; 1C
15-d Embryos; 5C
15-d Embryos; 10C
15-d Embryos; 20C
15-d Embryos; 30C
Percentage
0
20
40
60
80
100
Duration (Weeks)
0
2
4
6
8
Category 5
Category 8
Vitreous
Roots
1
2
3
4
5

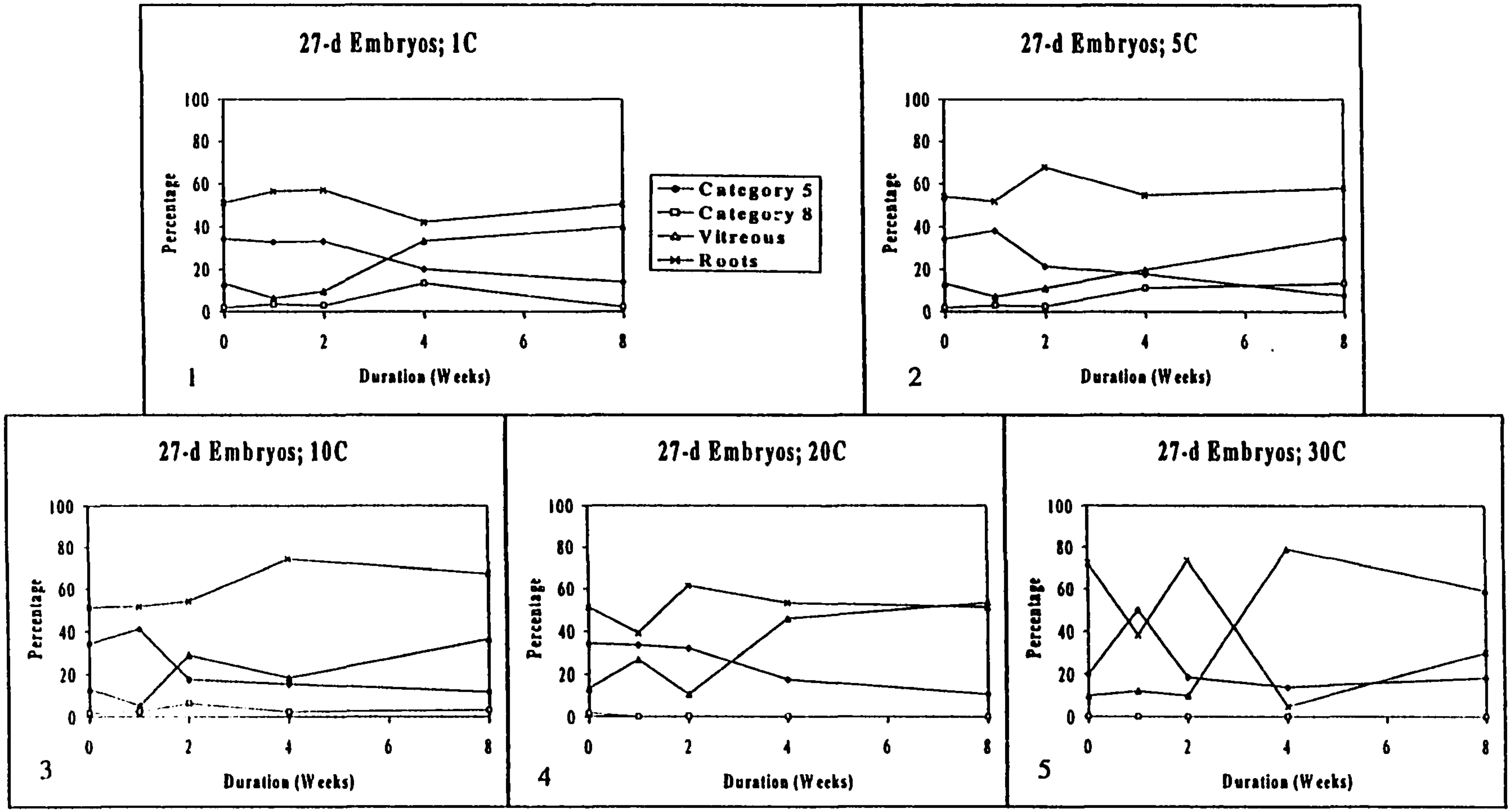
Graph 5.3. The percentage of temperature treated 27-d embryos that produced category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots). The embryos were subjected to five temperatures for varying lengths of time. Each data point is the average of 4 replicates. The experiment was repeated 3 times and the results averaged. The 30°C treatment was not repeated.

The following temperatures were used:

Graph 5.3.1. 1°C
Graph 5.3.2. 5°C
Graph 5.3.3. 10°C
Graph 5.3.4. 20°C
Graph 5.3.5. 30°C

See Graph 5.3.1 for a description of the symbols.

Graph 5.3


27-d Embryos; 1C
27-d Embryos; 5C
27-d Embryos; 10C
27-d Embryos; 20C
27-d Embryos; 30C
Percentage
100
80
60
40
20
0
Duration (Weeks)
0
2
4
6
8
Category 5
Category 8
Vitreous
Roots
1
2
3
4
5

Graph 5.4. The percentage of temperature treated 39-d embryos that produced category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots). The embryos were subjected to five temperatures for varying lengths of time. Each data point is the average of 4 replicates. The experiment was repeated 3 times and the results averaged. The 30°C treatment was not repeated.

The following temperatures were used:

Graph 5.4.1. 1°C

Graph 5.4.2. 5°C

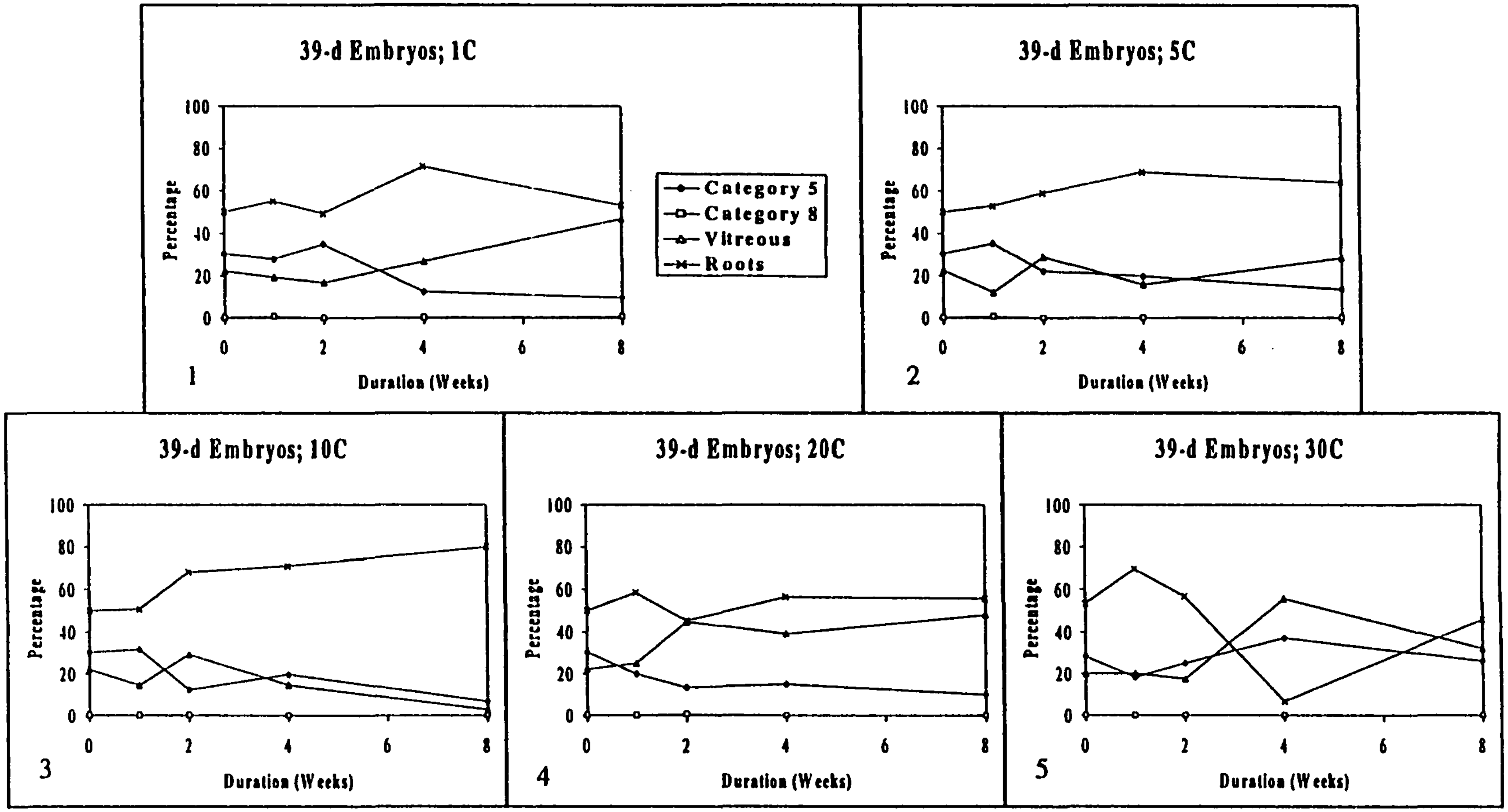
Graph 5.4.3. 10°C

Graph 5.4.4. 20°C

Graph 5.4.5. 30°C

See Graph 5.4.1 for a description of the symbols.

Graph 5.4


39-d Embryos; 1C
39-d Embryos; 5C
39-d Embryos; 10C
39-d Embryos; 20C
39-d Embryos; 30C
Percentage
0
20
40
60
80
100
Duration (Weeks)
0
2
4
6
8
Category 5
Category 8
Vitreous
Roots
1
2
3
4
5

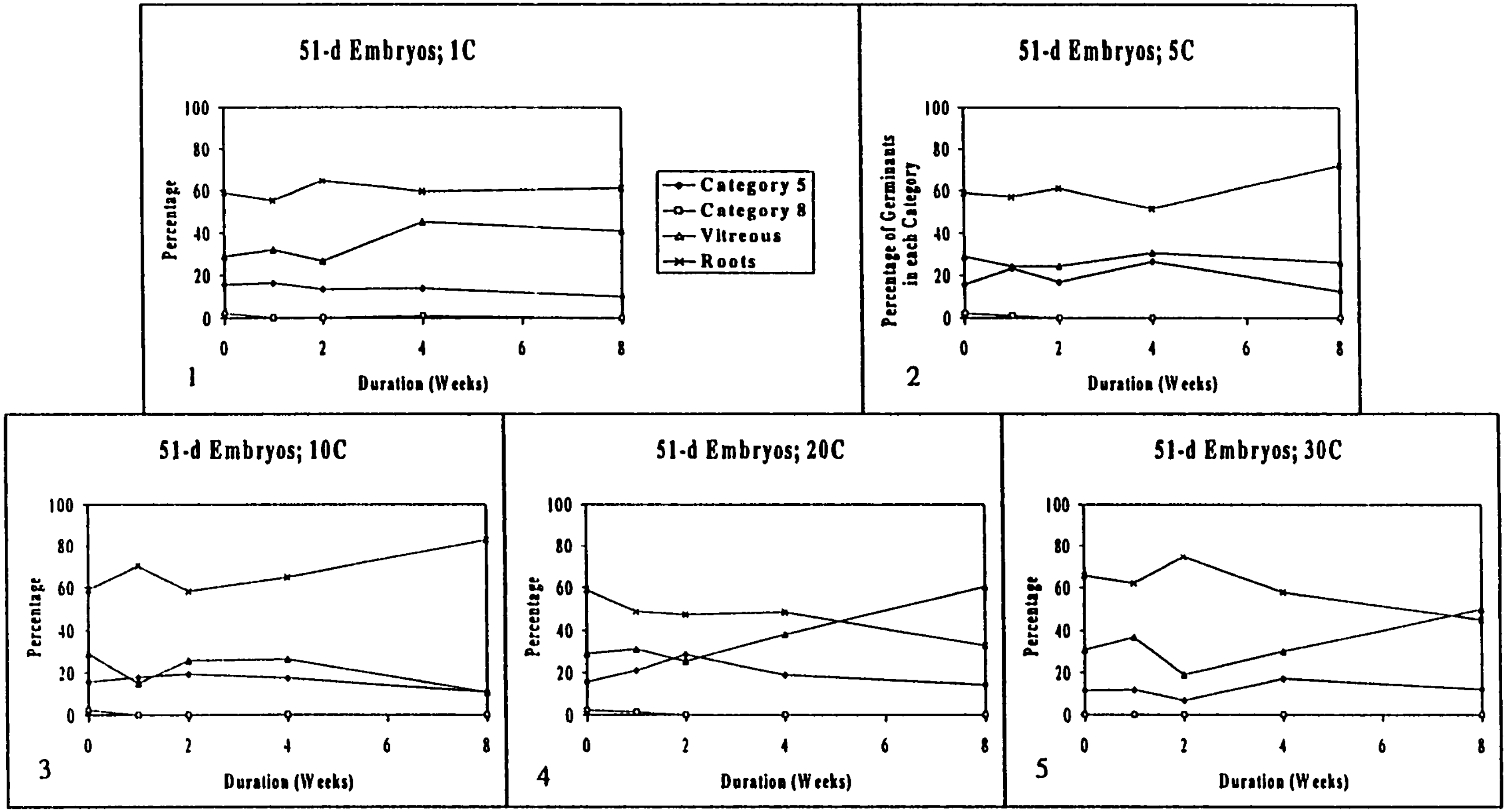
Graph 5.5. The percentage of temperature treated 51-d embryos that produced category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots). The embryos were subjected to five temperatures for varying lengths of time. Each data point is the average of 4 replicates. The experiment was repeated 3 times and the results averaged. The 30°C treatment was not repeated.

The following temperatures were used:

Graph 5.5.1. 1°C
Graph 5.5.2. 5°C
Graph 5.5.3. 10°C
Graph 5.5.4. 20°C
Graph 5.5.5. 30°C

See Graph 5.5.1 for a description of the symbols.

Graph 5.5


51-d Embryos; 1C
Percentage
Duration (Weeks)
1
Category 5
Category 8
Vitreous
Roots
51-d Embryos; 5C
Percentage of Germinants in each Category
Duration (Weeks)
2
51-d Embryos; 10C
Percentage
Duration (Weeks)
3
51-d Embryos; 20C
Percentage
Duration (Weeks)
4
51-d Embryos; 30C
Percentage
Duration (Weeks)
5
0
20
40
60
80
100
0
2
4
6
8

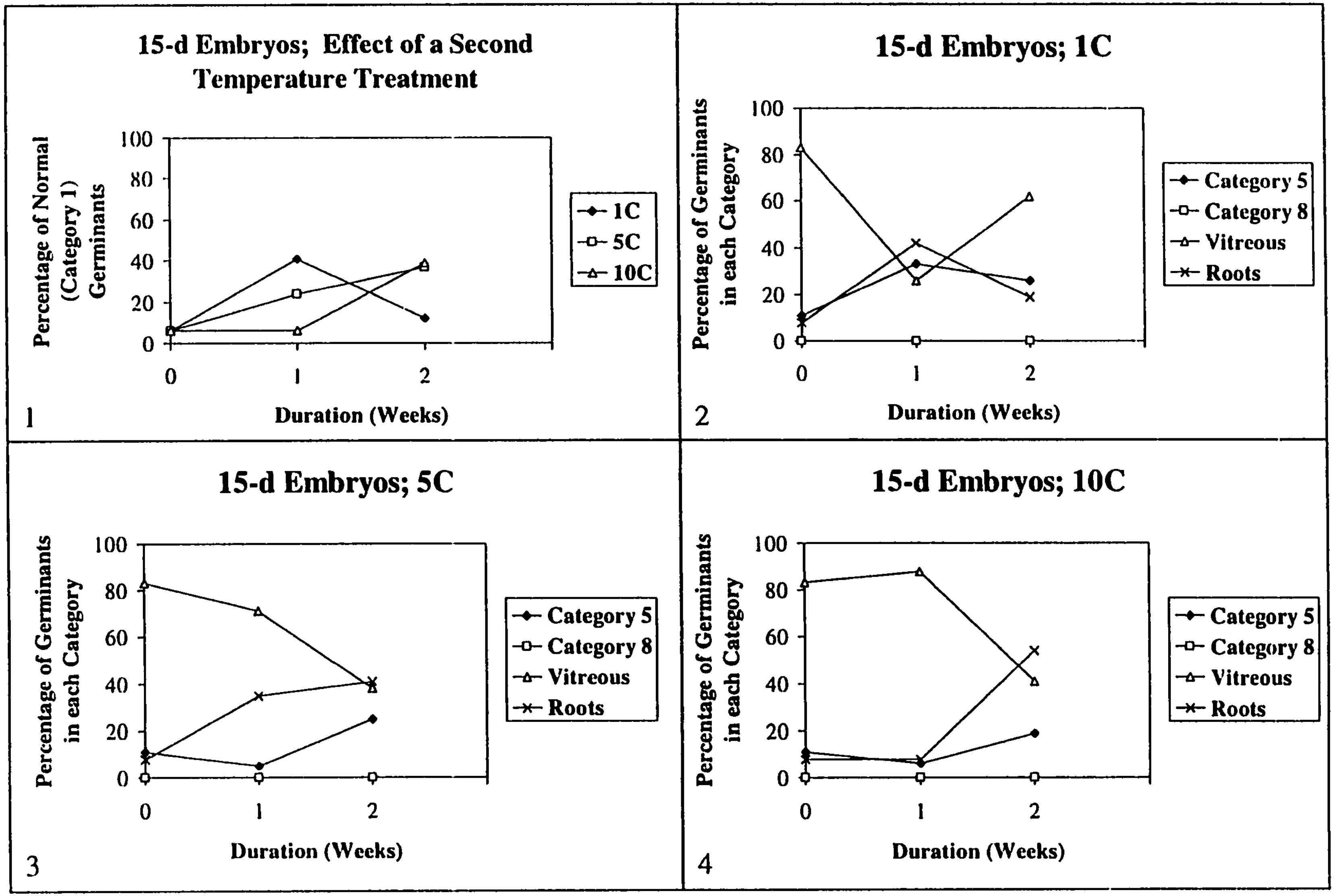
Graph 5.6.1. The effect of a second temperature treatment on the percentage of normal germinants produced by 15-d embryos. The embryos were initially matured at 30°C for eight weeks before exposure to the second temperature treatment for a period of 0, 1 or 2 weeks.

Graph 5.6.2. The effect of a second temperature treatment of 1°C on the percentage of category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots) produced by 15-d embryos. The embryos were initially matured at 30°C for eight weeks before exposure to the second temperature treatment for a period of 0, 1 or 2 weeks.

Graph 5.6.3. The effect of a second temperature treatment of 5°C on the percentage of category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots) produced by 15-d embryos. The embryos were initially matured at 30°C for eight weeks before exposure to the second temperature treatment for a period of 0, 1 or 2 weeks.

Graph 5.6.4. The effect of a second temperature treatment of 10°C on the percentage of category 5, category 8 and vitreous germinants and germinants with elongating roots (Roots) produced by 15-d embryos. The embryos were initially matured at 30°C for eight weeks before exposure to the second temperature treatment for a period of 0, 1 or 2 weeks.

Graph 5.6.


15-d Embryos; Effect of a Second Temperature Treatment
Percentage of Normal (Category 1) Germinants
100
80
60
40
20
0
0
1
2
Duration (Weeks)
1C
5C
10C
1
15-d Embryos; 1C
Percentage of Germinants in each Category
100
80
60
40
20
0
0
1
2
Duration (Weeks)
Category 5
Category 8
Vitreous
Roots
2
15-d Embryos; 5C
Percentage of Germinants in each Category
100
80
60
40
20
0
0
1
2
Duration (Weeks)
Category 5
Category 8
Vitreous
Roots
3
15-d Embryos; 10C
Percentage of Germinants in each Category
100
80
60
40
20
0
0
1
2
Duration (Weeks)
Category 5
Category 8
Vitreous
Roots
4

the second temperature treatment produced germinants of poor quality with only 6% of these germinants being normal (Category 1)(Graph 5.6.1) while 83% were vitreous (Graphs 5.6.2-4). A one-week temperature treatment of 1°C increased the percentage of normal germinants and concurrently decreased the percentage of vitreous germinants. Two weeks of exposure had the opposite effect. A two-week temperature treatment of 5 and 10°C increased the percentage of normal and rooted germinants and decreased the percentage of vitreous germinants.

5.4 Discussion

A low temperature treatment can improve germinant quality by stimulating rooting and decreasing vitreousness.

Overall, growth of the embryos at a temperature of 10°C resulted in the most consistent improvement in the percentage of normal (category 1) germinants produced by all developmental stages of the embryos. The results are significantly better than the maximum produced by the mature untreated controls and compare favorably with the maximum achieved by the desiccation treatment in Chapter 4. A temperature treatment of 5°C and, to a lesser extent, 1°C also improved the quality of the germinants produced by the 27-51 d embryos while temperatures of 20 and 30°C resulted in little improvement in germinant quality.

A similar temperature response is seen with vernalization (i.e. "the acquisition or acceleration of the ability to flower by a chilling treatment") of wheat (Brooking 1996) and stratification of apple and peach seeds (Seeley 1996). Brooking exposed wheat seeds to various temperatures from 1 to 18°C and found that the rate of vernalization increased linearly with temperatures from 1 to 11°C and then decreased with increasing temperature. Stratification of peach and apple seed resulted in a sigmoidal temperature response curve. Seeley found that the optimum stratification temperature for peach was 4-6°C. The percentage of germinating seeds gradually declined with stratification at temperatures of 6 to 16°C and quickly declined if stratified at temperatures below 2°C.

The Utah Chill Unit model (Richardson et al. 1974, revised Seeley 1996) has been used to describe endodormancy release of peach and apple seeds by stratification. A chill unit (CU) is 1 h at the optimum chilling temperature at the optimum chilling time. Each hour of chilling at temperatures above and below the optimum chilling temperature (6°C) results in partial positive chill unit accumulation (chilling promotion) with smaller fractions of CUs accumulating the farther the temperature is from the optimum. Temperatures above 14-15°C result in negative CU accumulation (chilling negation), with larger fractions of negative CUs accumulating as the temperature increases up to a maximum of -1 CU at approximately 24°C.

The Utah Chill Unit model may partially explain the effects of the various temperatures on germinant quality in somatic embryos. The optimum temperature range for improving germination in white spruce somatic embryos is probably between 5 and 10°C as shown by the increase in the percentage of normal germinants and the decrease in the percentage of vitreous germinants produced from embryos subjected to these two temperatures. According to this model, a temperature of 1°C would result in only partial positive CU accumulation. This assumption is substantiated by the slight improvement in the percentage of normal germinants and the slightly larger percentage of vitreous germinants produced. The model also suggests that temperature treatments of 20 and 30°C would have a negative effect on germination and again this was the result seen in the 20 and 30°C treated embryos. The percentage of normal germinants produced was lower and the percentage of vitreous germinants produced was much higher.

Our results can also be explained on the basis of acclimation of the embryos to low temperatures. The results suggest that the embryos are able to acclimate to temperatures of 5-10°C. Therefore, improvement in germination response would be expected to increase with length of exposure and extent of acclimation (or positive CU accumulation). This improvement in germination with length of exposure was seen for both the 5 and 10°C treatments in our experiment. At the lower temperature of 1°C, short-term exposure improved germination in the older embryos, but longer exposure inhibited germination and increased vitreousness. It may be that the embryos cannot partially or completely acclimate to such low temperatures. The improvement in germination may be the result of the initial shock response to the temperature (i.e. short-term changes in gene expression (Howarth and Ougham 1993)). Beardmore and Charest (1995) improved germination in black spruce somatic embryos with a 2 d 2°C cold treatment. They found that the cold treatment increased root growth but did not decrease the percentage of vitreous germinants. This again may be the result of the initial shock response. The results of the experiment by Kott and Beaversdorf (1990) on canola (*Brassica napus*) also show a similarity to our results. At the temperatures of 0 and 2°C, short-term exposure improved germination while longer exposure inhibited germination.

The higher temperatures of 20 and 30°C are close to the temperature at which somatic embryos are normally grown (25°C) and therefore exposure to these temperatures would not be expected to improve germinant quality. Moreover, the Utah Chill Unit model suggests that exposure to these temperatures results in the accumulation of negative CUs, which may be inhibitory to germination. This was the case in our experiments. Germinant quality decreased with length of exposure of the mature embryos to temperatures of 20 and 30°C. The 39- to 51-d embryos also became vitreous by 8 weeks. This observation was substantiated by the large increase in fresh weight coupled with the concomitant decrease in dm/fm. The decrease in germinant quality with increased age of the embryo has also been noted by Lelu et al. 1995 in larch and by Kott and Beaversdorf (1990) in canola. This suggests that mature embryos grown at the normal growth temperature of 24-25°C must be germinated before the embryos become vitreous and before germinant quality starts to decline.

Therefore, another potential use of the low temperature treatments is for storage of the embryos. A temperature of 5°C is the best temperature for short-term storage of mature embryos. Although storage at a temperature of 10°C improves the quality of the germinants, the embryos continue morphological development and may germinate precociously. At a temperature of 5°C, the embryos are arrested morphologically, although physiologically changes are still occurring. This is substantiated by the increase seen in the dry mass/fresh mass ratio, especially in the 39-d embryos. The physiological changes that are occurring in the embryos during this cold storage improve their subsequent germination and reduce the percentage of vitreous germinants. This, therefore, has practical applications for SE production systems. Embryos can be cold stored at a temperature of 5°C as they are produced and saved until sufficient numbers have been produced to do a large germination run.

Cold may reduce the level of (Takeno et al. 1983) or decrease the sensitivity of the embryo to (Williams et al. 1973) endogenous ABA, which may partially explain the improvement in germination. This is one of the ways that mild desiccation is thought to improve germination (Chapter 4). Garciarrubio et al. (1997) showed that ABA inhibition of germination in *Arabidopsis* can be alleviated by adding sugars and amino acids to the medium. Cold acclimation causes increases in sugars, proteins, amino acids, etc. (discussed in section 2.7). These additional sugars and amino acids may help alleviate germination inhibition by ABA.

The use of cold treatments to improve germination has been reported in the literature. Profumo et al. (1991) improved germination in *Aesculus hippocastanum* L. embryos by chilling them in the dark at 6°C for 6 months. Kott and Beversdorf (1990) applied cold treatments (0-4°C) to *Brassica napus* embryos for 3-12 d and found 9-12 d at 4°C to be optimal.

5.5 Conclusion

Germinant quality can be manipulated without a desiccation treatment simply by controlling the temperature at which the embryos are matured and when the temperature treatment is applied. Temperature treatments are easy to apply and require no individual handling of the embryos.

Growing immature embryos (15-d embryos with cotyledonary buttresses visible) at a temperature of 10°C increased the percentage of normal germinants produced. Germination of the mature embryos (39 d+) was improved by treating the embryos at a temperature of 5 or 10°C for a minimum of 2 weeks.

Mature 39 and 51-d embryos were kept for 8 weeks at a temperature of 5°C without any loss of germinant quality. A temperature 5°C can therefore be used for short-term storage of mature embryos.

Chapter 6

Improving Tolerance to Flash Desiccation with a Cold Treatment

6.1 Introduction

Somatic embryos are produced continuously throughout the year. The ability to store somatic embryos will give the forest industry the flexibility to bulk up mature embryos and germinate them when required. The stored embryos can be mass germinated to provide uniform planting stock.

Mature somatic embryos can be stored in a hydrated state for several months on semi-solid medium at a temperature of 5°C without any loss of viability (Chapter 5). However, longer-term storage using this method is risky. Condensation occurs in the plates of semi-solid medium at a temperature of 5°C. This may allow sufficient water to build up to drown the embryos and to promote contamination. Loss of viability or the development of abnormal characteristics may also occur with long-term storage in the hydrated state, as embryos are still metabolically active at a temperature of 5°C.

Zygotic embryos of orthodox seeds are able to withstand long-term storage because they undergo desiccation on the plant. As a result, they are metabolically quiescent (Leopold and Vertucci 1989, Bewley 1995). Somatic embryos dried to levels equivalent to those of zygotic embryos could, theoretically, be kept in the dried state either at room temperature, frozen, or encapsulated (Gray et al. 1987, Attree and Fowke 1993). In particular, encapsulation of dried mature somatic embryos (i.e. synthetic seed) would significantly decrease production costs as they could be used in seeding machines. This dispenses with the need to develop specialized equipment for lab-germinated propagules.

To survive desiccation to low water contents, an organism must be able to maintain its physiological/cellular integrity during desiccation, and to repair any damage that occurs upon rehydration (Bewley 1995, Oliver et al. 1998). Many lichens, algae and bryophytes are able to withstand rapid desiccation. It is postulated that their desiccation tolerance is attributable to both constitutive cellular protection and rehydration activated repair systems (Oliver 1991, Scott and Oliver 1994). However, constitutive protection properties require that a great deal of the available energy resources be used to keep the plants ready for desiccation. The majority of desiccation tolerant plants have stress-inducible cellular protection systems (see section 2.4). These plants can usually survive slow, but not rapid water loss, as there is insufficient time during rapid desiccation to induce and establish the protection systems (Oliver et al. 1998).

Desiccation tolerance in somatic embryos can be induced by the application of ABA in combination with a sublethal stress. Mild water stress occurs in response to polyethylene glycol (PEG) (Attree et al. 1991, 1995) or high sucrose (Anandarajah and McKersie 1990, Tetteroo et al. 1995). Thermal stress (cold shock (Beardmore and Charest 1995, Anandarajah et al. 1991) or heat shock (Anandarajah et al. 1991, Anandarajah and McKersie 1990)) have also been used.

Although stress treatments improved the ability of the embryos to withstand a gradual drying to low water contents, they had little impact on tolerance to rapid desiccation. Slow desiccation usually involves transferring the embryos to chambers with progressively lower relative humidity (Senaratna et al. 1989, Attree et al. 1991, Anandarajah and McKersie 1990, Beardmore and Charest 1995), and requires 6-14 d. The process is labour intensive and time consuming and requires specialized equipment. Ideally, the quickest and least expensive method of drying embryos to low water contents would be to air-dry them in a laminar flow hood. Preliminary experiments in this study showed that the embryos are killed. In other studies in which white spruce embryos had been matured on medium with sucrose as the osmoticum, they were shown to be sensitive to harsh desiccation (Beadmore and Charest 1995), Roberts et al. 1990, Senaratna et al. 1989).

This chapter describes work undertaken to develop a method for improving the tolerance of white spruce somatic embryos to flash desiccation to low water contents. Plants that grow in temperate regions are adapted to sub-optimal temperatures and to survive below-freezing temperatures in winter (See section 2.7). Cells of plants that are sensitive to cold die. Cells of tolerant plants survive because of changes that occur in their metabolism (Howarth and Ougham 1993). Cells subjected to actual freezing temperatures undergo changes similar to that imposed by desiccation (Palta 1990, Hallgren and Oquist 1990). As the extracellular water begins to freeze, the increasing solute concentration causes water to diffuse from the inside to the outside of the cell, essentially dehydrating the cell (see section 2.7). Therefore, the changes that enable plants to survive freezing should theoretically also protect plants from the stress of desiccation.

To test the hypothesis that cold acclimation will prepare somatic embryos to survive flash desiccation, white spruce somatic embryos at four stages of development were subjected to various temperatures. The effects on desiccation tolerance were determined by first air-drying the embryos in a laminar flow hood, then germinating them and scoring the quality of the resulting seedlings.

6.2 Materials and Methods

6.2.1 Culture Conditions

Embryogenic tissue from cryopreserved white spruce clone 6 x 5, obtained as described before in section 3.2.1, was used to produce the somatic embryos. The tissue was proliferated on initiation medium, then transferred to maintenance medium for two successive periods. They were then transferred to maturation medium 1 and grown at 24°C with a 24-h photoperiod. For the experiment, 320 plates of embryogenic tissue on maturation medium 1 (four tissue masses per plate) were required. After 2 weeks, the tissue was subcultured monthly to fresh maturation medium 2. Temperature treatments were applied to embryos at four stages of development (15, 27, 39 and 51 d after their initial transfer to maturation medium 1)(designated as maturity).

6.2.2 Temperature Treatment

Plates of maturing embryos on maturation medium 2 were put in incubators in the dark at 1, 5, 10, 20°C for 0, 1, 2, 4, or 8 weeks.

6.2.3 Desiccation

For each of the four maturity levels (i.e. 15, 27, 39, 51-d embryos), 100 embryos (when available)/temperature/duration were put on a sterilized polypropylene screen, 25 embryos/screen (four screens per maturity/treatment/duration combination). The screens were put on top of sterile petri dishes with unobstructed exposure to the airflow in the laminar flow hood and the embryos were dried for 2 h.

6.2.4 Germination

Each screen with 25 dried embryos was placed on a plate of germination medium. Germinant quality (non-vitreous quality categories 1-8 (Appendix 2) and vitreous quality categories 1v-7v (Appendix 2)) was assessed after 4 weeks on germination medium. The effect of the temperature treatments on the ability of the embryos to survive desiccation was assessed by examining the change in the percentage of the germinants within the various quality categories.

Each plate was considered as a sample unit and the percentage of embryos in each quality category was calculated as a percentage of total embryos for that plate. For statistical purposes, each plate was considered a replication and the replications were averaged to give the overall results for each treatment. There were up to four replications.

6.2.5 Rate of Water Loss

A preliminary experiment was done to determine the rate at which the mature embryos lost water when exposed to the airflow in a laminar flow hood. This was done to confirm that this method of desiccation quickly desiccated the embryos (flash desiccation) and that the embryos had lost all free cytoplasmic water within the 2 h-desiccation period used for the main experiment. Mature embryos were weighed (M) after being exposed to an unobstructed airflow in a laminar flow hood for a period of 0, 0.03, 0.06, 0.13, 0.25, 0.50, 1, 2, 4, 8, 16, and 32 h. The embryos were then rehydrated in moist air at 5°C for 7 d to obtain the saturated mass (M_t). The dried mass (M_d) was obtained after drying the embryos at 65°C for 48h. The relative water content (RWC) (Livingston et al. 1992) was determined using the formula:

$$RWC = (M - M_d) / (M_t - M_d) \qquad [6.1]$$

The RWC from 40 embryos was averaged for each period of desiccation. The rate of water loss was then determined by plotting the average RWC vs. length of exposure to the airflow using Microsoft Excel.

6.2.6 Water Content (g H_2O g^{-1} dm)

The water content (g H_2O g^{-1} dm) of a subset of 40 embryos was determined at the end of each temperature treatment to establish the water content of the embryos before they were desiccated (initial water content- WC_i) and to determine the effect of the temperature treatments on water content. The water content (g H_2O g^{-1} dm) of the embryos was also determined after they had been air-dried in the laminar flow hood for 2 h to determine the level to which the embryos had been dried during flash desiccation (final water content- WC_f).

The fresh mass (M_i) was obtained by weighing the embryos after the temperature treatments, but before they were desiccated. The air-dried mass (or final mass- M_f) was determined by weighing the embryos after they had been dried for 2 h in the airflow of a laminar flow hood. The embryos were then dried at 65°C for 48h and weighed to obtain the dry mass (M_d).

The initial water content (g H_2O g^{-1} dm) of the embryos at the end of each temperature treatment was calculated using the formula:

$$WC_i = (M_i - M_d) / M_d \qquad [6.2]$$

The final water content (g H_2O g^{-1} dm) to which the embryos from each temperature treatment had been dried during 2 h of desiccation in the airflow of a laminar flow hood was determined using the formula:

$$WC_f = (M_f - M_d) / M_d \quad [6.3]$$

The results from 40 embryos were averaged for each water content calculation (in some cases only 20 embryos were available for water content determination).

6.2.7 Statistical models and analysis

The germination responses, expressed as the percentage of germinants in each quality category, were subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + M_i + T_j + D_k + MT_{ij} + MD_{ik} + TD_{jk} + MTD_{ijk} + e_{ijkl}$$

Where Y_{ijkl} is the percentage of germinated embryos of the *i*th maturity (developmental stage) stored at the *j*th temperature for the *k*th duration. M_i is the effect of the *i*th maturity of the embryo (15, 27, 39 or 51 d); T_j is the effect of the *j*th temperature (1, 5, 10, 20°C); D_k is the effect of the *k*th duration (0, 1, 2, 4, 8 weeks); MT_{ij} is the interaction effect of the *i*th maturity and the *j*th temperature; MD_{ik} is the interaction effect of the *i*th maturity and the *k*th duration; TD_{jk} is the interaction effect of the *j*th temperature and the *k*th duration; MTD_{ijk} is the interaction effect of the *i*th maturity, the *j*th temperature and the *k*th duration; e_{ijkl} is the random error component. All main effect terms were considered to be fixed effects. The data for each quality category was tested for normality. Computations of analysis of variance were performed on the transformed non-zero data for each quality category separately using the SAS GLM procedure. The percentage data in categories 1 and dead were transformed using the formula log(x+1). The percentage data in category 4 was transformed using the formula sqrt(x). The percentage data in category 8 was transformed using the formula arcsin(sqrt(x/100)). The means data are based on actual percentages. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

The percentage of germinants in all vitreous categories (1v-3v and 5v-7v) was summed to examine the change in the total percentage of vitreous germinants. Prior to statistical analysis using the SAS GLM procedure, the non-zero vitreous data was transformed using the formula log(x+1).

The combined response of the cotyledons and hypocotyls (C+H) to desiccation was examined by summing the percentage of germinants with undamaged hypocotyls and cotyledons (categories 1 + 5). The non-zero data was normalized prior to analysis using the formula log(x+1).

The effect of desiccation on the percentage of germinants with elongating roots (roots) was examined by summing the percentage of germinants in categories 1, 1v, 2, 2v, 3, 3v and 4. The non-zero data was normalized prior to analysis using the formula sqrt(x).

Over 95% of the embryos were dead or produced germinants that were restricted to categories 1, 4, 8 or the combined category of vitreous (Appendix 4). Therefore the data from the remaining individual quality categories was not statistically analyzed.

6.3 Results

6.3.1 Embryo development

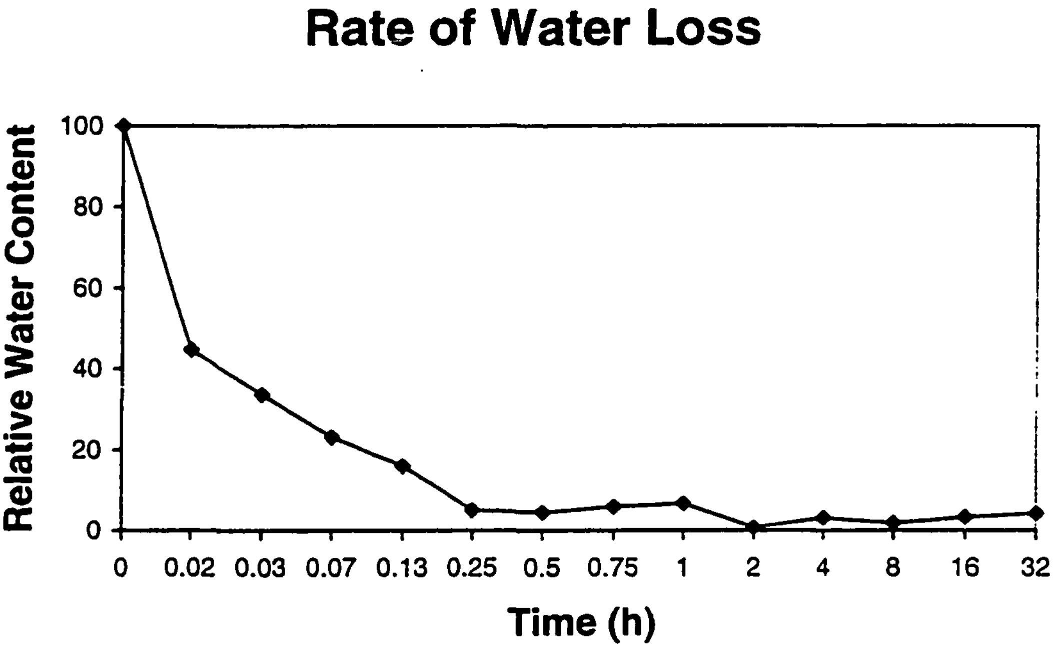
The temperature treatments had a significant effect on embryo development. Temperatures of 1 and 5°C halted any further morphological development as discussed previously in section 5.3.1, but dry matter accumulation (dm/fm), as previously presented in section 5.3.2, increased with duration of the temperature treatment. Morphological development and dry matter accumulation continued in the embryos subjected to the 10 or 20°C temperature treatments.

6.3.2 Desiccation

The embryos dried very quickly in the laminar flow hood. They had lost the majority of their water within the first 15 minutes (Graph 6.1). All embryos that were dried for 2 h in the laminar flow hood were dry, hard and brittle and had to be handled gently as the least amount of pressure broke them. However, the water content (g H_2O g^{-1} dm) of the

Graph 6.1. The rate of water loss (i.e. change in the relative water content (RWC)) in mature embryos when exposed to an unobstructed air-flow in a laminar flow hood for a period of 0 to 32 h. Each data point is the average of the RWC determinations of 40 embryos.

Graph 6.1.


Rate of Water Loss
Relative Water Content
100
80
60
40
20
0
0
0.02
0.03
0.07
0.13
0.25
0.5
0.75
1
2
4
8
16
32
Time (h)

2 h dried embryos was variable depending on the maturity of the embryos (Table 6.1). The immature embryos retained substantially more water than the more mature embryos. Although the 15 and 27-d embryos did not change morphologically when stored at temperatures of 1 and 5°C, their water content after 2 h of desiccation decreased with length of storage.

6.3.3 Germination

The analysis of variance showed that the maturity (developmental stage) of the embryos, the treatment temperature, treatment duration and their interactions had a significant influence on the distribution of embryos within the various quality categories that were analyzed (Table 6.2).

6.3.3.1 Maturity

With maturity, the embryos were better able to withstand the desiccation treatment as shown by the Duncan's test results in Table 6.3.1. The majority of the mature embryos were not killed by desiccation (74.1% dead for the 15-d embryos vs. 5.4% dead for the 51-d embryos), but organized growth was disrupted in over half of the mature embryos (15% category 8 germinants for the 15-d embryos vs. 49.4% for the 51-d embryos). The percentage of category 1, vitreous and rooted germinants also significantly increased with maturity. The percentage of germinants with undamaged cotyledons + hypocotyls also increased with maturity, but the increase was not statistically significant.

6.3.3.2 Temperature

The temperature treatment of 5°C had the most positive influence on the ability of the embryos to survive air-drying (Table 6.3.2). More of the 5°C treated embryos produced normal (category 1) and rooted germinants, and germinants with undamaged cotyledons + hypocotyls than any of the other three temperature treatments. The temperature treatment of 20°C resulted in severe damage to the embryos with 53.6% of the 20°C treated embryos being killed and only 25.7% of the germinants being classed as category 8.

Table 6.1. The initial water content (WC_i), (g H_2O g^{-1} dry mass), of the four developmental stages of embryos after 0, 1, 2, 4 and 8 weeks of storage at a temperature of 1, 5, 10 and 20°C and the final water content (WC_f) of the same embryos after 2 h of air-drying in the laminar flow hood. Each weight is the average of 40 embryos when available.

Table 6.1.

	1°C		5°C		10°C		20°C	
15-d Embryos	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f
0 Weeks	7.85	0.69						
1 Week	5.38	1.23	-	-	3.26	1.29	3.16	0.96
2 Weeks	5.42	0.46	5.96	1.08	5.35	0.74	3.52	0.07
4 Weeks	5.63	0.28	4.29	0.13	3.63	0.22	4.09	0.03
8 Weeks	8.88	0.32	7.12	0.14	3.75	0.20	2.63	0.12

27-d Embryos	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f
0 Weeks	4.93	0.69						
1 Week	4.42	0.26	4.28	0.22	4.58	0.18	4.05	0.05
2 Weeks	4.12	0.12	4.04	0.14	4.62	0.14	6.09	0.13
4 Weeks	4.11	0.05	1.03	0.04	2.93	0.06	3.22	0.04
8 Weeks	3.28	0.19	4.30	0.25	2.30	0.14	3.15	0.09

39-d Embryos	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f
0 Weeks	7.15	0.12						
1 Week	6.79	0.15	4.60	0.09	4.84	0.08	5.97	0.21
2 Weeks	3.53	0.05	3.28	0.07	3.58	0.15	3.06	0.06
4 Weeks	3.35	0.08	2.92	0.11	3.54	0.09	3.54	0.09
8 Weeks	2.69	0.19	2.34	0.19	2.41	0.13	2.06	0.10

51-d Embryos	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f	WC_i	WC_f
0 Weeks	2.34	0.08						
1 Week	3.83	0.05	3.69	0.07	4.32	0.10	5.11	0.14
2 Weeks	3.17	0.10	3.08	0.07	3.05	0.04	3.12	0.05
4 Weeks	2.63	0.08	2.73	0.07	1.95	0.07	2.25	0.02
8 Weeks	2.80	0.15	1.96	0.16	3.62	0.23	3.49	0.32

Table 6.2. Analysis of variance summary table for the effect of embryo maturity, treatment temperature and duration of the treatment on the ability of the embryos to survive flash desiccation as measured by the subsequent percentage of germinants in each germinant quality category. The change in the percentage of germinants in each quality category was analyzed separately. F-values are significant at the 0.05(*), 0.01(**), and 0.001(***) level.

Table 6.2

	Germinant Quality Category							
	Category 1		**Category 4**		**Category 8**		**Dead**	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Maturity (M)	3	4.68**	3	5.42**	3	11.61***	3	143.63***
Temperature (T)	3	6.16**	3	2.49	3	5.54**	3	7.64***
Duration (D)	4	5.90***	4	2.41	4	1.88	4	102.44***
M x T	7	1.41	8	2.31*	8	5.18***	9	2.19*
M x D	4	1.70	8	3.80***	8	12.93***	11	16.32***
T x D	11	1.03	12	1.42	12	3.01***	12	4.63***
M x T x D	2	0.29	12	0.42	19	1.36	29	2.35***
Error	39		109		137		134	
Corrected total	73		159		194		205	

Table 6.2 cont'd

	Combined Quality Categories					
	Roots		**Vitreous**		**C+H**	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Maturity (M)	3	3.79*	3	13.55***	3	2.42
Temperature (T)	3	7.59***	3	5.14**	3	6.16**
Duration (D)	4	11.13***	3	8.05***	4	5.58***
M x T	8	2.44*	5	3.00*	7	1.76
M x D	7	1.87	2	6.72**	4	0.59
T x D	12	2.93**	6	5.69***	11	0.99
M x T x D	14	0.93	1	1.96	2	0.42
Error	118		34		49	
Corrected total	170		57		83	

Table 6.3. Duncan's Multiple Range Test for the effects of embryo maturity on the ability of the embryos to survive flash desiccation as measured by the subsequent percentage of germinants in each germinant quality category. The percentage data for each quality category were analyzed separately. Data are means averaged over all treatment temperatures and durations. Means within the same quality category with different letters are significantly different (p=0.05) from each other.

Table 6.3.2. Duncan's Multiple Range Test for the effects of the treatment temperature on the ability of the embryos to survive flash desiccation as measured by the subsequent percentage of germinants in each germinant quality category. The percentage data for each quality category were analyzed separately. Data are means averaged over the four stages of embryo maturity and all treatment durations. Means within the same quality category with different letters are significantly different (p=0.05) from each other.

Table 6.3.3. Duncan's Multiple Range Test for the effects of treatment duration on the ability of the embryos to survive flash desiccation as measured by the subsequent percentage of germinants in each germinant quality category. The percentage data for each quality category were analyzed separately. Data are means averaged over the four stages of embryo maturity and all treatment temperatures. Means within the same quality category with different letters are significantly different (p=0.05) from each other.

Table 6.3.1.

	Maturity			
Quality Category	15	27	39	51
1	1.1bc	0.8c	6.2a	11.0ab
4	2.7b	11.7a	12.0a	15.7a
8	15.0b	28.4b	33.3b	49.4a
Dead	74.1a	56.1b	34.1c	5.4d
Combined Categories				
Roots	5.2b	13.2ab	22.0a	31.5a
Vitreous	6.4a	0.2c	7.7b	9.7b
C+H	1.6a	1.6a	7.9a	13.7a

Table 6.3.2.

	Temperature			
Quality Category	1C	5C	10C	20C
1	2.9b	10.2a	4.5b	1.7b
4	12.9a	13.9a	12.0a	6.7a
8	38.3a	33.6bc	34.6ab	25.7c
Dead	34.2b	33.0b	39.9b	53.6a
Combined Categories				
Roots	18.2bc	26.7a	19.5ab	11.4c
Vitreous	9.0a	3.8b	3.5ab	6.3a
C+H	4.1bc	12.7ab	5.9ab	2.6c

Table 6.3.3.

	Duration				
Quality Category	0 Weeks	1 Week	2 Weeks	4 Weeks	8 Weeks
1	0.3c	0.6bc	5.6ab	6.7ab	13.3a
4	7.6a	9.4b	12.3ab	16.1ab	12.4b
8	20.8ab	38.0a	32.9a	43.2a	30.7b
Dead	70.7a	47.5b	41.1b	19.2c	15.7c
Combined Categories					
Roots	8.8b	11.8c	20.9ab	26.7ab	30.0a
Vitreous	0.0	1.6b	2.7b	5.5b	20.5a
C+H	0.5b	0.7b	5.9ab	9.9ab	17.1a

6.3.3.3 Duration

The longer the temperature treatments were applied, the better the embryos were able to withstand desiccation as shown by the Duncan's test results in Table 6.3.3. The percentage of category 1 and rooted germinants and germinants with undamaged cotyledons + hypocotyls increased with duration of the treatments while the percentage of dead embryos decreased from 70.7% for a treatment duration of 1 week to 15.7% for a treatment duration of 8 weeks. The percentage of vitreous germinants also increased with treatment duration.

6.3.4 Maturity x Temperature x Duration Interactions

6.3.4.1 Effect on the percentage of Normal (Category 1) Germinants

The temperature treatments had a significant effect on the percentage of normal (category 1) germinants produced by the desiccated embryos (Graphs 6.2.1-4). Pre-treatment of the 39-d embryos at a temperature of 5 or 10°C for 4 to 8 weeks resulted in an increase in the percentage of category 1 germinants produced. Exposure of the 51-d embryos to a temperature of 5°C for 8 weeks increased the percentage of category 1 germinants produced to 58%. However, a 10 and 20°C temperature treatment for 8 weeks resulted in the 51-d embryos germinating precociously. These embryos were therefore not subjected to the desiccation treatment, which is why there are no data points on these graphs. The temperature treatments had little effect on the percentage of normal germinants produced by the 15 and 27-d embryos.

6.3.4.2 Effect on Embryo Survival

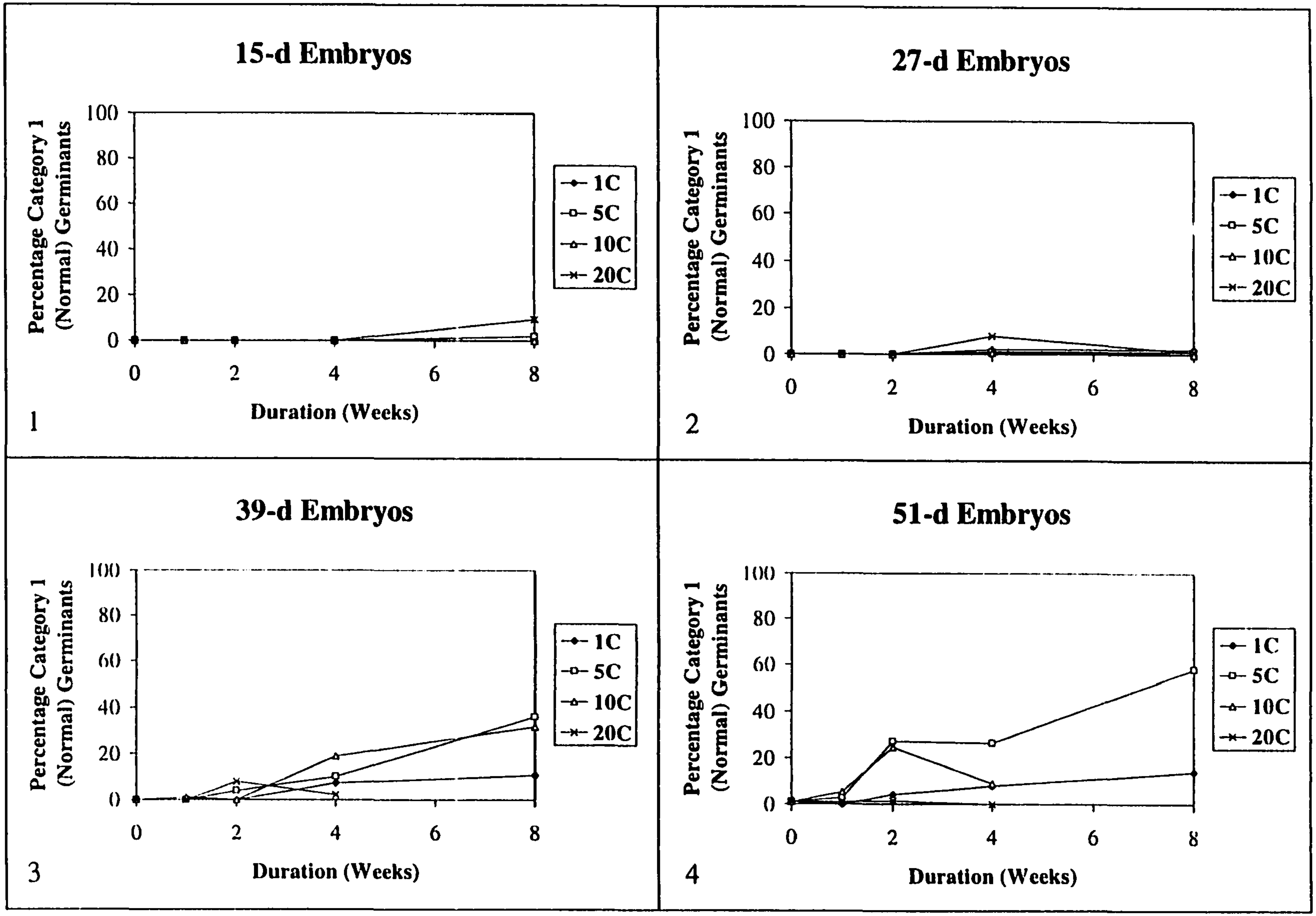
The embryos became more tolerant to desiccation as they matured. The untreated 15 and 27-d embryos (Graphs 6.3.1-4 and 6.4.1-4 respectively) could not withstand desiccation at all and were killed. By comparison, mortality of untreated 39 and 51-d embryos was less at 83 and 11% respectively (Graphs 6.5.1-4 and 6.6.1-4). The temperature treatments substantially improved the ability of the 15, 27 and 39-d embryos to survive desiccation. Eight weeks of exposure of the 15-d embryos to a temperature of 5 or 10°C decreased the percentage of dead embryos in favour of category 8 germinants (Graphs 6.3.1-2) while

Graph 6.2. The percentage of category 1 (normal) germinants produced from temperature treated, flash dried embryos. Embryos at four stages of development were subjected to four temperatures for varying lengths of time (0, 1, 2, 4, 8 weeks) before they were flash dried. Each data point is the average of 4 replicates of 25 embryos.

Four developmental stages of embryos were used:

Graph 6.2.1. 15-d embryos
Graph 6.2.2. 27-d embryos
Graph 6.2.3. 39-d embryos
Graph 6.2.4. 51-d embryos

Graph 6.2.

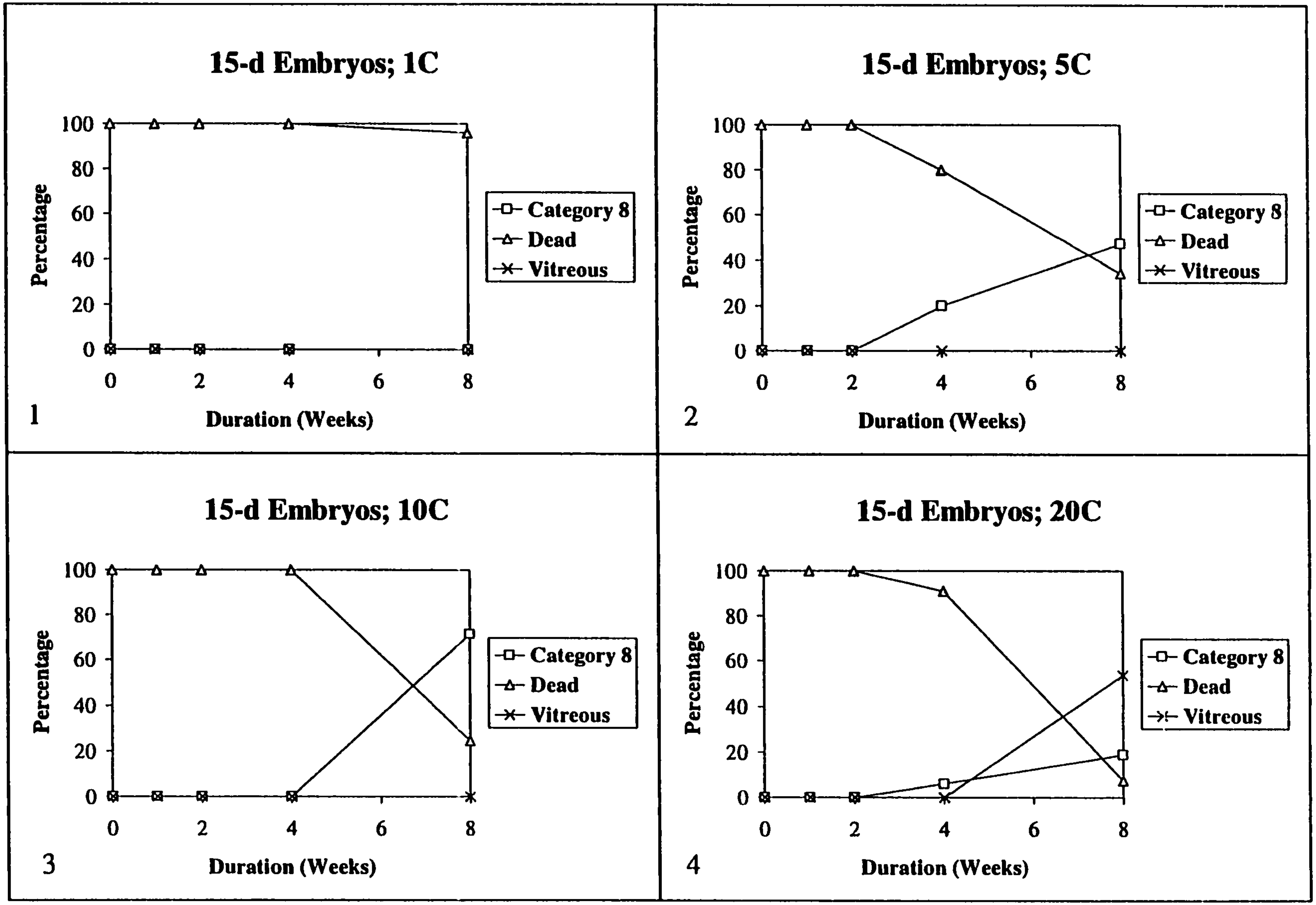

15-d Embryos
27-d Embryos
39-d Embryos
51-d Embryos
Percentage Category 1 (Normal) Germinants
0
20
40
60
80
100
Duration (Weeks)
0
2
4
6
8
1C
5C
10C
20C
1
2
3
4

Graph 6.3. The percentage of temperature treated 15-d embryos that had been killed by flash desiccation or had germinated and were scored as category 8 or vitreous. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.3.1. 1°C
Graph 6.3.2. 5°C
Graph 6.3.3. 10°C
Graph 6.3.4. 20°C

Graph 6.3.

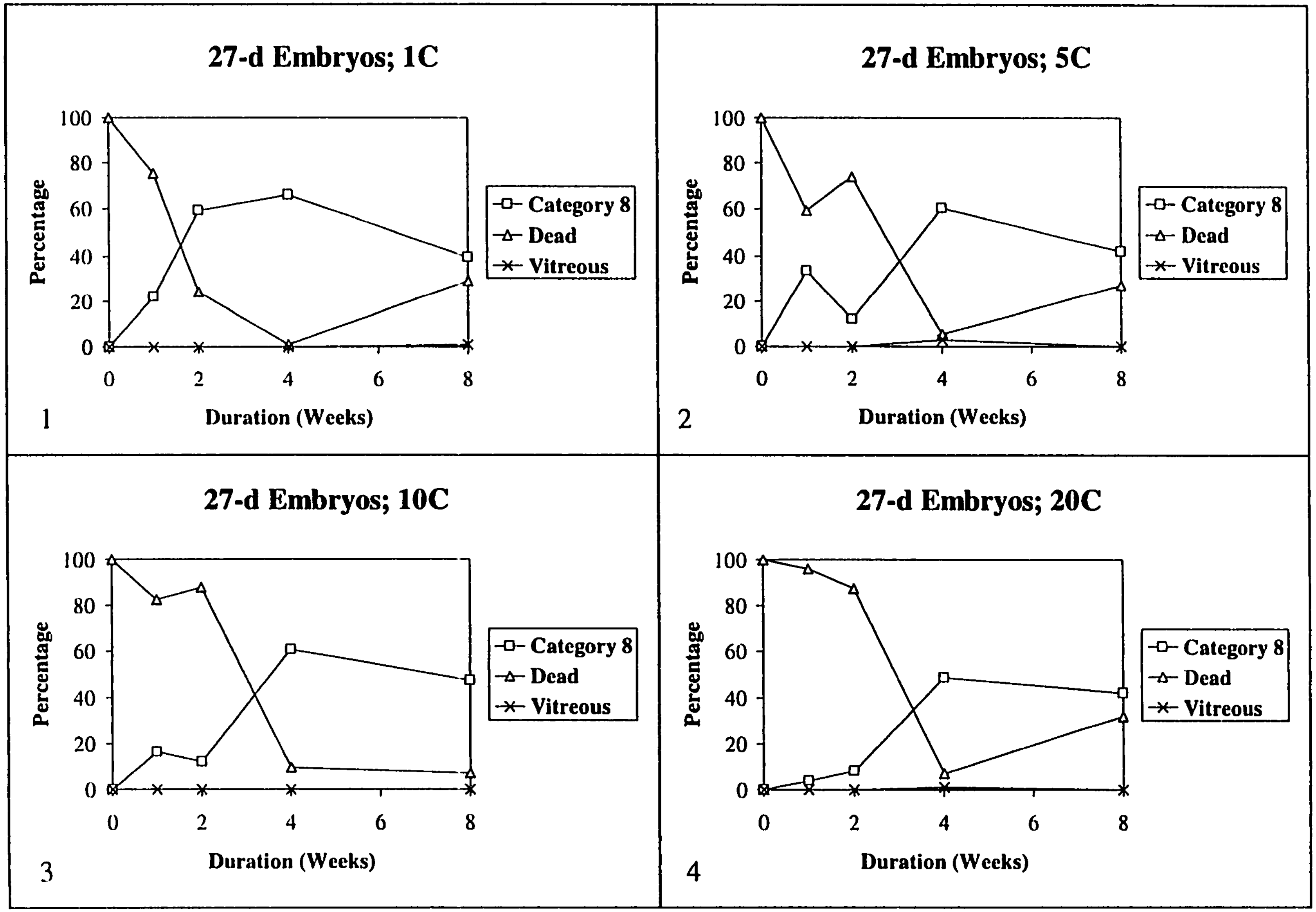

15-d Embryos; 1C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
1
15-d Embryos; 5C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
2
15-d Embryos; 10C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
3
15-d Embryos; 20C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
4

Graph 6.4. The percentage of temperature treated 27-d embryos that had been killed by flash desiccation or had germinated and were scored as category 8 or vitreous. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.4.1. 1°C
Graph 6.4.2. 5°C
Graph 6.4.3. 10°C
Graph 6.4.4. 20°C

Graph 6.4.

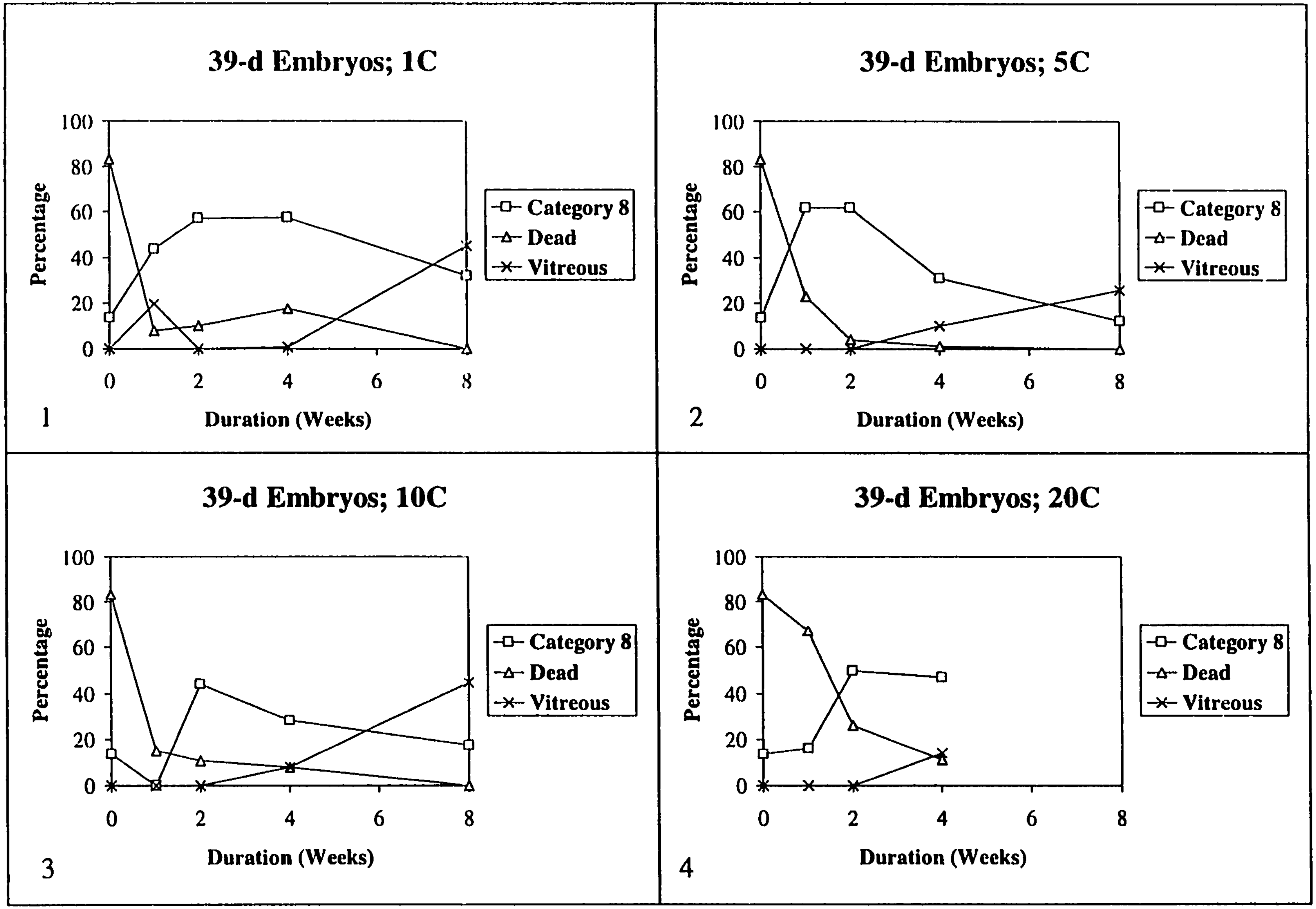

27-d Embryos; 1C
100
80
60
40
20
0
Percentage
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
1
27-d Embryos; 5C
100
80
60
40
20
0
Percentage
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
2
27-d Embryos; 10C
100
80
60
40
20
0
Percentage
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
3
27-d Embryos; 20C
100
80
60
40
20
0
Percentage
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
4

Graph 6.5. The percentage of temperature treated 39-d embryos that had been killed by flash desiccation or had germinated and were scored as category 8 or vitreous. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.5.1. 1°C
Graph 6.5.2. 5°C
Graph 6.5.3. 10°C
Graph 6.5.4. 20°C

Graph 6.5.

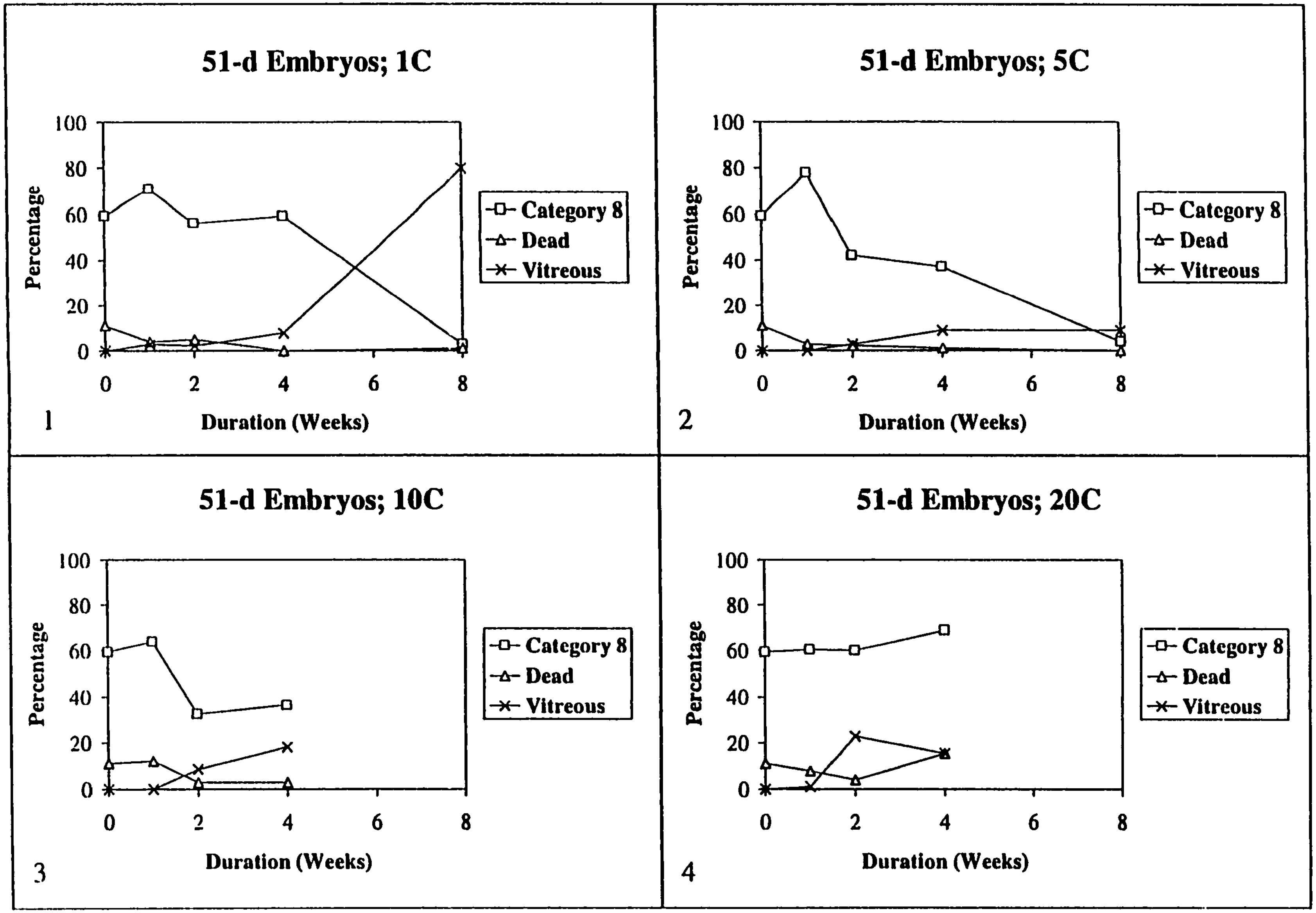

39-d Embryos; 1C
39-d Embryos; 5C
39-d Embryos; 10C
39-d Embryos; 20C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Category 8
Dead
Vitreous
1
2
3
4

Graph 6.6. The percentage of temperature treated 51-d embryos that had been killed by flash desiccation or had germinated and were scored as category 8 or vitreous. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.6.1. 1°C
Graph 6.6.2. 5°C
Graph 6.6.3. 10°C
Graph 6.6.4. 20°C

Graph 6.6.



51-d Embryos; 1C

51-d Embryos; 5C

51-d Embryos; 10C

51-d Embryos; 20C

exposure to a temperature of 20°C increased the percentage of vitreous germinants (Graph 6.3.4). The 27-d embryos required 4 weeks of exposure to any of the temperatures for maximum improvement in embryo survival (Graphs 6.4.1-4). A temperature treatment of 1, 5 or 10°C improved desiccation tolerance in 39-d embryos significantly after only 1 week of exposure (Graphs 6.5.1-3). The mature 51-d embryos survived desiccation without a temperature treatment, but a temperature treatment of 1, 5, and to a lesser extent, 10°C resulted in a decrease in the percentage of category 8 germinants produced (Graphs 6.6.1-4).

6.3.4.3 Differential Survival of Roots and Cotyledon+Hypocotyls

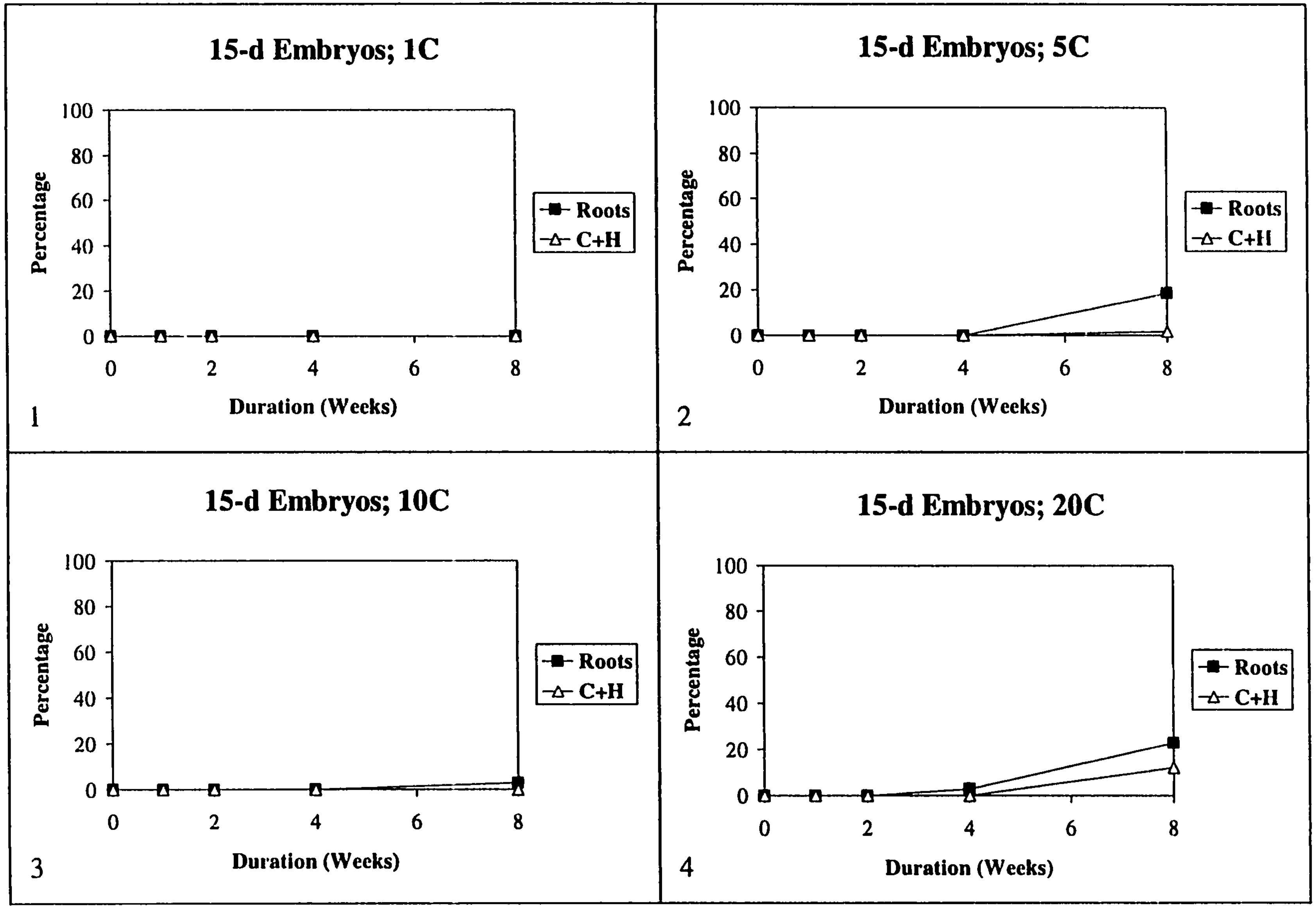
Mature untreated embryos (51-d) were able to survive desiccation but showed differential survival with the roots being less sensitive to damage than the cotyledons+hypocotyls. Exposure to a temperature of 5°C and, to a lesser extent, temperatures of 10 and 1°C improved root and cotyledon+hypocotyl survival for all embryo developmental stages, with the temperature treatments having a more positive effect on the mature embryos (Graphs 6.7.1-4). The roots developed tolerance to desiccation at a younger age and also after a shorter period of exposure to the temperature treatments than did the cotyledons+hypocotyls. The temperature treatments increased the percentage of germinants from the 15 and 27-d embryos with undamaged roots, but there was little increase in the percentage of germinants with undamaged cotyledons+hypocotyls (Graphs 6.7.1-4 and 6.8.1-4). A temperature treatment of 5°C, and to a lesser extent 10°C, improved survival of both the roots and cotyledons+hypocotyls of the 39 and 51-d embryos (Graphs 6.9.1-4 and 6.10.1-4). The majority of the 10 and 20°C treated 51-d embryos had started to germinate precociously after 4 and 8 weeks duration. Therefore, the embryos from the 8 weeks duration were not desiccated and the data points are missing on the graphs.

Graph 6.7. The percentage of temperature treated 15-d embryos that produced germinants with undamaged roots (Roots) or cotyledons+hypocotyls (C+H) after flash desiccation. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.7.1. 1°C
Graph 6.7.2. 5°C
Graph 6.7.3. 10°C
Graph 6.7.4. 20°C

Graph 6.7.

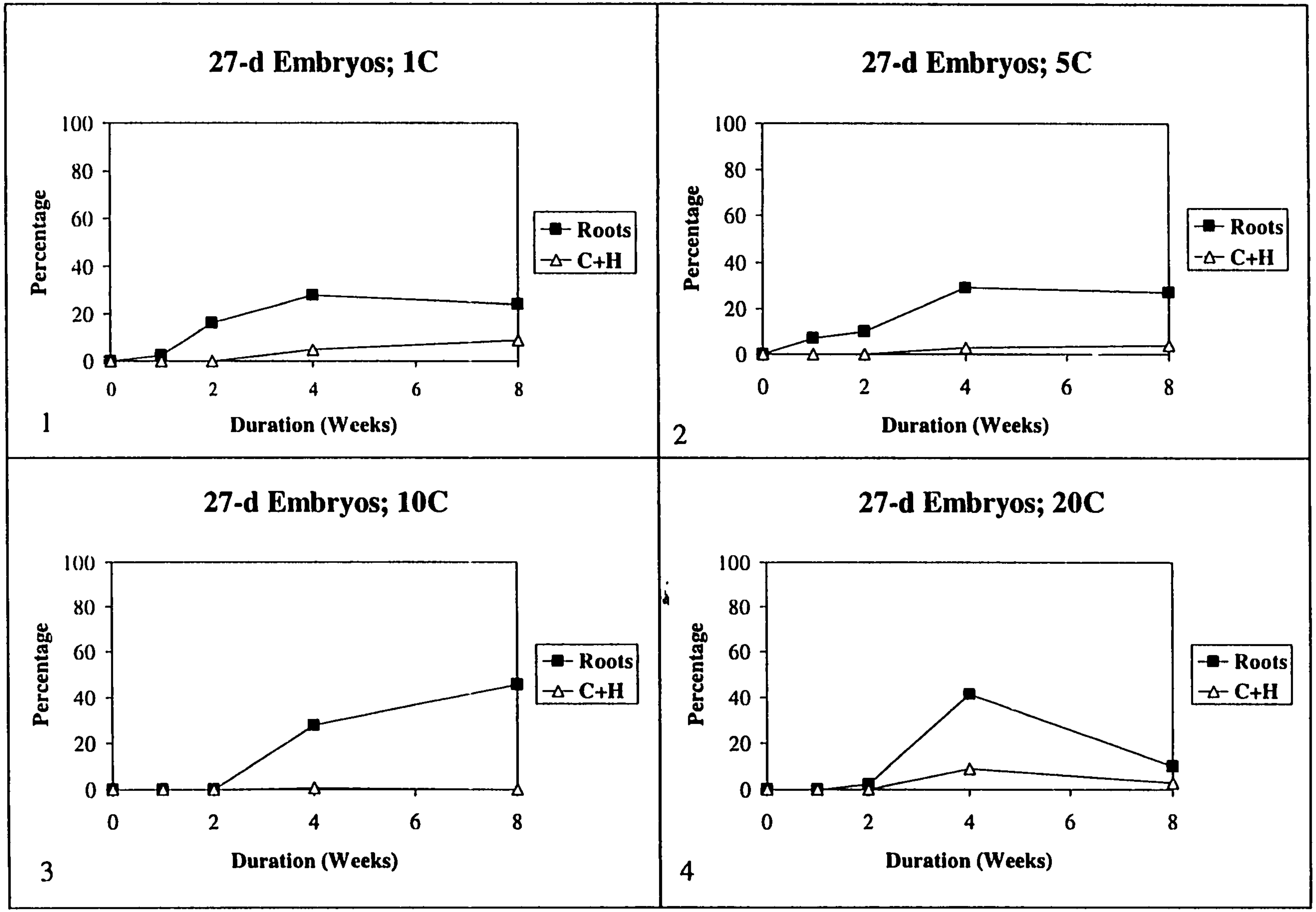

15-d Embryos; 1C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
1
15-d Embryos; 5C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
2
15-d Embryos; 10C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
3
15-d Embryos; 20C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
4

Graph 6.8. The percentage of temperature treated 27-d embryos that produced germinants with undamaged roots (Roots) or cotyledons+hypocotyls (C+H) after flash desiccation. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.8.1. 1°C
Graph 6.8.2. 5°C
Graph 6.8.3. 10°C
Graph 6.8.4. 20°C

Graph 6.8.

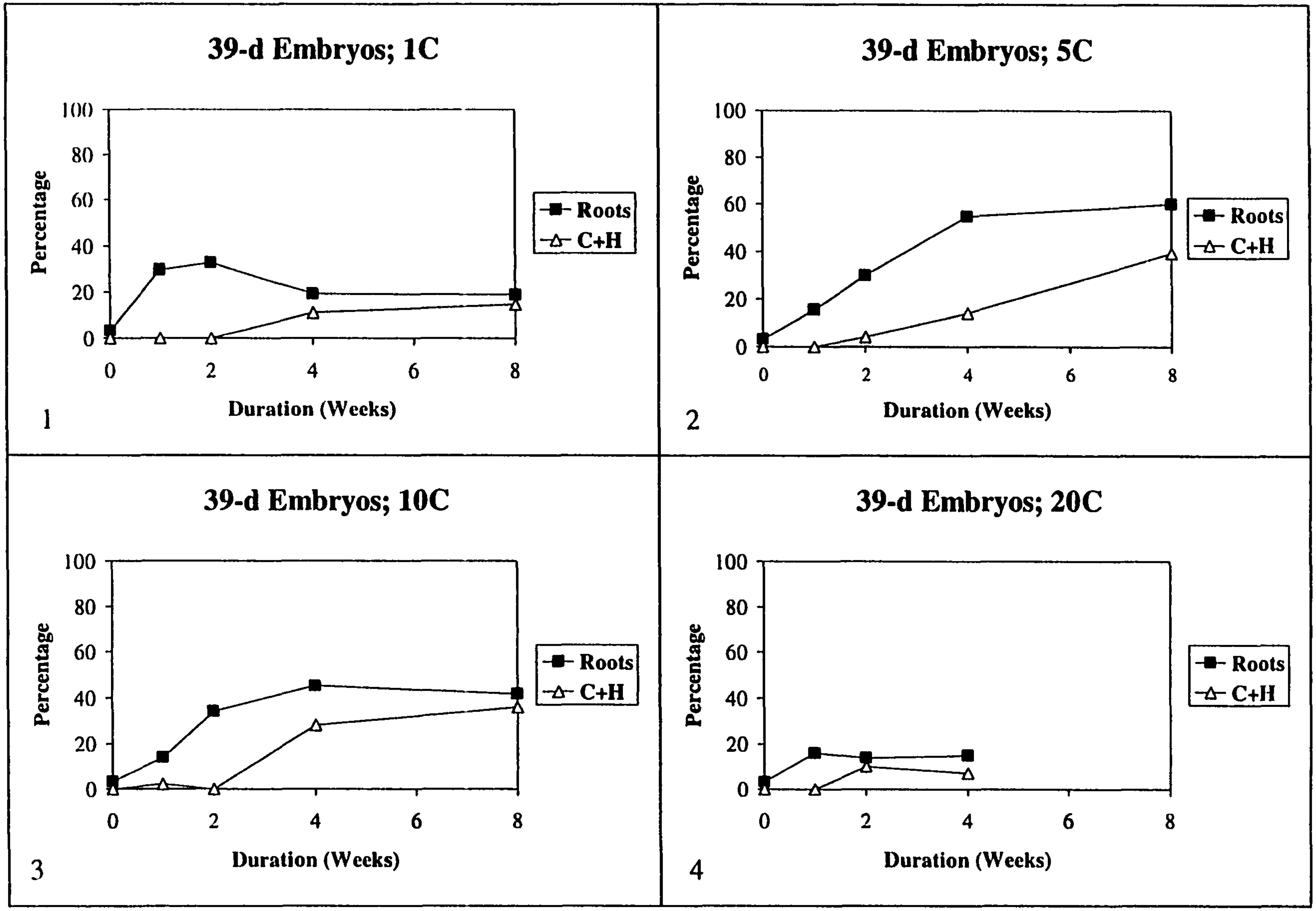

27-d Embryos; 1C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
1
27-d Embryos; 5C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
2
27-d Embryos; 10C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
3
27-d Embryos; 20C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
4

Graph 6.9. The percentage of temperature treated 39-d embryos that produced germinants with undamaged roots (Roots) or cotyledons+hypocotyls (C+H) after flash desiccation. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.9.1. 1°C
Graph 6.9.2. 5°C
Graph 6.9.3. 10°C
Graph 6.9.4. 20°C

Graph 6.9.

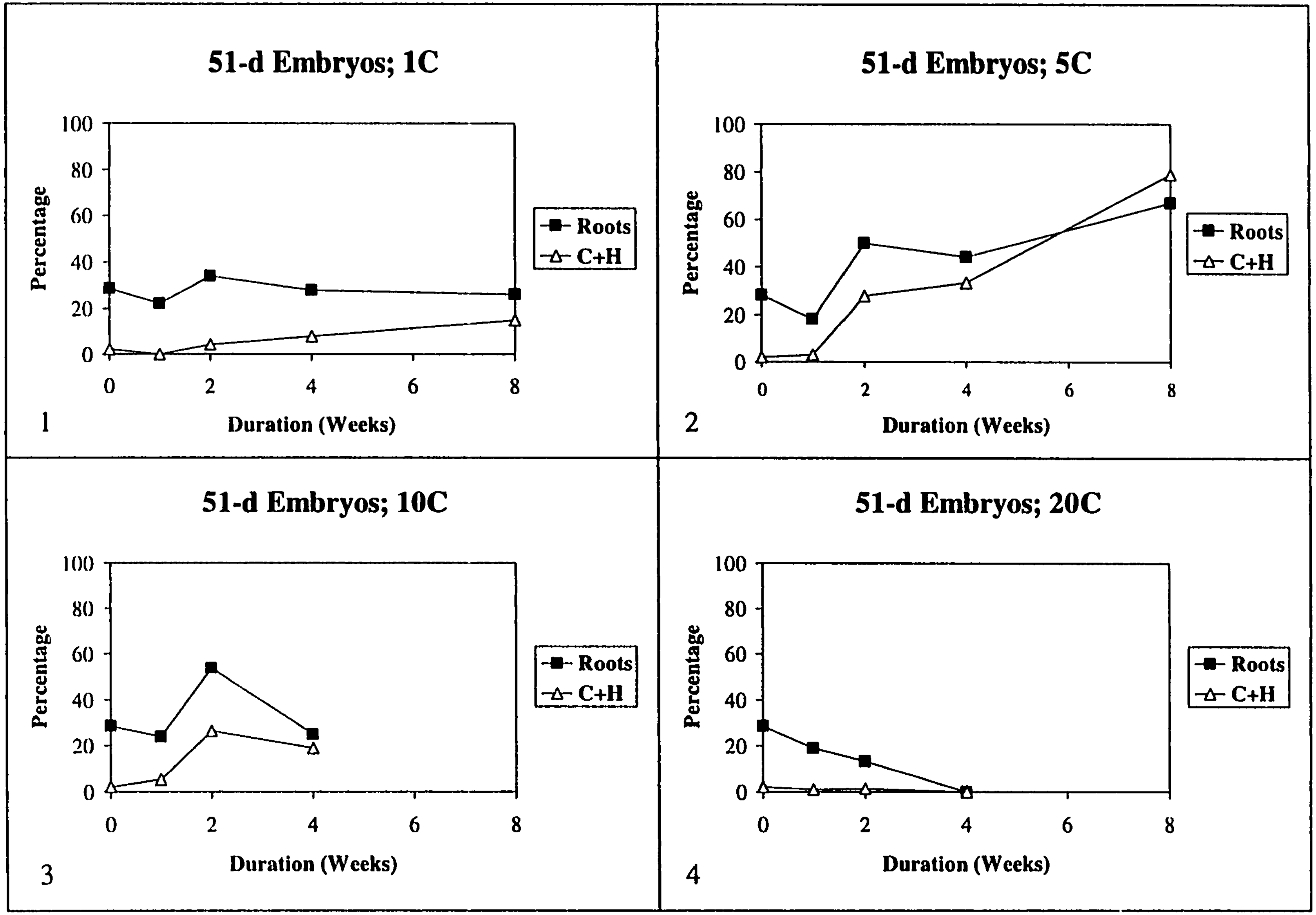

39-d Embryos; 1C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
1
39-d Embryos; 5C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
2
39-d Embryos; 10C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
3
39-d Embryos; 20C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
4

Graph 6.10. The percentage of temperature treated 51-d embryos that produced germinants with undamaged roots (Roots) or cotyledons+hypocotyls (C+H) after flash desiccation. The embryos were subjected to the temperature treatments for a period of 0-8 weeks prior to flash desiccation. Each data point is the average of four replicates of 25 embryos.

The following temperatures were used:

Graph 6.10.1. 1°C
Graph 6.10.2. 5°C
Graph 6.10.3. 10°C
Graph 6.10.4. 20°C

Graph 6.10.


51-d Embryos; 1C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
1
51-d Embryos; 5C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
2
51-d Embryos; 10C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
3
51-d Embryos; 20C
Percentage
100
80
60
40
20
0
0
2
4
6
8
Duration (Weeks)
Roots
C+H
4

6.4 Discussion

Air-drying somatic embryos in a laminar flow hood is a quick and easy method to remove all free cytoplasmic water from the embryos. Embryos lose most of their water within the first 15 minutes of exposure to the airflow. However, it is a harsh method of desiccation that can cause severe damage to untreated embryos. This experiment showed that a simple cold treatment will increase desiccation tolerance in embryos. A temperature treatment of 5°C applied to 51-d embryos for 8 weeks results in maximum desiccation tolerance.

Desiccation tolerance is acquired gradually during the in situ maturation of conifer and angiosperm seeds (reviewed in Vertucci and Farrant 1995). However, a discrete developmental stage must be achieved before desiccation tolerance is induced. Seeds undergoing histodifferentiation and in the early stages of cell expansion are not desiccation tolerant (Kermode 1995). Tolerance is gradually acquired throughout the subsequent maturation period only after dry matter accumulation has started. Embryos become tolerant to slow desiccation first (Kermode and Bewley 1985a). Slow drying is believed to allow metabolism to proceed and tolerance to continue developing as the embryos are drying (Oliver et al. 1998, Wolkers et al. 1998a). Maximum tolerance occurs within days of maximum dry matter accumulation (Sun and Leopold 1993). Only when all desiccation protection and rehydration-inducible repair mechanisms are in place can the embryos withstand flash desiccation.

This gradual acquisition of desiccation tolerance coupled with storage product accumulation (particularly sucrose and stachyose) has been observed in the seeds of many species. For example *Brassica campestris* seeds required 9 d for full desiccation tolerance to occur (Leprince et al.1990)) which was coupled with an increase in lipids, proteins, sucrose and stachyose and a decrease in starch content. Desiccation tolerance in *Glycine max* increased with maturation and was quantitatively associated with the increase in sucrose and stachyose (Sun et al. 1994). Similar correlation was found in pollen desiccation tolerance (Hoekstra and Roekel 1988, Hoekstra et al. 1989).

Mild water stress has been shown to induce the gradual development of desiccation tolerance in somatic embryos. Senaratna et al. (1989) were able to increase tolerance of alfalfa to rapid desiccation (1 d in an unsealed petri dish at lab atmosphere) by applying water stress for a 3-week period. The embryos were left on medium in an unsealed petri dish and allowed to dry out slowly over the course of 3 weeks. Attree et al. (1995) found that 50% of the white spruce somatic embryos grown on maturation medium with 40μM ABA and 7.5% PEG were able to withstand slow drying after 3 weeks of culture and that this percentage increased to 77% after 8 weeks of culture. The embryos required 5-6 weeks of culture to survive rapid drying (over a 24-48 hour period) at a conversion percentage of <30%. This percentage increased to >70% with 8 weeks of culture.

Cold shock has been used to improve tolerance of somatic embryos to slow desiccation, but tolerance to rapid desiccation was not significantly improved. The cold stress treatments were often applied for only short periods and/or possibly at too low a temperature. Cold shock was applied for 2 d at 2 or 4°C (Beardmore and Charest 1995, Lelu et al. 1995), for 2-10 d at 2°C (Anandarajah et al. 1991) or for 14 d at 2°C (Senaratna et al. 1989). In all cases, tolerance to slow desiccation was improved, but the treatments resulted in very little improvement in tolerance to rapid desiccation (Beardmore and Charest 1995).

Long-term exposure of plants to temperatures low enough to stress them without causing permanent damage results in cold acclimation in the plants (see section 2.7). This is brought about by changes in their metabolism to protect them from damage by the cold (Howarth and Ougham 1993). Cold stress results in changes similar to those associated with water stress. The mechanisms involved in desiccation tolerance are known to share many features, i.e. accumulation, or changes in composition, of carbohydrates, proteins, amino acids, nucleic acids and phospholipids (Hallgren and Oquist 1990, Gusta et al. 1996, Howarth and Ougham 1993).

A critical threshold in response in our experiments is at 3-4 weeks of development. The percentage of normal germinants produced by the immature (27 d of age or less), flash desiccated embryos was not improved by the temperature treatments. This corresponds with the findings in zygotic embryos that desiccation tolerance can only be induced after the deposition of storage products has begun. According to Attree et al. (1995), the bulk of storage product deposition starts in white spruce somatic embryos during week 3 on maturation medium (21 d +). Joy et al. (1991) found that starch accumulation in white spruce somatic embryos began immediately after the embryos were transferred to the maturation medium, lipid accumulation began 1 week later and protein deposition started after cotyledon initiation which occurred during week three. This correlation has also been suggested by Wolkers et al. (1998a) who correlated the acquisition of tolerance to rapid desiccation in *Zea mays* 22 DAP (Bochicchio et al. 1994) with the initiation of a Lea protein at 22 DAP (i.e. days after pollination) (Mao et al. 1995).

The ability of the mature embryos to withstand flash desiccation without severe damage (as measured by the increase in the percentage of normal germinants produced) increased with the duration of the temperature treatments. Exposure of 39 or 51-d embryos to a temperature of 5°C for a minimum of 4 weeks was required to significantly increase their tolerance to air-drying. Eight weeks of exposure more than doubled the percentage of normal germinants produced by the 51-d embryos as compared to the percentage produced after 4 weeks of exposure.

The results suggest that there is a delicate balance between temperature and length of exposure. The cold treatments (2-4°C) used by Beardmore and Charest (1995), Lelu et al. (1995), Anandarajah et al. (1991) and Senaratna et al. (1989) may have been too cold and too short in duration. Also, Ougham (1987) showed that a 24-h 5°C cold shock caused no visible change in protein synthesis and that longer term exposure of *Lolium temulentum* to a temperature of 5°C was required to affect the pattern of protein synthesis.

Desiccation tolerance developed differentially in the embryo: it appeared in the roots before it did in the hypocotyls and cotyledons. This was most noticeable in the 27 and 39-d embryos. Anandarajah et al. (1991) and Attree et al. (1995) found that in untreated embryos the roots survived desiccation more often than did the hypocotyls+cotyledons, which may be a consequence of differential storage product deposition in somatic embryos during maturation. Joy et al. (1991) studied storage product deposition in white spruce somatic embryos and detected a strong gradient in storage product accumulation with starch, lipid and protein appearing first and in higher concentrations near the suspensor end of the embryo. Storage product accumulation then proceeded acropetally. By full maturity, they were spread throughout the embryo.

6.5 Conclusion

Air-drying of mature somatic embryos in a laminar flow hood is a quick and easy method of removing all free cytoplasmic water from the embryos.

Tolerance of white spruce somatic embryos to this severe method of desiccation can be significantly improved by a simple cold treatment. The ability of the embryos to survive flash desiccation increases with the maturity of the embryos, but the mature embryos still cannot withstand flash desiccation without suffering severe damage unless they have been pre-treated with a cold treatment.

A temperature treatment of 10 or 5°C induces desiccation tolerance, but only a temperature of 5°C will inhibit precocious germination long enough to allow maximum desiccation tolerance to develop. The response of the embryos to the cold treatment also improved with the maturity of the embryo. Therefore, the treatment regime that produced the highest percentage of normal germinants after the flash drying treatment was storage of the 51-d (mature) embryos at a temperature of 5°C for 8 weeks. The percentage of normal germinants produced after air-drying these cold pre-treated embryos was similar to the percentage of normal germinants produced by undesiccated controls.

The advantage lies in the ability to store dried embryos. Our embryos could be desiccated, stored and germinated, with the resulting germinants of high quality. For artificial seed production, desiccation and a cold pre-treatment to make desiccation possible are essential. As pointed out elsewhere, production of artificial seed would greatly promote industrial application of SE technology.

Desiccation tolerance develops in the roots of the embryos first, then the hypocotyls and cotyledons become desiccation tolerant. This may be a consequence of where storage products are deposited within the somatic embryos.

This experiment confirms that the stress response to low temperatures (cold acclimation) can also increase tolerance to desiccation, but that sufficient time must be given for maximum desiccation tolerance to develop. This tolerance has a novel characteristic, in that it allows flash desiccation of plant embryos. These results again suggest that cold and desiccation stress response must share some of the same metabolic pathways.

Chapter 7

Rehydration of Desiccated Embryos

7.1 Introduction

Somatic embryos can be partially desiccated to improve germination (Attree and Fowke 1993, Roberts et al. 1990) or totally desiccated for potential use as synthetic seed (Gray et al. 1987), but must be rehydrated before they can germinate and can suffer severe damage if the process of water uptake (imbibition) is not strictly controlled.

During imbibition in zygotic embryos, unlike somatic embryos, the structures of the surrounding seed help to regulate the rate of water uptake and/or may act as barriers to solute leakage (Bewley and Black 1994). The testa controls water uptake in pea embryos. The embryos suffer severe damage when imbibed without their surrounding testa (Powell and Matthews 1978, 1979). In *Pinus sylvestris* L. and *Picea abies* (L.) Karst. seeds, water uptake is largely controlled by the megaspore membranes, especially the outer and inner exine (Tillman-Sutela and Kauppi 1995 a, b).

Somatic embryos are naked and do not have a protective seed coat or endosperm structures to govern water uptake. We predict that severe damage during rehydration will occur if the method of rehydration itself is not strictly controlled.

This chapter describes work undertaken to develop protocols to minimize imbibitional damage suffered during rehydration of desiccated white spruce somatic embryos.

The first objective was to develop a method to quantify damage to enable comparisons to be made between different rehydration methods. There are two ways to do this. The most reliable method for assessing damage is to germinate the desiccated embryos and assess the damage. However, this method is cumbersome and time consuming because all manipulations must be done under sterile conditions and a minimum of 3 weeks growth on germination medium is required before an assessment of damage can be done.

Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) is another reliable method of determining seed viability (Edwards 1987, Kruse 1996, Kovach and Bradford 1992). Aseptic conditions are not required and results are available within 18 h. Hydrogen released by the action of the dehydrogenase enzymes in the living tissue reduces the colourless TTC to a red-stained, stable, non-diffusable triphenyl formazan while the dead tissue remains colourless. Assessment of damage can either be determined visually (Kruse 1996, Percy 1997) or colour intensity can be quantified by measuring the absorbance at 485 nm after extraction in 95% ethanol (Towill and Mazur 1975).

To determine the most reliable method for quantifying damage, white spruce somatic embryos were dried and rehydrated using diverse methods. A visual TTC damage assessment method was developed and compared to results obtained from TTC absorbance readings and germination results.

The next objective was to develop rehydration protocols to minimize imbibitional damage. Several factors can influence the amount of damage suffered by embryos during imbibition: 1.) Method of rehydration- Chapter 4 showed that slowly desiccated embryos suffered less damage if they were rehydrated indirectly in an atmosphere approaching 100% RH rather than directly on germination medium, 2.) Temperature- It has been shown that organisms suffer more imbibitional damage if they are rehydrated at low temperatures (Hoekstra et al. 1992a), 3.) Embryo pre-treatment- Acclimation of the embryos to a temperature of 5°C was shown to increase desiccation tolerance (Chapter 6). It is our expectation that this build up of desiccation tolerance is linked to the ability to survive imbibition as well.

Therefore, the first experiment examined the effect of the method of imbibition (direct, indirect) and the temperature of imbibition (5, 24°C) on the amount of damage suffered by air-dried fresh and cold stored white spruce somatic embryos.

Tetteroo et al. (1995) showed that the amount of imbibitional damage suffered by carrot embryos was influenced by the length of time the embryos were indirectly rehydrated before they being placed on germination medium. Therefore, in a second experiment, slowly dried and flash desiccated fresh and cold stored embryos were indirectly rehydrated for varying periods of time at a temperature of 5 or 20°C.

7.2 Materials and Methods

7.2.1 Damage Assessment

7.2.1.1 Assessment of Damage using TTC

White spruce embryogenic tissue from cryopreserved clone 6 x 5 (as described previously in section 3.2.1) was used to produce approximately 600 embryos (~ 6 weeks of age). The embryos were matured using the method described in section 4.2.1.

Mature embryos were individually harvested, and subjected to various combinations of rapid (flash) or slow drying and indirect or direct rehydration treatments. The treatments were applied either immediately after the embryos were harvested or after they had been stored for up to 1 month at 5-10°C. For flash desiccation, the embryos were air-dried on a screen (1000 μm polypropylene mesh) on top of a sterile petri dish in the unobstructed airflow of a laminar flow hood for a period of 0 to 64 h. For slow desiccation, the embryos were equilibrated at a temperature of 5°C for a period of 7 d in a closed system over NaCl solutions generating atmospheres with water potentials ranging from 0 to -20 MPa (see section 4.2.2). The embryos were directly rehydrated by placing them on germination medium for 24 h or indirectly rehydrated for 24 h at 5°C in a cuvette which had an atmosphere approaching 100% RH. A dead control was also used. The embryos were killed by autoclaving them for 15 min at 121°C at 15 psi.

The rehydrated embryos were put in a 1.2-ml cryovial and covered with 1% TTC solution (Appendix 5). The cryovials were covered in tinfoil and left at room temperature for 18 to 20 h. The stained embryos were then washed in distilled water and examined for the presence of red stained and unstained areas indicating living and dead tissue respectively.

7.2.1.2 Correlation of TTC scoring method with spectrophotometer readings

A total of 760 mature embryos from clone 6 x 5 were used. They were produced as described previously in section 7.2.1.1. The embryos were air-dried in the laminar flow hood for 0.25, 0.50 1, 2, 4, 8, 16, 31 or 64 h. They were rehydrated by direct or indirect rehydration at a temperature of 5 or 25°C, (20 embryos per duration per rehydration per temperature combination). A dead control of 20 autoclaved embryos and an undamaged control of 20 undesiccated embryos were also used. The 25°C directly rehydrated embryos were placed on germination medium that had previously been warmed to room temperature and were then put in an incubator at 25°C for 24 h to rehydrate. The 5°C directly rehydrated embryos were placed on germination medium that had previously been cooled to 5°C, the plates being kept on ice while the dried embryos were transferred to them. They were then put in an incubator at 5°C to rehydrate for 24 h. The indirectly rehydrated embryos were rehydrated over water in a magenta jar and were held above the water on a wire mesh. The sides of the jar were lined with filter paper. The 25°C indirectly rehydrated embryos were placed in magenta jars that had previously been warmed to room temperature and then put in an incubator at 25°C for 24 h to rehydrate. The 5°C indirectly rehydrated embryos were placed in magenta jars that had previously been cooled to 5°C, the jars being kept on ice while the dried embryos were transferred to them and put in an incubator at 5°C to rehydrate for 24 h.

After rehydration, the embryos were stained with a 1% TTC solution as described previously in section 7.2.1.1. and the pattern of damage was scored according to Appendix 6.1 and 6.2. Next, the stained embryos were placed in 1.2-ml cryovials (one embryo/vial) and the colour extracted by soaking them in 1-ml of 95% ethanol for 18 h. The ethanol was then poured into the spectrophotometer cell (a 1.5-ml cell), topped up to 1.5 ml with 95% ethanol, and the absorbance at 485 nm was measured. The TTC score was totaled for each treatment and adjusted for a total of 20 embryos. The absorbance readings were also summed for each treatment and adjusted for a total of twenty embryos. The results were graphed using Microsoft Excel.

7.2.1.3 Correlation of TTC results with germination results

A total of 140 embryos of clone 6 x 5 were used. They were produced as discussed previously in section 7.2.1.1. The embryos were dried in the laminar flow hood for 5, 10, 15, 30, 60 and 240 min (20 embryos for every desiccation duration). Twenty embryos were killed by autoclaving to use as the dead control. After desiccation, the embryos were rehydrated directly at 24°C on germination medium, and after each desiccation period, one half of the embryos (10) were removed from the germination medium and stained with 1% TTC as previously described in section 7.2.1.1. After 18 h, the pattern of damage was scored according to Appendix 6.1 and 6.2. The remaining embryos were left on germination medium and placed in the growth room at 24°C with a 24h photoperiod, and after 21 d, the number of dead embryos was recorded. The living germinants were assessed for the presence of normal elongating cotyledons, hypocotyls and roots (quality categories 1-8 Appendix 2) while swollen germinants with bumpy or shiny surfaces were scored separately according to the vitreous categories in Appendix 3. Germination response was compared to TTC scores.

7.2.2 The effect of pre-treatment of the embryos and rehydration method on embryo viability and germinant quality

7.2.2.1 Effect of Rehydration Method on Embryo Viability and Germinant Quality

7.2.2.1.1 Experimental Method

White spruce embryogenic tissue from cryopreserved clones 6 x 5 and 6 x 3, as described previously in section 3.2.1, were used to produce mature embryos for the experiment. There were 2 runs. For run 1, approximately 620 mature embryos (~6 weeks of age) from clone 6 x 5 were harvested and used immediately. A total of 320 embryos were air-dried for 2h in the laminar flow hood. These were rehydrated (as described previously in section 7.2.1.2) either directly on germination medium at a temperature of 5 or 24°C, or indirectly in an atmosphere approaching 100% RH at 5 or 24°C. For each temperature and method of rehydration, there were 80 embryos. The remaining 300 embryos were used as controls. The control embryos were not desiccated, but 270 of them were put through the rehydration treatments (60 embryos per rehydration method x temperature). The remaining 60 embryos were not subjected to rehydration.

The effect of rehydration on embryo viability was determined by staining one half of the embryos from each rehydration method (40 embryos) with 1% TTC for 18 h (as described previously in section 7.2.1.1). Damage was assessed by scoring the pattern of damage (Appendix 6.1 and 6.2) and then measuring the intensity of the colour extracted in 95% ethanol (as described in section 7.2.1.2). The effect of the rehydration method on germinant quality was assessed by germinating the remaining embryos (40 from each rehydration method) on germination medium for 21 d and scoring the quality of the resulting germinants as described previously in section 7.2.1.3.

Run 2 used 360 embryos of each of clones 6 x 5 and 6 x 3. Prior to the experiment, the mature embryos were stored for 1 month on plates of maturation medium in a 5°C incubator. The embryos were dried and rehydrated following the method in run 1 except that only 60 embryos were used as the controls and these control embryos were not subjected to rehydration.

7.2.2.1.2 Statistical models and analysis

The germination responses expressed as the percentage of germinants in each quality category, were subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + C_i + R_j + T_k + CR_{ij} + CT_{ik} + RT_{jk} + CRT_{ijk} + e_{ijkl} \qquad [7.1]$$

Where Y_{ijkl} is the percentage of germinated embryos of the *i*th clone rehydrated by the *j*th rehydration method at the *k*th temperature. C_i is the effect of the *i*th clone (6 x 5 fresh, 6 x 5 cold stored, 6 x 3 cold stored); R_j is the effect of the *j*th rehydration method (direct, indirect); T_k is the effect of the *k*th temperature (5, 24°C); CR_{ij} is the interaction effect of the *i*th clone and the *j*th rehydration method; CT_{ik} is the interaction effect of the *i*th clone and the *k*th temperature; RT_{jk} is the interaction effect of the *j*th rehydration method and the *k*th temperature; CRT_{ijk} is the interaction effect of the *i*th clone, the *j*th rehydration method and the *k*th temperature; e_{ijkl} is the random error component. All main effect terms were considered to be fixed effects. The data for each germinant quality category was individually tested for normality. Computations of analysis of variance were performed on the transformed non-zero data for each quality category separately using the SAS GLM procedure. The percentage data in category 1 was transformed using the formula arcsin(sqrt(x/100)), in categories 3, 7 and dead using the formula log(x+1), in categories 4 and 8 using the formula sqrt(x) and in category 5 using the formula 1/(x+1). The means data are based on actual percentages. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

The percentage of germinants in all vitreous categories (1v-3v, 5v-7v) was summed to examine the change in the total percentage of vitreous germinants. Prior to statistical analysis using the SAS GLM procedure, the non-zero vitreous data was transformed using the formula arcsin(sqrt(x/100)).

The combined response of the cotyledons and hypocotyls (C+H) was examined by summing the percentage of germinants with undamaged hypocotyls and cotyledons (categories 1+5). The non-zero data was normalized prior to analysis using the formula sqrt(x).

The effect of the rehydration treatments on the percentage of germinants with elongating roots was examined by summing the percentage of germinants in categories 1, 1v, 2, 2v, 3, 3v and 4. The non-zero data was normalized prior to analysis using the formula arcsin(sqrt(x/100)).

The viability data were also individually subjected to analysis of variance using the model in equation 7.1. The TTC scores and absorbance readings were tested for normality and computations of analysis of variance using the SAS GLM procedure were performed on the untransformed data. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

7.2.2.2 Effect of the length of the indirect rehydration treatment on embryo viability and subsequent germinant quality of slowly dried and flash desiccated fresh and cold stored embryos.

7.2.2.2.1 Culture Conditions

The experiment used 3360 mature white spruce somatic embryos (~5 weeks of age) from clone 6 x 5. The mature embryos were randomly picked from those produced by new and old embryogenic tissue during run 2 of the squash experiment (see section 3.2.5) and transferred to fresh plates of maturation medium 2 (50 embryos per plate for a total of 68 plates). The embryos from the new and old tissue were kept separate during the experiment. Prior to desiccation, the embryos were stored in an incubator at 5°C in the dark for a period of 0, 2, 4, or 8 weeks (cold storage treatment).

7.2.2.2.2 Desiccation

After each cold storage treatment, 840 of the embryos (420 from new tissue and 420 from old tissue) were flash desiccated or dried slowly. A total of 420 of the embryos (210 from new tissue and 210 from old tissue) were flash desiccated by placing the embryos on sterilized screens (approximately 25 embryos per screen) on top of petri dishes in the unobstructed airflow of a laminar flow hood for 4h. The remaining embryos were slowly dried by suspending them in a closed system at 5°C over a -10 MPa NaCl solution (Appendix 1) for 7 d as described previously in section 4.2.2. There were 70 vials of embryos from new tissue and 70 vials of embryos from old tissue with 3 embryos per vial.

7.2.2.2.3 Rehydration and Germination

The desiccated embryos were rehydrated over water for 0, 1, 2, 3, 4, 16, and 32 h using a closed system (as previously described in section 4.2.2). Rehydration was carried out at 5 or 20°C. The embryos were then placed on germination medium (15 from new tissue, 15 from old tissue for each rehydration period) and allowed to grow in the growth room for 21 d at 24°C under continuous light. The effect of the rehydration method on germinant quality was assessed by scoring the quality of the resulting germinants as described previously in section 7.2.1.3.

7.2.2.2.4 Statistical models and analysis

The flash desiccation and slow desiccation results were analyzed separately. Preliminary analysis indicated that there was no significant difference in the response to the rehydration treatments of the embryos produced by new or old tissue. Therefore, the results from the old and new tissue were treated as replications and analyzed as such. The germination responses, expressed as the percentage of germinants in each quality category, were subjected to analysis of variance using the model:

$$Y_{ijkl} = \mu + D_i + R_j + T_k + DR_{ij} + DT_{ik} + RT_{jk} + DRT_{ijk} + e_{ijkl} \qquad [7.2]$$

Where Y_{ijkl} is the percentage of germinated embryos stored for the *i*th duration at 5°C prior to desiccation and rehydrated for *j* h at the *k*th temperature. D_i is the effect of the *i*th duration of the cold treatment (0, 2, 4, 8 weeks); R_j is the effect of the *j*th length of the rehydration treatment (0, 1, 2, 4, 8, 16, 32 h); T_k is the effect of the *k*th temperature (5, 20°C); DR_{ij} is the interaction effect of the *i*th duration and the *j*th rehydration treatment; DT_{ik} is the interaction effect of the *i*th duration and the *k*th temperature; RT_{jk} is the interaction effect of the *j*th rehydration treatment and the *k*th temperature; DRT_{ijk} is the interaction effect of the *i*th duration, the *j*th rehydration treatment and the *k*th temperature; e_{ijkl} is the random error component. All main effect terms were considered to be fixed effects. The data for each quality category was individually tested for normality. Computations of analysis of variance were performed on the transformed non-zero data for each quality category separately using the SAS GLM procedure.

For the flash desiccated embryos (air-dried), the non-zero percentage data in category 8 was transformed using the formula arcsin(sqrt(x/100)); in categories 1v, 2v, 3, 5v, 7v and dead using the formula log(x+1), in categories 1 and 4 using the formula sqrt(x); in categories 2, 3v, 5, 6 and 6v using the formula 1/(x+1). Computations of analysis of variance were also performed on the non-transformed non-zero category 7 data. The means data are based on actual percentages. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

The percentage of germinants in all vitreous categories (1v-3v, 5v-7v) was summed to examine the change in the total percentage of vitreous germinants. Prior to statistical analysis using the SAS GLM procedure, the non-zero vitreous data was transformed using the formula arcsin(sqrt(x/100)).

The combined response of the cotyledons and hypocotyls (C+H) was examined by summing the percentage of germinants with undamaged hypocotyls and cotyledons (categories 1+5). The non-zero data was normalized prior to analysis using the formula sqrt(x).

For the slowly desiccated embryos, the percentage data in category 1 was transformed using the formula arcsin(sqrt(x/100)). The non-zero percentage data in categories 1v, 3v, 4 and 5 was transformed using the formula log(x+1), in category 5v using the formula sqrt(x), in categories 2, 2v, 3, 6v, 7, 7v, 8 and dead using the formula 1/(x+1). Computations of analysis of variance were performed on the non-transformed non-zero category 6 data. The means data are based on actual percentages. The Duncan's multiple range test at the significance level of $\alpha \leq 0.05$ was performed on all main-effect means.

The percentage of germinants in all vitreous categories (1v, 2v, 3v, 5v, 6v, and 7v) was summed to examine the change in the total percentage of vitreous germinants. Prior to statistical analysis using the SAS GLM procedure, the vitreous data was transformed using the formula arcsin(sqrt(x/100)).

The combined response of the cotyledons and hypocotyls (C+H) was examined by summing the percentage of germinants with undamaged hypocotyls and cotyledons (categories 1+5). The data was normalized prior to analysis using the formula arcsin(sqrt(x/100)).

The effect of the rehydration treatments on the percentage of germinants with elongating roots was examined by summing the percentage of germinants in categories 1, 1v, 2, 2v, 3, 3v and 4. The data was transformed using the formula arcsin(sqrt(x/100)).

7.3 Results

7.3.1 Damage Assessment

7.3.1.1 Assessment of Damage using TTC

TTC stain showed which tissues have been killed by the various desiccation and rehydration treatments. The dead areas in the embryos were clearly delineated by the absence of red colouration enabling the pattern of damage to be divided into 5 main categories based on the extent of the colour loss (Appendix 6.1, 6.2).

7.3.1.2 Correlation of TTC scoring method with spectrophotometer readings

The undesiccated control embryos stained completely red with TTC while the embryos killed by autoclaving did not stain at all. Progressive damage was shown by a decrease in the percentage of the embryo that stained red. Extraction and measurement of the intensity of this colour gave a good indication of the amount of the embryo that was damaged by each desiccation and rehydration treatment with higher absorbance values indicating less damage (Graph 7.1.1). These results suggest that desiccation of embryos for more than 0.5h causes severe damage to the embryos as shown by the sharp drop in absorbance readings in embryos desiccated for 1h and longer. The readings for the 0.25 and 0.5h desiccated embryos were also higher for the directly rehydrated embryos than for the indirectly rehydrated embryos suggesting that direct rehydration on germination medium causes less damage than indirect rehydration in an atmosphere approaching 100% RH. This difference is less pronounced in the embryos that have been desiccated for longer periods of time.

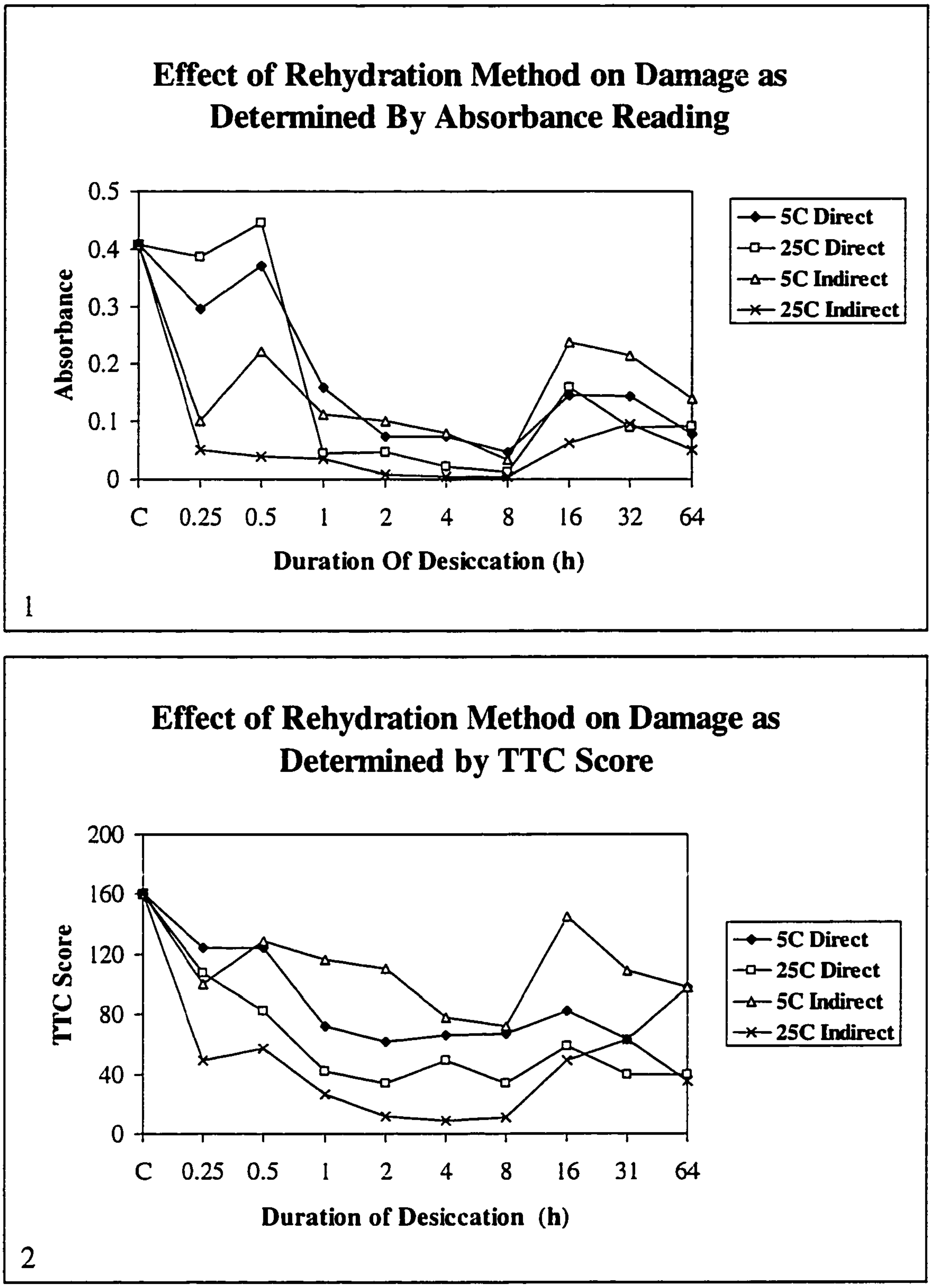
Examination of the pattern of damage before the colour was extracted separated the individual embryos into one of the damage categories. A damage value was arbitrarily assigned to each damage class (Appendix 6.2). Multiplying the number of embryos in each category by the damage value for that category, summing the results for each treatment, and then adjusting the scores to give a value out of a total of 20 embryos gave the TTC damage score for that treatment (Appendix 7). This allowed comparisons to be made between the damage caused by different treatments (Graph 7.1.2). Again, the results showed that damage increased with prolonged exposure to the airflow, but the

Graph 7.1. Comparison of two different methods of using TTC staining to quantify rehydration damage. The air-dried embryos were rehydrated using different rehydration methods.

Graph 7.1.1. The effect of rehydration method on damage as determined by absorbance reading. The red colour was extracted from the embryos and the absorbance at 485 nm measured.

Graph 7.1.2. The effect of rehydration method on damage as determined by TTC score. The pattern of the living and dead tissue was scored according to the TTC scoring system.

Graph 7.1.


Effect of Rehydration Method on Damage as Determined By Absorbance Reading
0.5
0.4
0.3
0.2
0.1
0
Absorbance
C
0.25
0.5
1
2
4
8
16
32
64
Duration Of Desiccation (h)
5C Direct
25C Direct
5C Indirect
25C Indirect
1
Effect of Rehydration Method on Damage as Determined by TTC Score
200
160
120
80
40
0
TTC Score
C
0.25
0.5
1
2
4
8
16
31
64
Duration of Desiccation (h)
5C Direct
25C Direct
5C Indirect
25C Indirect
2

results were not as striking as with the absorbance readings. The TTC scores suggested that rehydration at 5°C, whether direct or indirect, caused less damage than rehydration at 24°C. Comparison of the absorbance readings and the TTC damage scores (Graphs 7.2a-4) show that the two scoring methods show similar trends in damage assessment, but the TTC scoring method may under assess the damage exhibited by embryos rehydrated at 5°C, especially by indirect rehydration.

7.3.1.3 Correlation of TTC results with germination results

The damage assessment with TTC and the visual damage seen in germinated embryos correlated. Both the viability and quality declined if the embryos were desiccated longer than 15 min. The TTC scores ranged from 80-66 from the embryos that were desiccated for 5-15 min to 32 and 20 for the 30 and 60 min desiccated embryos (Table 7.1.1). The percentage of quality category 8 germinants (i.e. severely damaged germinants exhibiting unorganized growth) increased from 0 for the 5-15 min desiccated embryos to 10, 60 and 90% for the 30, 60 and 240 min desiccated embryos (Table 7.1.2).

However, the most interesting result from this experiment was that although the TTC viability test shows that the embryo is viable and has suffered no damage, this does not foretell the quality of the germinant. For example, all of the 5 min desiccated embryos were rated as category A (undamaged) by the TTC test (Table 7.1.1), but none of the germinated 5-min desiccated embryos produced germinants that were classed as quality category 1 (i.e. with normal elongating cotyledons, hypocotyls and roots). Sixty percent of them had normal elongating cotyledons and hypocotyls, but no roots. The remaining 40% were vitreous (Table 7.1.2). However, according to the TTC test, they showed no visible signs of damage.

Graph 7.2. Comparison of two different methods of using TTC staining to quantify rehydration damage. The air-dried embryos were rehydrated using different rehydration methods and temperatures. The colour was extracted with ethanol and the absorbance measured at 485 nm (Abs.), or the TTC scoring method was used to score the pattern of living and dead tissue (TTC). The embryos were rehydrated by the following methods:

Graph 7.2.1. Direct rehydration at 5°C
Graph 7.2.2. Direct rehydration at 25°C
Graph 7.2.3. Indirect rehydration at 5°C
Graph 7.2.4. Indirect rehydration at 25°C

Each data point is the average value of 20 embryos.

Graph 7.2.

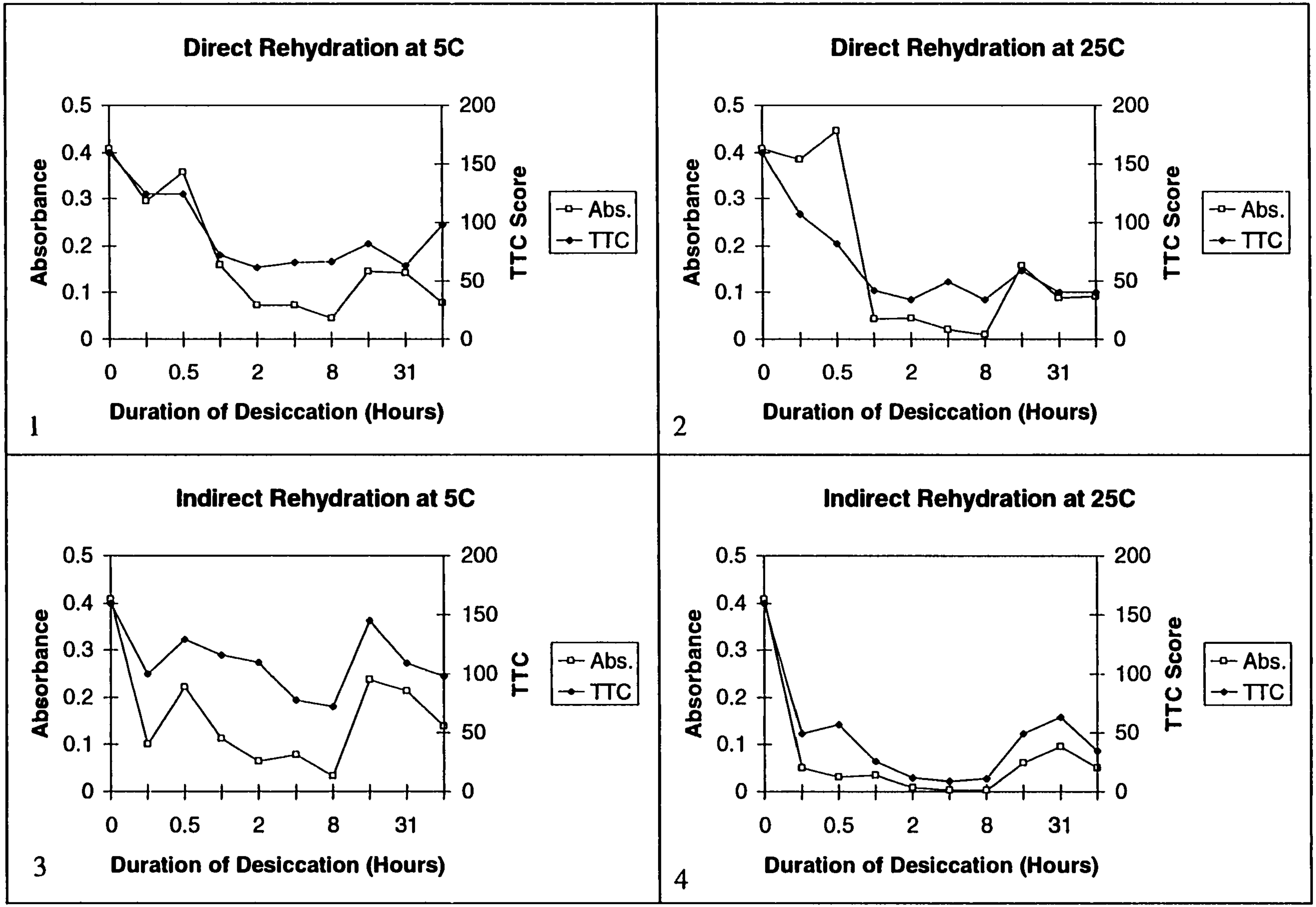

Direct Rehydration at 5C
Absorbance
TTC Score
0.5
0.4
0.3
0.2
0.1
0
200
150
100
50
0
0
0.5
2
8
31
Duration of Desiccation (Hours)
Abs.
TTC
1
Direct Rehydration at 25C
Absorbance
TTC Score
Duration of Desiccation (Hours)
Abs.
TTC
2
Indirect Rehydration at 5C
Absorbance
TTC
Duration of Desiccation (Hours)
Abs.
TTC
3
Indirect Rehydration at 25C
Absorbance
TTC Score
Duration of Desiccation (Hours)
Abs.
TTC
4

Table 7.1. The comparison of two different methods of quantifying damage caused to embryos by air-drying them for various lengths of time followed by direct rehydration at 25°C:

Table 7.1.1. TTC Scoring Method (the TTC score was determined by multiplying the number of embryos in each TTC damage category (A-E) by the damage category score (8), (4), (2), (1) or (0), adding all category values and adjusting for a total of 10 embryos).

Table 7.1.2. Germinant Quality Assessment (the percentage of the 10 embryos that were in each quality category).

Table 7.1.1.

	TTC Damage Category and Score ( )							
Duration of Desiccation	A (8)	B (4)	C (2)	D (1)	E (0)	Total # Embryos	Score	Adjusted Score
5 min	10	0	0	0	0	10	80	80
10 min	8	1	1	0	0	10	70	70
15 min	7	2	1	0	0	10	66	66
30 min	2	0	8	0	0	10	32	32
60 min	0	0	8	0	0	8	16	20
240 min	0	0	10	0	0	10	20	20

Table 7.1.2.

	Germinant Quality Category								
Duration of Desiccation	1	2	3	4	5	6	7	8	Vitreous
5 min	-	-	-	-	60	-	-	-	40
10 min	-	-	-	-	80	-	-	-	20
15 min	-	-	-	-	60	-	10	-	30
30 min	-	-	-	-	-	-	-	10	90
60 min	-	-	-	-	-	-	40	60	-
240 min	-	-	-	-	-	-	10	90	-

7.3.2 The effect of pre-treatment of the embryos and rehydration method on embryo viability and germinant quality

7.3.2.1 Effect of rehydration method on embryo viability and germinant quality

Germinants of 10 of the 15 possible quality categories were produced (Appendix 8). However, because the percentage of all vitreous germinants only totaled 5.79%, they will be discussed as one group.

The analysis of variance showed that clone (6 x 5 fresh, 6 x 5 cold stored and 6 x 3 cold stored) had a significant effect at the 0.01% level on the percentage of germinants in quality category 8 (unorganized growth) and on the percentage of embryos killed by the treatments, and at the 0.05% level on the percentage of category 3 germinants (Table 7.2). The TTC score and absorbance assessment of viability also showed that clone had a significant effect on an embryo's ability to survive desiccation. As well, the method of rehydration (direct or indirect) had a significant effect at the 0.01% level on the percentage of dead embryos. TTC assessment showed that method of rehydration influenced embryo viability, but temperature had no significant influence on the results, although the clone x temperature interaction had a significant effect at the 0.05% level on the percentage of dead embryos. The TTC method of viability assessment showed that temperature, clone x temperature and clone x temperature x rehydration method interactions had a significant influence on embryo viability, but the absorbance reading did not duplicate these results.

The Duncan's test results on the effect of the clone (averaged over all treatment combinations) suggest that cold stored embryos are better able to withstand air-drying than fresh embryos (Table 7.3.1). Over 48% of the embryos from fresh clone 6 x 5 were dead, while the remaining 51.6% produced severely damaged germinants classed as quality category 8. Conversely, both of the cold stored clones (6 x 5 and 6 x 3) produced a few category 8 germinants (6.2 and 9.9%) and even fewer dead embryos (3.8% and 0.0%). In fact, the majority of the cold-stored germinants suffered none to slight damage

Table 7.2. Analysis of variance summary table for the effects of clone, temperature at which the embryos were rehydrated and rehydration method on the ability of flash desiccated embryos to survive various rehydration treatments as measured by embryo viability and the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category was analyzed separately. Viability, as assessed by the TTC scoring method and measurement of the absorbance at 485 nm were also analyzed separately. F-values are significant at the 0.05(*), 0.01(**), and 0.001(***) level.

Table 7.2.

	Quality Category							
	Category 1		Category 3		Category 4		Category 5	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Clone (C)	1	0.18	1	6.51*	1	0.00	1	0.00
Temperature (T)	1	0.21	1	0.01	1	1.14	1	0.57
Rehydration (R)	1	0.02	1	4.17	1	0.06	1	1.39
C x T	1	0.01	1	0.02	1	0.08	1	0.00
C x R	1	0.18	1	5.51	1	0.06	1	0.12
T x R	0		1	1.00	1	1.80	0	
C x T x R	0		1	0.47	1	0.08	0	
Error	3		6		4		3	
Corrected total	8		13		11		8	

	Quality Category							
	Category 7		Category 8		Dead		Vitreous	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Clone (C)	1	3.82	2	18.87**	1	42.91**	1	0.00
Temperature (T)	1	0.02	1	1.93	1	3.33	0	
Rehydration (R)	1	3.97	1	7.30*	1	42.98**	1	2.75
C x T	0		2	0.04	1	12.02*	0	
C x R	1	1.58	1	1.15	0		0	
T x R	0		1	0.01	1	0.14	0	
C x T x R	0		1	0.85	0		0	
Error	2		5		4		2	
Corrected total	6		14		9		4	

	Quality Category				Viability Assessment			
	Roots		C+H		TTC		Absorbance	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Clone (C)	1	2.77	1	0.01	2	28.56***	2	36.6***
Temperature (T)	1	0.06	1	0.04	1	23.83***	1	1.23
Rehydration(R)	1	4.41	1	0.37	1	23.03***	1	0.03
C x T	1	0.01	1	0.05	2	6.41*	2	0.42
C x R	1	4.01	1	0.00	2	0.05	2	0.08
T x R	1	0.00	1	0.01	1	0.01	1	0.12
C x T x R	1	0.30	1	0.02	2	10.16**	2	0.34
Error	8		6		12		12	
Corrected total	15		13		23		23	

Table 7.3.1. Duncan's Multiple Range Test for the effects of clone on the ability of flash desiccated embryos to survive the various rehydration methods as measured by embryo viability and the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Viability as assessed by the TTC scoring method and measurement of the absorbance at 485 nm were also analyzed separately. Data are means averaged over both rehydration temperatures and methods. Means within the same quality category (or viability assessment method) with different letters are significantly different (p=0.05) from each other.

Table 7.3.2. Duncan's Multiple Range Test for the effects of rehydration temperature on the ability of flash desiccated embryos to survive the various rehydration methods as measured by embryo viability and the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Viability as assessed by the TTC scoring method and measurement of the absorbance at 485 nm were also analyzed separately. Data are means averaged over three clones and both rehydration methods. Means within the same quality category (or viability assessment method) with different letters are significantly different (p=0.05) from each other.

Table 7.3.1.

	Clone		
Quality Assessment (Germination Test)			
Quality Category	6 x 5 Fresh	6 x 5 Cold Stored	6 x 3 Cold Stored
1	0.0	16.5a	10.6a
3	0.0	31.1b	15.2a
4	0.0	16.7a	24.7a
5	0.0	13.9a	10.7a
7	0.0	5.0a	17.1a
8	51.6a	6.2b	9.9b
Dead	48.4a	3.8b	0.0
Combined Categories			
Vitreous	0.0	6.8a	10.6a
Roots	0.0	68.3a	51.7a
C+H	0.0	30.4a	21.4a
Viability Assessment			
TTC Score	68b	136a	128a
Absorbance	1.819b	10.752a	10.095a

Table 7.3.2.

	Temperature	
Quality Assessment (Germination Test)		
Quality Category	5°C	24°C
1	7.5a	10.6a
3	12.4a	18.5a
4	16.6a	11.0a
5	8.4a	8.0a
7	8.9a	5.8a
8	27.7a	17.4a
Dead	13.9a	20.8a
Combined Categories		
Vitreous	4.5a	7.0a
Roots	39.2a	40.9a
C+H	15.9a	18.6a
Viability Assessment		
TTC Score	130a	91b
Absorbance	8.082	7.028

and were classed as categories 1, 3, 4, 5, or 7. The results from both of the methods of viability testing also supported this conclusion.

Rehydration at a temperature of 24°C resulted in fewer dead embryos, but the difference was not statistically significant (Table 7.3.2). Overall, the temperature at which the embryos were rehydrated did not significantly affect the quality of the embryos. This finding was supported by the absorbance readings, with a temperature of 5°C producing a slightly higher, but not significantly different absorbance reading than at 24°C. However, this difference was significantly higher when measured with the TTC scoring method.

The method of rehydration significantly affected the quality of the embryos. Indirect rehydration produced significantly fewer category 8 germinants and more dead embryos (Table 7.4). There was no significant difference between the percentage of embryos in the other quality categories, but direct rehydration produced more category 1 germinants and germinants with elongating roots and undamaged cotyledons + hypocotyls. The TTC viability assessment methods showed the opposite results, with indirect rehydration producing embryos with a higher TTC score. The absorbance readings from the indirectly rehydrated embryos, although slightly higher, were not significant.

Comparison of the TTC viability test results with the germinant quality results (Table 7.5) again shows that TTC is not useful for predicting the quality of the germinated embryos. For example, all undesiccated control embryos were classed as TTC category A (no damage), but in all three clones, 35% or less of the germination test control embryos germinated into quality category 1 germinants. The majority of the germinants were vitreous. The remainder had normal cotyledons and hypocotyls, but no elongating roots (quality category 5). The other point to note from this table is that, for both cold stored clones, desiccation decreased the percentage of vitreous germinants (eg. for cold stored clone 6 x 5 the percentage of vitreous germinants decreased from 80% for the control to 0 to 27.2% for the desiccated embryos).

Table 7.4. Duncan's Multiple Range Test for the effects of rehydration method on the ability of flash desiccated embryos to survive the various rehydration methods as measured by embryo viability and the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Viability as assessed by the TTC scoring method and measurement of the absorbance at 485 nm were also analyzed separately. Data are means averaged over three clones and both rehydration temperatures. Means within the same quality category (or viability assessment method) with different letters are signifiantly different (p=0.05) from each other.

Table 7.4.

	Rehydration Method	
Quality Assessment (Germination Test)		
Quality Category	Direct	Indirect
1	12.7a	5.4a
3	22.2a	8.6a
4	11.8a	15.8a
5	7.5a	8.9a
7	4.2a	10.6a
8	29.4a	15.8b
Dead	8.9b	25.8a
Combined Categories		
Vitreous	2.5a	9.1a
Roots	47.5a	32.5a
C+H	20.2a	14.3a

Viability Assessment		
TTC Score	91b	130a
Absorbance	7.476a	7.634a

Table 7.5. Comparison of three different methods of quantifying damage caused by air-drying embryos followed by direct and indirect rehydration at a temperature of 5 or 24°C: a.) TTC Scoring Method (the TTC score was determined by multiplying the number of embryos in each TTC damage category (A-E) by the damage category score (8), (4), (2), (1) or (0), adding all category values and adjusting for a total of 20 embryos), b.) The TTC absorbance reading was the average of 40 embryos, c.) Germinant Quality Assessment (the percentage of the 40 embryos that were in each quality category).

Table 7.5.

Clone 6 x 5 Fresh	TTC Damage Category and Score ( )							
Rehydration Method (RM)	A (8)	B (4)	C (2)	D (1)	E (0)	Total # Embryos	Score (/ 20)	Absorbance (average)
Control	150	0	0	0	0	150	160	9.058
Direct 5C	2	24	0	12	2	40	62	2.584
Direct 24C	0	5	18	7	10	40	32	1.202
Indirect 5C	35	3	0	0	0	38	154	3.210
Indirect 24C	0	0	18	8	10	36	24	0.258

Clone 6 x 5 Fresh	Germinant Quality Category									
RM	1	3	4	5	7	8	Dead	Vitr.	Roots	C+H
Control	35.0	0.0	0.0	8.0	0.0	0.0	2.5	54.5	41.6	43.0
Direct 5C	0.0	0.0	0.0	0.0	0.0	78.9	21.1	0.0	0.0	0.0
Direct 24C	0.0	0.0	0.0	0.0	0.0	67.5	32.5	0.0	0.0	0.0
Indirect 5C	0.0	0.0	0.0	0.0	0.0	47.5	52.5	0.0	0.0	0.0
Indirect 24C	0.0	0.0	0.0	0.0	0.0	12.5	87.5	0.0	0.0	0.0

Clone 6 x 5 Cold Stored	TTC Damage Category and Score ( )							
Rehydration Method (RM)	A (8)	B (4)	C (2)	D (1)	E (0)	Total # Embryos	Score (/ 20)	Absorbance (average)
Control	14	0	0	0	0	14	160	11.226
Direct 5C	18	3	0	0	0	21	148	11.636
Direct 24C	3	21	0	0	0	24	89	9.932
Indirect 5C	17	3	0	0	0	20	148	10.796
Indirect 24C	19	0	0	0	0	19	160	10.646

Clone 6 x 5 Cold Stored	Germinant Quality Category									
RM	1	3	4	5	7	8	Dead	Vitr.	Roots	C+H
Control	20.0	0.0	0.0	0.0	0.0	0.0	0.0	80.0	0.0	20.0
Direct 5C	25.0	40.0	20.0	15.0	0.0	0.0	0.0	0.0	85.0	40.0
Direct 24C	31.1	58.3	5.6	0.0	5.0	0.0	0.0	0.0	95.0	31.1
Indirect 5C	10.0	11.1	11.1	20.6	0.0	10.0	10.0	27.2	48.3	30.6
Indirect 24C	0.0	15.0	30.0	12.9	15.0	15.0	5.0	0.0	45.0	20.0

Clone 6 x 3 Cold Stored	TTC Damage Category and Score ( )							
Rehydration Method (RM)	A (8)	B (4)	C (2)	D (1)	E (0)	Total # Embryos	Score (/ 20)	Absorbance (average)
Control	10	1	0	0	0	11	153	5.972
Direct 5C	11	9	0	0	0	20	124	9.303
Direct 24C	3	16	0	0	0	19	93	10.200
Indirect 5C	13	2	0	0	0	15	146	10.945
Indirect 24C	17	3	0	0	0	20	148	9.932

Clone 6 x 3 Cold Stored	Germinant Quality Category									
RM	1	3	4	5	7	8	Dead	Vitr.	Roots	C+H
Control	30.0	0.0	0.0	20.0	0.0	0.0	0.0	50.0	80.0	50.0
Direct 5C	0.0	15.0	40.0	15.0	5.0	25.0	0.0	0.0	55.0	15.0
Direct 24C	20.0	20.0	5.0	15.0	15.0	5.0	0.0	15.0	50.0	35.0
Indirect 5C	10.0	8.3	28.3	0.0	48.3	5.0	0.0	0.0	46.7	10.0
Indirect 24C	12.5	17.4	25.4	12.9	0.0	4.5	0.0	27.3	55.3	25.4

TTC is a method of showing where death has occurred in embryos. It is possible to correlate the damage seen in the five TTC damage classes with damage shown in germinated embryos. Undamaged TTC category A embryos (Appendix 6.1) usually germinate into undamaged germinants. They may be normal (Fig.7.1.1), without an elongating root (Fig. 7.1.2), vitreous with an elongating root (Fig. 7.1.3) or vitreous without an elongating root (Fig. 7.1.4). The TTC Category B embryos frequently germinate with damage visible somewhere on the seedling, either on the cotyledons (Fig. 7.1.5-F), or on the cotyledons and hypocotyl (Fig. 7.2.1-2). The TTC category C embryos usually have a layer of dead tissue on the outside of the embryo and living shoot and root meristem (as shown by the pattern of red colouration (Appendix 6.1)), a pattern which also shows up very clearly in some of the germinants (Fig. 7.2.3). The TTC stain suggests that the shoot meristem is still alive. A normal shoot (Fig. 7.2.4) or shoot and root (Fig. 7.2.5) will often grow from the damaged embryo even though the hypocotyl is severely damaged or dead. TTC damage class D embryos have a living root meristem producing germinants that have a living root, but dead hypocotyl and cotyldeons (Fig.7.2.6). TTC damage class E embryos are dead.

7.3.2.2 Effect of the length of the indirect rehydration treatment on embryo viability and subsequent germinant quality of slowly dried and flash desiccated fresh and cold stored embryos.

7.3.2.2.1 Flash desiccated (air-dried) embryos

The GLM statistical analysis showed that of the three main-effect terms, the duration of the cold treatment prior to the air-drying of the embryos had the most significant influence on the germination response of the embryos. The influence of duration (0, 2, 4, 8 weeks) on the percentage of embryos in categories 4 and 8 and on the percentage in the combined categories vitreous, undamaged roots, and undamaged cotyledons+hypocotyls (C+H) was significant at the 0.001% level; on the percentage in categories 1 and dead at the 0.01% level; and on the percentage of germinants in category 5 at the 0.05% level (Table 7.6).

Figure 7.1. Germinants showing various types of damage as a result of different desiccation and rehydration treatments. The cotyledons (C) and hypocotyl (H) were usually the most seriously affected. Some germinants had an elongating root (R) or only a root apex (RA).

Figure 7.1.1. Undamaged germinant with a normal elongating root. Magnification is [3.5x].

Figure 7.1.2. Undamaged germinant without an elongating root. Magnification is [3.5x].

Figure 7.1.3. Vitreous germinant with a normal elongating root. Magnification is [4.8x].

Figure 7.1.4. Vitreous germinant without an elongating root. Magnification is [3.5x].

Figure 7.1.5. Germinant with damaged cotyledons and a normal elongating root. Magnification is [3.5x].

Figure 7.1.6. Germinant with damaged cotyledons and without an elongating root. Magnification is [3.5x].

Figure 7.1.

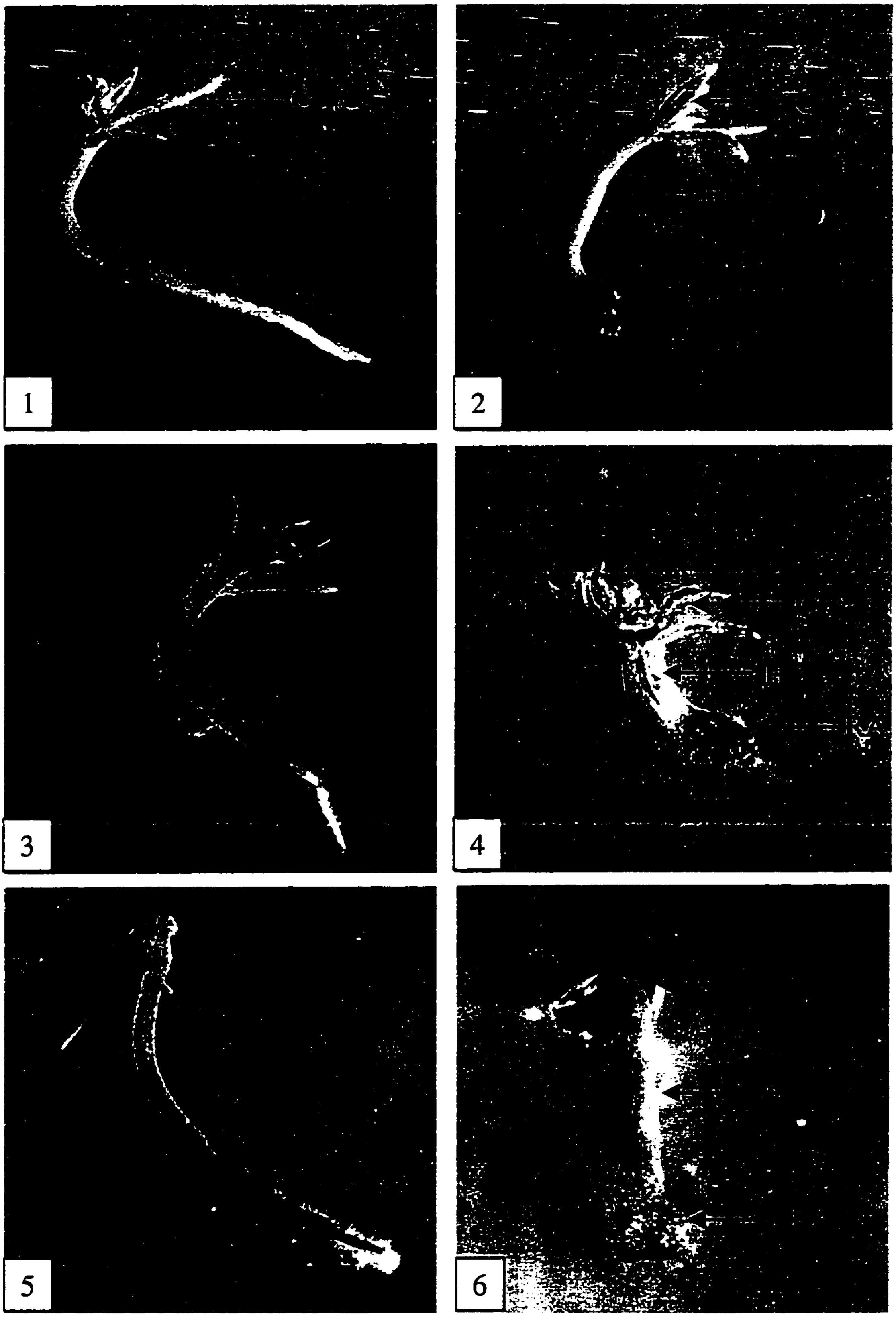


Figure 7.2. Germinants showing various types of damage as a result of different desiccation and rehydration treatments. The cotyledons (C) and hypocotyl (H) were usually the most seriously affected. Some germinants had an elongating root (R) or only a root apex (RA).

Figure 7.2.1. Germinant with cotyledon and hypocotyl damage but with an elongating root. Magnification is [3.5x].

Figure 7.2.2. Germinant with cotyledon and hypocotyl damage and without an elongating root. Magnification is [7x].

Figure 7.2.3. Germinant with severe cotyledon and hypocotyl damage and without an elongating root. Magnification is [9.4x].

Figure 7.2.4. Germinant with severe cotyledon and hypocotyl damage and without an elongating root. A normal shoot (S) is growing from the shoot apex. Magnification is [7x].

Figure 7.2.5. Germinant with severe cotyledon and hypocotyl damage and without an elongating root. A normal shoot (S) is growing from the shoot apex. Magnification is [3.5x].

Figure 7.2.6. Germinant with dead cotyledons and hypocotyl but with a growing root. Magnification is [3.5x].

Figure 7.2.

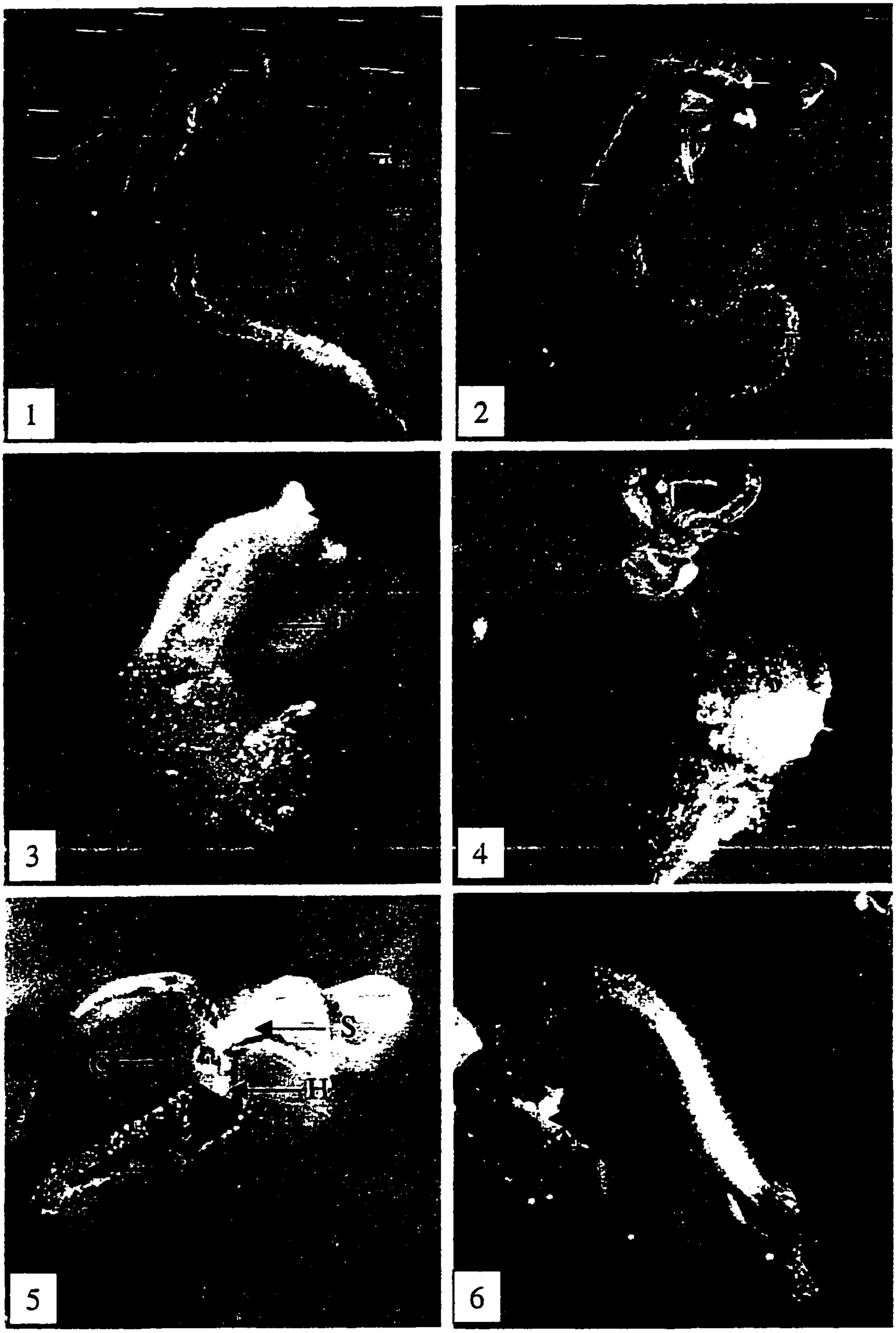


Table 7.6. Analysis of variance summary table for the effects of duration of the cold treatment (D), temperature of rehydration (T) and length of time the flash desiccated embryos were indirectly rehydrated (R) on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category was analyzed separately. Viability, as assessed by the TTC scoring method and measurement of the absorbance at 485 nm were also analyzed separately. F-values are significant at the 0.05(*), 0.01(**), and 0.001(***) level.

Table 7.6.

	Quality Category											
	Category 1		Category 2		Category 3		Category 4		Category 5		Category 6	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Duration (D)	3	5.26**	2	2.01	3	2.12	3	20.89***	2	6.72*	2	0.12
Temperature (T)	1	0.11	1	1.12	1	0.48	1	8.37**	1	1.69	1	0.25
Rehydration(R)	6	2.75*	6	4.30*	6	0.32	6	2.58*	6	2.23	4	0.39
D x T	3	1.36	1	4.78	2	0.67	2	1.15	2	0.29	1	0.03
D x R	8	0.23	7	2.26	6	0.38	14	1.75	9	0.64	2	0.17
T x R	6	0.55	5	0.89	1	0.09	6	0.70	4	1.07	2	0.91
D x T x R	6	0.84	1	1.70	0		12	0.50	1	7.90*	0	
Error	23		6		2		37		12		1	
Corrected total	56		29		21		81		37		13	

Table 7.6 cont'd

	Quality Category									
	Category 8		Dead		Vitreous		Roots		C+H	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Duration (D)	3	14.91***	3	9.81**	3	9.69***	3	18.47***	3	18.47***
Temperature (T)	1	13.17***	1	0.05	1	0.19	1	4.49*	1	0.12
Rehydration(R)	6	1.60	6	1.48	6	1.41	6	3.30**	6	3.60**
D x T	3	5.04**	2	0.40	3	0.33	3	3.29*	3	1.02
D x R	18	0.86	6	0.15	17	0.75	18	1.06	11	0.62
T x R	6	1.28	5	2.04	6	1.14	6	1.15	6	0.69
D x T x R	17	1.96*	1	0.72	14	0.68	18	0.60	8	0.87
Error	51		12		42		55		27	
Corrected total	105		36		92		110		65	

The temperature of rehydration only had a significant influence on the percentage of the germinants classed as category 4 (0.01%), category 8 (0.001%) and in the combined category of germinants with undamaged roots (0.01%) (Table 7.6).

The method of rehydration (0, 1, 2, 4, 8, 16, 32 h of indirect rehydration in moist air) had a significant influence at the 0.01% level on the percentage of germinants in the combined categories of roots and C+H, and at the 0.05% level on the percentage of germinants in the categories 1, 2 and 4 (Table 7.6).

The Duncan's test of effect of duration, temperature and rehydration method averaged over all treatments showed that tolerance to desiccation increased with the duration of the cold treatment and that the percentage of germinants in category 1 (normal) increased while the percentage of germinants in categories 4, 8, and dead decreased with duration (Table 7.7.1). Temperature had little influence on the distribution of the germinants within the various quality categories with only significantly more germinants of quality categories 4 and roots, and significantly less of category 8 being produced from rehydration at 5°C than at 24°C (Table 7.7.2). Overall, direct rehydration (0 rehydration) appeared to be the best method of rehydration. Rehydration of the air-dried embryos directly on germination medium produced more category 1 (normal) germinants and germinants with undamaged roots and cotyledons + hypocotyls but the differences were not statistically significant in most cases (Table 7.8). Direct rehydration also produced fewer category 8 and dead embryos although only the difference in percentage of dead embryos produced was significant.

Graphs of data from four of the germinant quality categories (Graphs 7.3-7.4) showed similar trends. There were only slight differences between the two rehydration temperatures for the same quality category data (e.g. graphs 7.3.1-2). Major differences were seen between the germination response of the embryos that had been cold-stored for various periods of time. Furthermore, whether the embryos had been directly rehydrated (0 rehydration) or not made a distinct difference. Desiccation tolerance increased with duration of the cold temperature with the largest numbers of normal germinants being

Table 7.7.1. Duncan's Multiple Range Test for the effect of the duration of the cold treatment prior to flash desiccation and indirect rehydration of the embryos on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Data are means averaged over four cold treatment durations and all methods of rehydration (h). Means within the same quality category with different letters are signifiantly different (p=0.05) from each other.

Table 7.7.2. Duncan's Multiple Range Test for the effect of the temperature at which flash desiccated embryos were indirectly rehydrated on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Data are means averaged over four cold treatment durations and all methods of rehydration (h). Means within the same quality category with different letters are signifiantly different (p=0.05) from each other.

Table 7.7.1.

	Duration			
Quality Category	0 Weeks	2 Weeks	4 Weeks	8 Weeks
1	0.2b	1.0ab	18.8ab	27.2a
2	0.0	0.8a	4.7a	6.5a
3	0.2a	2.1a	4.8a	1.6a
4	24.0a	31.6a	12.1b	1.1b
5	0.0	2.5ab	1.9a	10.2b
6	0.0	1.3a	0.2a	2.3a
8	44.5a	37.0a	22.7b	22.0b
Dead	25.6a	4.1b	0.8b	0.5b
Combined Categories				
Vitreous	5.4c	17.4b	30.9a	26.4ab
Roots	27.8c	46.0b	61.6a	47.0b
C+H	0.2b	3.5b	20.7a	37.3a

Table 7.7.2.

	Temperature	
Quality Category	5°C	24°C
1	11.5a	12.1a
2	3.2a	2.7a
3	1.5a	2.9a
4	21.1a	13.3b
5	4.0a	3.3a
6	0.8a	1.1a
8	26.9b	36.1a
Dead	8.9a	6.5a
Combined Categories		
Vitreous	19.6a	20.5a
Roots	49.1a	42.1b
C+H	15.8a	15.4a

Table 7.8. Duncan's Multiple Range Test for the effect of the length of time that flash desiccated embryos were indirectly rehydrated on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Data are means averaged over four cold treatment durations and all methods of rehydration (h). Means within the same quality category with different letters are signifiantly different (p=0.05) from each other.

Table 7.8.

	Rehydration (h)						
Quality Category	0	1	2	4	8	16	32
1	23.2a	9.8ab	10.6ab	6.4b	8.9ab	10.5ab	13.3ab
2	2.1ab	3.1a	1.3a	3.6ab	2.5ab	7.2b	1.1a
3	4.8a	0.9a	0.9a	1.4a	3.6a	1.2a	2.5a
4	17.4b	11.6b	13.8b	16.5b	18.0b	18.7ab	24.3a
5	5.3ab	4.0b	1.8a	4.0ab	2.7a	2.3ab	5.3ab
6	0.4a	0.4a	0.9a	2.7a	1.3a	0.0	0.9a
8	22.8ab	36.6ab	36.6a	33.9ab	32.5ab	33.2ab	24.9b
Dead	0.4b	10.8a	6.7a	4.2a	10.3a	9.5a	12.3a
Combined Categories							
Vitreous	19.2ab	20.2ab	26.4a	24.8ab	18.8ab	16.1ab	14.9b
Roots	57.0a	35.6b	44.6ab	39.2b	44.4ab	47.1ab	51.2a
C+H	30.4a	13.8a	12.5ab	10.3b	11.7ab	12.8ab	18.6ab

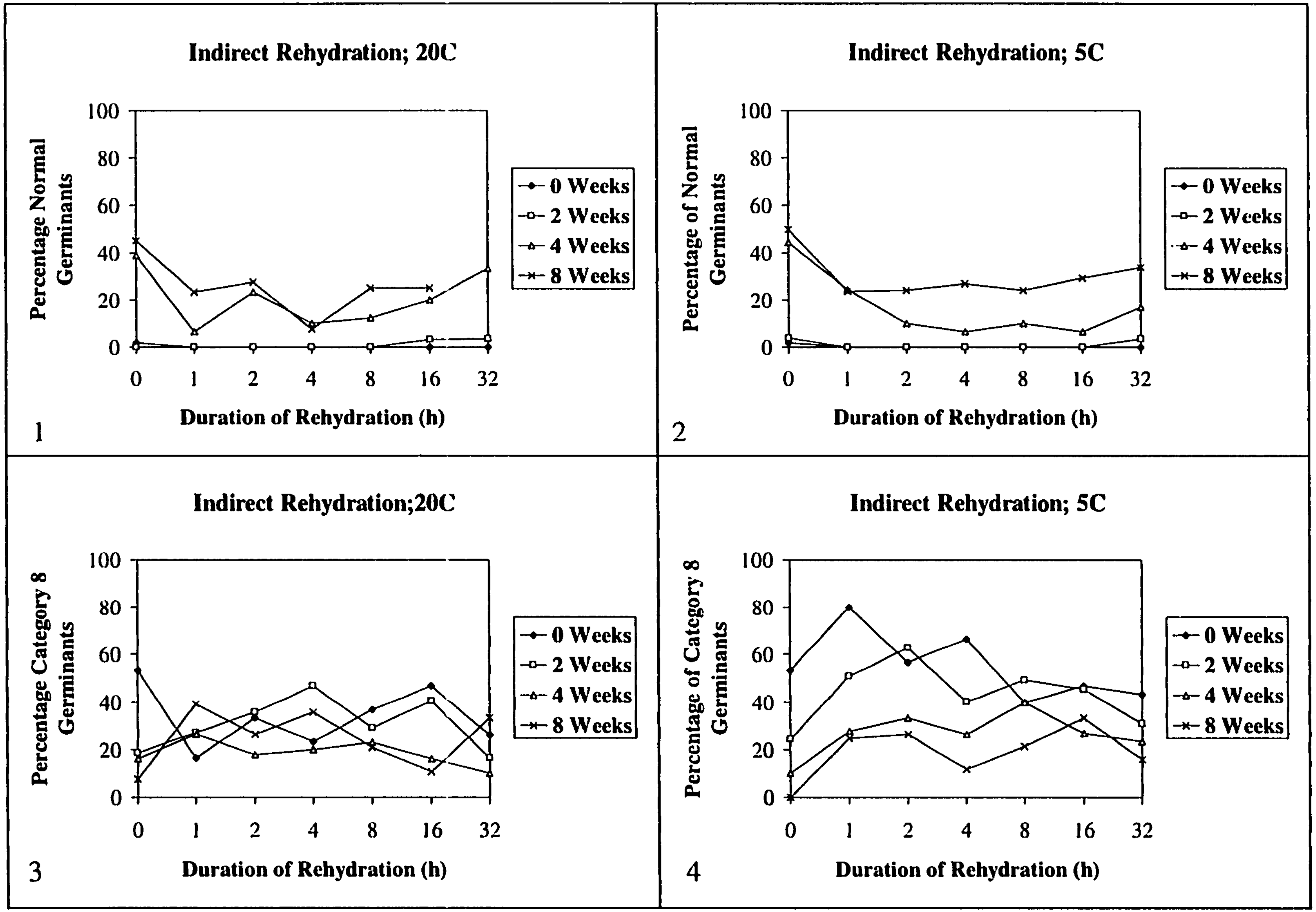
Graph 7.3.1. The effect of indirect rehydration of flash desiccated embryos at 20°C on the percentage of normal germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.3.2. The effect of indirect rehydration of flash desiccated embryos at 5°C on the percentage of normal germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.3.3. The effect of indirect rehydration of flash desiccated embryos at 20°C on the percentage of category 8 germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.3.4. The effect of indirect rehydration of flash desiccated embryos at 5°C on the percentage of category 8 germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.3.


Indirect Rehydration; 20C
Percentage Normal Germinants
Duration of Rehydration (h)
1
Indirect Rehydration; 5C
Percentage of Normal Germinants
Duration of Rehydration (h)
2
Indirect Rehydration;20C
Percentage Category 8 Germinants
Duration of Rehydration (h)
3
Indirect Rehydration; 5C
Percentage of Category 8 Germinants
Duration of Rehydration (h)
4
0 Weeks
2 Weeks
4 Weeks
8 Weeks
0
20
40
60
80
100
0
1
2
4
8
16
32

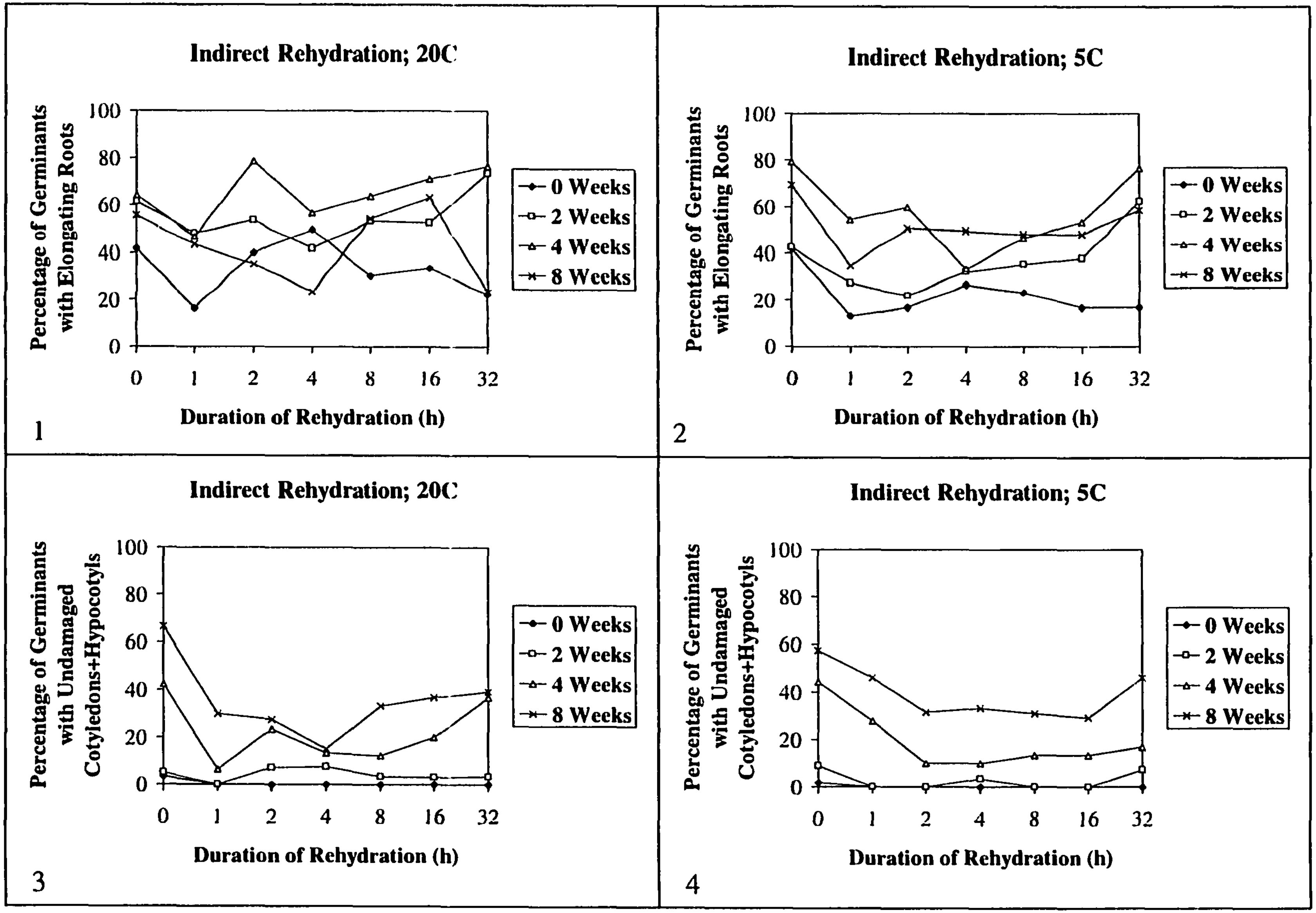
Graph 7.4.1. The effect of indirect rehydration of flash desiccated embryos at 20°C on the percentage of germinants with elongating roots. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.4.2. The effect of indirect rehydration of flash desiccated embryos at 5°C on the percentage of germinants with elongating roots. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.4.3. The effect of indirect rehydration of flash desiccated embryos at 20°C on the percentage of germinants with undamaged cotyledons+hypocotyls. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.4.4. The effect of indirect rehydration of flash desiccated embryos at 5°C on the percentage of germinants with undamaged cotyledons+hypocotyls. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were flash desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.4.


Indirect Rehydration; 20C
Percentage of Germinants with Elongating Roots
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
1
Indirect Rehydration; 5C
Percentage of Germinants with Elongating Roots
Duration of Rehydration (h)
2
Indirect Rehydration; 20C
Percentage of Germinants with Undamaged Cotyledons+Hypocotyls
Duration of Rehydration (h)
3
Indirect Rehydration; 5C
Percentage of Germinants with Undamaged Cotyledons+Hypocotyls
Duration of Rehydration (h)
4

produced from the directly rehydrated embryos (graphs 7.3.1-2). A slight increase in the percentage was also seen with duration of the indirect rehyration and in the percentage of germinants with undamaged roots and cotyledons+hypocotyls (graphs 7.4.1). The results from the different lengths of indirect rehydration at 24°C were more erratic than those from the 5°C rehydrated embryos.

7.3.2.2.2 Slowly dried embryos

The GLM statistical analysis showed that duration of the cold pre-treatment only had a significant effect on the percentage of germinants in category 1 (at the 0.01% level) and in the combined category of germinants with undamaged roots (at the 0.001% level) (Table 7.9). The temperature at which the embryos were rehydrated had no significant effect on the quality of the germinants, but the method of rehydration (0, 1, 2, 4, 8, 16, 32 h indirect rehydration in moist air) had a significant effect at the 0.001% level on the percentage of germinants in quality category 1 and in the combined categories of germinants with undamaged roots and undamaged cotyledons+hypocotyls (C+H); and at the 0.05% level on the percentage of germinants in category 8.

The Duncan's test showed the effects of duration, temperature of rehydration, and the rehydration method on the germination response of the embryos (results averaged over all treatments). The percentage of normal germinants produced was improved by two weeks of exposure to the cold pre-treatment (53.5% vs. 67.9% for 0 and 2 weeks exposure) (Table 7.10.1), but decreased with longer exposure to a low of 49.6% for 8 weeks exposure. The percentage of germinants with elongating roots and undamaged cotyledons+hypocotyls followed the same general pattern, but only the results of the roots category were significant. The temperature at which the embryos were rehydrated had no significant influence (Table 7.10.2), but the method of rehydration did. The directly rehydrated embryos (0 h rehydration) produced the lowest percentage of category 1 germinants (36.9%) (Table 7.10.3) which increased with indirect rehydration to a maximum of 75.7% category 1 germinants after 32 h. The percentage of germinants with elongating roots (roots) and undamaged cotyledons+hypocotyls (C+H) also

Table 7.9. Analysis of variance summary table for the effects of duration of the cold treatment (D), temperature of rehydration (T) and length of time the slowly desiccated embryos were indirectly rehydrated (R) on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category was analyzed separately. F-values are significant at the 0.05(*), 0.01(**), and 0.001(***) level.

Table 7.9.

	Germinant Quality Category							
	Category 1		Category 2		Category 5		Category 8	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Duration (D)	3	5.84**	3	1.44	3	2.94	3	1.65
Temperature (T)	1	0.10	1	0.46	1	0.00	1	2.07
Rehydration(R)	6	6.57***	6	1.08	6	1.26	6	12.64*
D x T	3	1.29	2	0.88	3	0.54	1	0.45
D x R	17	1.04	8	0.78	15	0.71	6	4.47
T x R	6	0.61	3	1.10	6	1.12	2	0.48
D x T x R	17	0.50	0		11	1.24	1	0.76
Error	53		5		22		14	
Corrected total	106		28		67		24	

Table 7.9 cont'd

	Combined Categories					
	Vitreous		Roots		C+H	
Source	**DF**	**F-Value**	**DF**	**F-Value**	**DF**	**F-Value**
Duration (D)	3	1.05	3	15.32***	3	1.38
Temperature (T)	1	0.15	1	1.10	1	0.01
Rehydration(R)	6	2.15	6	4.77***	6	4.62***
D x T	3	0.09	3	2.02	3	0.73
D x R	17	0.66	17	0.77	17	0.91
T x R	6	0.52	6	1.36	6	0.63
D x T x R	17	0.71	17	0.49	17	0.59
Error	53		53		53	
Corrected total	106		106		106	

Table 7.10.1. Duncan's Multiple Range Test for the effect of the duration of the cold treatment prior to slow desiccation and indirect rehydration of the embryos on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Data are means averaged over four cold treatment durations and all methods of rehydration (h). Means within the same quality category with different letters are signifiantly different (p=0.05) from each other.

Table 7.10.2. Duncan's Multiple Range Test for the effect of the temperature at which slowly desiccated embryos were indirectly rehydrated on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Data are means averaged over four cold treatment durations and all methods of rehydration (h). Means within the same quality category with different letters are signifiantly different (p=0.05) from each other.

Table 7.10.3. Duncan's Multiple Range Test for the effect of the length of time that slowly desiccated embryos were indirectly rehydrated on the percentage of germinants in each germinant quality category. The change in percentage of germinants in each quality category were analyzed separately. Data are means averaged over four cold treatment durations and all methods of rehydration (h). Means within the same quality category with different letters are signifiantly different (p=0.05) from each other.

Table 7.10.1.

	Duration			
Quality Category	0 Weeks	2 Weeks	4 Weeks	8 Weeks
1	53.5b	67.9a	52.9b	49.6b
2	1.0a	4.0a	3.4a	2.5a
5	11.4a	3.7b	8.9ab	14.3a
8	6.4a	0.5a	3.6a	2.1a
Combined Categories				
Vitreous	21.8a	22.0a	27.9a	26.7a
Roots	70.7b	88.1a	74.9b	60.1c
C+H	64.9a	71.6a	61.8a	63.8a

Table 7.10.2.

	Temperature	
Quality Category	20°C	5°C
1	55.9a	56.3a
2	2.2a	3.2a
5	10.1a	8.8a
8	3.5a	2.9a
Combined Categories		
Vitreous	24.1a	25.0a
Roots	72.9a	74.7a
C+H	66.0a	65.2a

Table 7.10.3.

	Rehydration (h)						
Quality Category	0	1	2	4	8	16	32
1	36.9d	48.8cd	53.7bc	55.5bc	60.2bc	66.6ab	75.7a
2	2.1a	1.8a	4.6a	4.3a	2.2a	1.9a	1.8a
5	10.2a	13.7a	11.8a	11.3a	7.4a	6.2a	4.7a
8	11.0b	3.8b	1.8ab	1.2a	1.7a	1.8b	0.6a
Combined Categories							
Vitreous	33.2a	28.7ab	23.4abc	22.0abc	28.0ab	19.0bc	15.5c
Roots	57.7c	67.9bc	74.7b	73.8b	77.4b	79.7b	88.6a
C+H	47.0c	62.4b	65.4b	66.8b	67.6b	72.8ab	80.4a

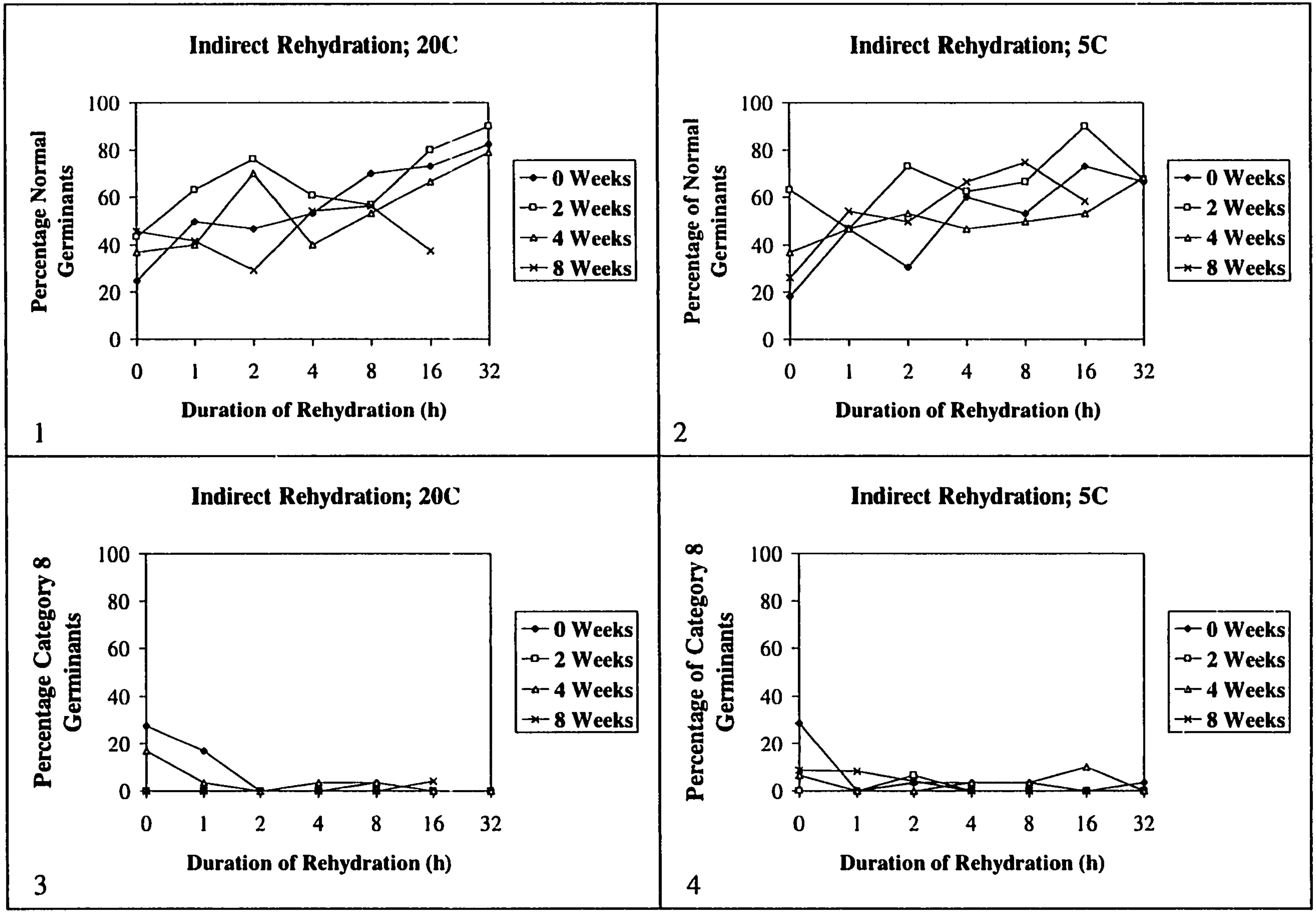
Graph 7.5.1. The effect of indirect rehydration of slowly desiccated embryos at 20°C on the percentage of normal germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.5.2. The effect of indirect rehydration of slowly desiccated embryos at 5°C on the percentage of normal germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.5.3. The effect of indirect rehydration of slowly desiccated embryos at 20°C on the percentage of category 8 germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.5.4. The effect of indirect rehydration of slowly desiccated embryos at 5°C on the percentage of category 8 germinants. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.5


Indirect Rehydration; 20C
Percentage Normal Germinants
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
1
Indirect Rehydration; 5C
Percentage of Normal Germinants
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
2
Indirect Rehydration; 20C
Percentage Category 8 Germinants
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
3
Indirect Rehydration; 5C
Percentage of Category 8 Germinants
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
4

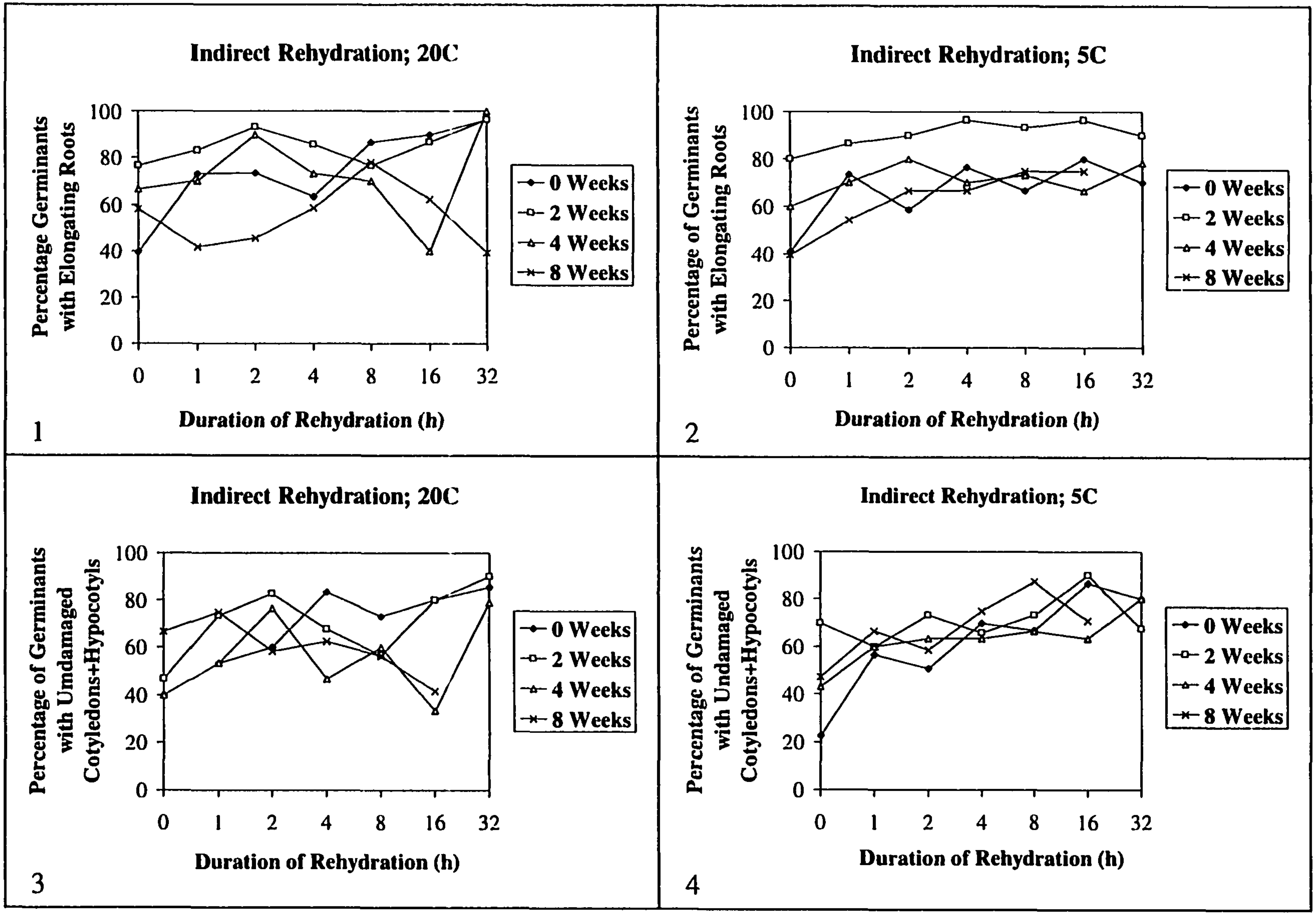
Graph 7.6.1. The effect of indirect rehydration of slowly desiccated embryos at 20°C on the percentage of germinants with elongating roots. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.6.2. The effect of indirect rehydration of slowly desiccated embryos at 5°C on the percentage of germinants with elongating roots. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.6.3. The effect of indirect rehydration of slowly desiccated embryos at 20°C on the percentage of germinants with undamaged cotyledons+hypocotyls. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 20°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.6.4. The effect of indirect rehydration of slowly desiccated embryos at 5°C on the percentage of germinants with undamaged cotyledons+hypocotyls. Embryos that had been cold stored at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks were slowly desiccated and indirectly rehydrated at a temperature of 5°C for various periods of time (0, 1, 2, 4, 8, 16 or 32 h). Each data point is the average of 30 embryos.

Graph 7.6.


Indirect Rehydration; 20C
Percentage Germinants with Elongating Roots
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
1
Indirect Rehydration; 5C
Percentage of Germinants with Elongating Roots
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
2
Indirect Rehydration; 20C
Percentage of Germinants with Umdamaged Cotyledons+Hypocotyls
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
3
Indirect Rehydration; 5C
Percentage of Germinants with Undamaged Cotyledons+Hypocotyls
100
80
60
40
20
0
0
1
2
4
8
16
32
Duration of Rehydration (h)
0 Weeks
2 Weeks
4 Weeks
8 Weeks
4

increased with indirect rehydration, while the percentage of category 8 and vitreous germinants decreased.

The graphs of the individual results (averaged over 2 replications) (Graphs 7.5-7.6) showed the same general patterns; the quality of the germinants increased with the length of time the embryos were indirectly rehydrated in moist air before they were transferred to germination medium. Also, those that had been pre-treated with a cold treatment for 2 weeks were shown to produce the best quality embryos.

7.4 Discussion

7.4.1 Comparison of TTC and germination tests as a method of damage assessment

Tetrazolium testing is an accepted standardized biochemical method for testing the viability of agricultural (ISTA Rules, 1985) and forest tree seed (Edwards 1987). Our experiments showed that tetrazolium testing is an easy way to assess damage in somatic embryos, but that germination tests are more accurate for actual quality assessment.

A viable seed or somatic embryo is one that is capable of producing a seedling (i.e. a category 1 germinant with normal elongating cotyledons, hypocotyls and roots). There are various staining criteria in tetrazolium viability testing that must be met for a seed to be deemed viable. Because cereal seeds have the ability to repair small necrotic areas in its tissue and germinate subsequently, both totally stained embryos and embryos with small unstained areas (as described by Kruse 1996) are assessed as being viable. In the Canadian tree species described by Edwards (1987), seeds of all the species, except *Acer rubrum,* are required to contain embryos with no unstained areas in order to be assessed as viable. According to these standards, only the embryos in our experiments assessed as TTC Damage Category A would be deemed viable. This fits in well with comparative germination tests as embryos classed as Category A (i.e. undamaged) have the greatest potential to produce normal seedlings. However, as shown in this experiment and previously in Chapters 4 and 5, normal shaped, undamaged embryos have the potential to produce normal seedlings, but, in fact, very few of them produce normal germinants unless they have had a germination pre-treatment. Therefore, the use of TTC staining alone as a method of viability assessment overestimates the number of normal germinants that will be produced. Tetrazolium testing is known to overestimate viability in seeds (Kruse 1996).

Tetrazolium testing is an excellent way of distinguishing between living and dead tissue in somatic embryos. However, assessment of damage in seeds has been shown to be subjective and assessments can vary between labs (Kruse 1996). Undamaged and dead seeds are easy to assess. The problem lies in the assessment of damaged seeds, as there can be varying intensities of the red colouration present, making assessment difficult. This was also a problem with the somatic embryos. Flash desiccated embryos rehydrated at a temperature of 20-25°C, produced very sharply defined areas of living (red) and dead (white) tissue, but the embryos rehydrated at a temperature of 5°C did not show this same demarcation. The living areas stained red, but damaged areas stained varying intensities of pink making interpretation of the results difficult. Possibly embryos rehydrated at 5°C did not suffer damage severe enough to totally prevent TTC reduction from occurring, but the damage was severe enough to affect germination. Therefore, the TTC scores tended to overestimate the viability of the embryos rehydrated at 5°C.

Extraction of the colour and measurement of its absorbance gives an objective assessment of the amount of red colour present in the tissue. This method was shown to give a good qualitative assessment of the percentage of living *Acer saccharum* cells in a cell suspension mixture of living and dead cells (Towill and Mazur 1975). In our experiments, absorbance readings were an excellent way of comparing undamaged and severely damaged embryos as there were large differences in their absorbance readings. However, the differences were less striking when comparing embryos that suffered only slight damage with undamaged ones. There are two problems with the use of absorbance readings for viability testing in white spruce somatic embryos: 1.) The embryos are variable in size. Therefore, some of the variation in the colour intensity reading in a set amount of ethanol may be due to variation in size of the embryos and not due to a variation in the percent of living tissue present 2.) Absorbance readings will determine the total amount of red colour present in an embryo, but it will not pinpoint where the damage has occurred in the embryo and will not distinguish between areas that stain with varying intensity. Determination of the percentage of damaged tissue present is therefore difficult.

The variation in colour intensity as a result of variation in embryo size can be overcome by weighing the embryos prior to TTC staining and expressing the absorbance on a per gram basis. This would allow comparisons to be made between equivalent weights of tissue. With this modification, comparisons would be more accurate. The problem of overestimation of viability would still be present, however. High absorbance readings would indicate undamaged or minimally damaged embryos with the potential to germinate normally, but could not be used to predict the quality of the germinants.

In summary, tetrazolium testing in somatic embryos is an excellent method of determining damage, but does not predict the quality of the germinant. Therefore, for tests aimed at improving the quality of the germinants, TTC staining will not suffice as a method of damage assessment. Actual germination tests and assessments of the quality of the resulting germinants are required.

7.4.2 Effect of pre-treatment and rehydration method on germinant quality

7.4.2.1 Flash desiccated embryos

These experiments confirmed the previous results from Chapter 6 that mature embryos could survive air-drying only after they had been subjected to a cold treatment for a minimum of 4 weeks. Direct rehydration of these desiccation tolerant embryos produced more normal germinants than indirect rehydration did.

These results suggest that the membrane phospholipids of desiccation tolerant embryos do not undergo a phase change when they are air-dried. They, therefore, would not need to be rehydrated in moist air before being placed on germination medium as they would not be subjected to a phase transition during rehydration and theoretically should not suffer any leakage of cell constituents. The idea that desiccation tolerant embryos do not exhibit a phase change with desiccation is supported by Hoekstra et al. (1993) who found that desiccation tolerant alfalfa embryoids did not show a change in their phase transition temperature when they were desiccated. Tetteroo et al. (1996) reported similar findings in carrot somatic embryos. As discussed in Chapter 6, a minimum of four weeks of exposure to a temperature of 5°C may have increased the sucrose concentration and/or changed the lipid composition of the membranes lowering their T_m and thus preventing the phospholipids from undergoing a phase change as they are desiccated.

It is also possible that the lack of phase change is a result of the method of desiccation. Air-drying results in flash desiccation (i.e. total desiccation within 15 min). Tetteroo (1998) has suggested that flash desiccation maintains the embryo components in the position they were in when initially subjected to the airflow. One possible explanation is that the phoshpolipids may not have had time to undergo a phase transition and lateral phase separations. The proteins are also thought to be locked in their original conformations. The lack of phase change may also explain why the temperature at which the embryos were rehydrated made little difference.

Another more plausible explanation is that as a result of flash desiccation metabolism would have stopped almost immediately and none of the protective events associated with slow desiccation would have occurred as would very few of the destructive metabolic events (Pammenter et al. 1991). This concept is supported by the research of Leprince et al. (1995). They concluded that desiccation damage can be decreased by finding ways of limiting metabolism during desiccation thereby reducing free radical formation which they postulate to be a major cause of desiccation damage. Based on this assumption, the embryos would probably be better off to rehydrate quickly. With slow rehydration, metabolism would resume almost immediately, but those reactions less sensitive to low water content would resume first. The result could be an unbalanced metabolism with destructive reactions (eg. free radical formation and subsequent oxidative damage) occurring faster than the damage can be repaired. It is therefore possible that, during slow indirect rehydration, the oxidation by-products are accumulating in the membranes, causing phase changes in the membranes (Tetteroo et al.

1996) and actually preventing those membranes in the gel phase from returning to the liquid crystalline phase.

Hoekstra et al. (1992a) found that very dry pollen (<0.05%g H_2O g^{-1} dry weight) did not behave according to the phase transition hypothesis. They found that when the pollen was partially hydrated it then followed expectations of the phase transition hypothesis. Perhaps there may be other factors responsible for imbibitional damage in very dry organisms besides a simple gel-liquid phase change.

7.4.2.2 Slowly dried embryos

Imbibitional damage can be prevented in slowly dried embryos by rehydrating them in moist air before they are placed on germination medium. The embryos were relatively dry (24% RWC) and showed severe damage when rehydrated directly on germination medium (shown by the low percentage of normal germinants that were produced). The percentage of normal germinants produced increased with the duration of indirect rehydration. This suggests that 1.) the majority of the damage occurs during imbibition and not during desiccation and/or 2.) any damage that has occurred during desiccation is being repaired during the slow rehydration.

The pattern of decreasing damage with increasing length of indirect rehydration suggests that phase transitions may be one possible explanation for the damage. Logically, the membrane phospholipids of the embryos dried at -10 MPa would exist in the solid gel phase and directly placing these embryos on germination medium would result in extensive leakage of cell constituents and therefore severe imbibitional damage. Leakage of cell contents does not occur in 100% RH. The embryos would rehydrate gradually when indirectly rehydrated in moist air with the outside surfaces of the embryo rehydrating first, followed by gradual rehydration inwards. The result would be a gradual phase change from the solid gel to the liquid crystalline phase as the cells rehydrate.

The temperature at which the embryos are rehydrated did not significantly influence their germination response. There were slightly more normal germinants when they were rehydrated at a temperature of 20°C. The transition temperature (T_m) of the hydrated and dried embryos would have to be known to explain the lack of effect of temperature on imbibition damage. The T_m of white spruce somatic embryos at full hydration and various levels of desiccation have not been reported in the literature as of yet. However, Hoekstra et al. (1993) estimate that the T_m of membrane phospholipids in the membranes of dry soybean seeds fall in the range of 7 to 27°C, and that the T_m of hydrated seeds falls in the range of -5 to 15°C. Tetteroo et al. (1996) determined the T_m of isolated membrane preparations from slowly dried desiccation tolerant somatic embryos to be 8°C and that of rapidly dried desiccation intolerant embryos to be 22°C. It is possible that spruce somatic embryos may exhibit similar transition temperatures.

Desiccation tolerant embryos are thought to be able to prevent damage by avoiding membrane phase transitions (as discussed in section 7.4.2.1). Therefore the desiccation tolerant embryos should rely less on indirect rehydration in moist air to reverse the phase transitions and prevent imbibitional damage. As discussed previously in Chapter 6, embryos become more desiccation tolerant with exposure to cold temperatures. As expected, the cold pre-treatment at a temperature of 5°C for a period of 0, 2, 4, or 8 weeks resulted in less damage in the directly rehydrated embryos. However, indirect rehydration still increased the percentage of normal germinants that were produced, even after 8 weeks of the cold treatment. Phase transitions must still occur to some extent, although there may be other explanations for the positive effects of indirect rehydration in the cold-pretreated, slowly desiccated embryos. One possible explanation is that during slow rehydration, radical scavengers and damage repair mechanisms are activated allowing repairs to be made before germination starts. It is hypothesized that similar repair activities and germination preparations are occurring during phase II (osmopriming)(see section 2.5) in imbibing orthodox seeds (reviewed in Bray 1995). Therefore, indirect rehydration may also act as a short priming treatment for dried somatic embryos. This would help explain the increase in the percentage of normal germinants produced as a result of longer indirect rehydration.

These desiccation and rehydration results confirmed the findings from earlier desiccation experiments for slowly dried embryos (Chapter 4) namely that indirect rehydration increases the percentage of normal germinants produced and that mild desiccation may improve germinant quality by decreasing the percentage of vitreous germinants.

7.4.2.3 Future Research

The mechanisms behind the effect of rehydration method on germinant quality of dried embryos should be further investigated to determine the actual physiological basis for these observations. Phase transitions are easily observed using Fourier transform infrared spectroscopy (FT-IR) in model lipid systems (Blazyk and Rana 1987), isolated membranes (Hoekstra et al. 1991, Tetteroo et al. 1996) in intact pollen (Crowe et al. 1989c) but they are not easily observed in dry seeds of pea and soybean and in somatic embryos of alfalfa (Hoekstra et al. 1993). Hoekstra et al. (1993) found that the storage oils in the whole seeds interfered with the signals from the membrane lipids and Mantsch et al. 1988 found that isolation of the membranes affected their behaviour. Therefore, some technical difficulties are going to have to be worked out before FT-IR can be applied to whole white spruce somatic embryos. We have done some preliminary experiments using FT-IR white spruce somatic embryos to attempt to sort out the mechanisms behind the rehydration behaviour, but some further studies will have to be done to ensure that the results can be correctly interpreted. Further research should also be done to further define the role free radical damage plays in desiccation and rehydration.

7.5 Conclusion

Germination tests are the best method of determining the effect of various desiccation and rehydration treatments on the quality of the resulting germinants. Tetrazolium testing is an excellent method of showing where dead tissue is located in the embryo. However, it significantly overestimates the viability of somatic embryos.

Slowly and rapidly dried embryos require different methods of rehydration to improve the quality of the resulting germinants. Those slowly dried to relatively low water contents require indirect rehydration in moist air for at least 16 h. Embryos flash desiccated to very low water contents suffer less damage if they are rehydrated quickly by direct dehydration on germination medium.

The slowly dried embryos were dried for 7 d over a –10 MPa NaCl salt solution and survived desiccation. The cold treatment decreased the amount of damage that the embryos suffered when they were directly rehydrated, but a period of indirect rehydration still improved the quality of the germinants. The flash desiccated embryos only survived desiccation after they had been pre-treated with a cold treatment for a total of 4 to 8 weeks. Indirect rehydration decreased the germinant quality.

The proper treatment of embryos both during desiccation and rehydration results in a very large percentage of good quality germinants. Those slowly desiccated to 24% RWC and then prehydrated for 32h before being germinated produced almost 90% normal germinants which is over the 80% germination rate required for certification of most seeds (Cowan 1972). The embryos flash desiccated to 4% RWC produced 50% normal germinants if they were rehydrated directly on germination medium. This percentage can probably be improved upon by further improving the quality of the embryos before they are desiccated.

The mechanisms by which embryos are able to survive water loss and its re-introduction are not fully understood. All research points to the fact that there is not one simple explanation and that survival is probably a result of the interaction of many protection and repair mechanisms. Rehydration experiments suggest that membrane phase transitions may not be totally responsible for imbibitional damage and as suggested by other researchers, free radical damage may be implicated as a source of damage during desiccation and rehydration. The activation of damage repair mechanisms during rehydration probably plays an important role.

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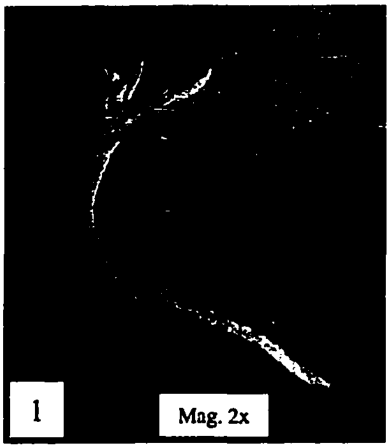
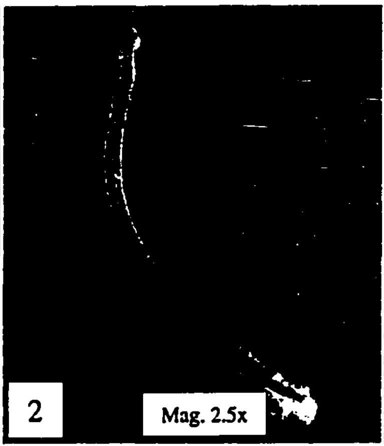
Appendix 1. Water Potential (at 5°C), Molality, Mass of NaCl (g) per 500g water and RH%

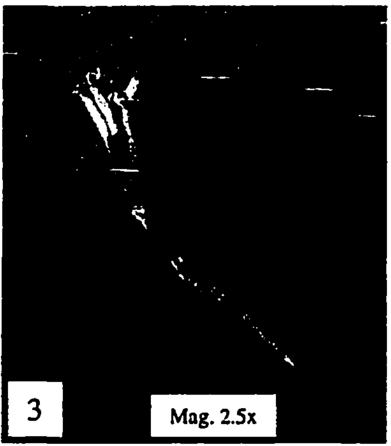
Water Potential (-MPa)	Molality	Mass of NaCl	RH%
0		0.0	100.00
1	0.2500	7.3054	99.24
2	0.4800	14.0263	98.47
3	0.7108	20.7706	97.73
4	0.9343	27.5733	96.98
5	1.1719	34.3858	96.24
8	1.8269	53.3858	94.05
10	2.2371	65.3716	92.63
15	3.1735	92.73314	89.14
20	3.9989	116.8555	85.80

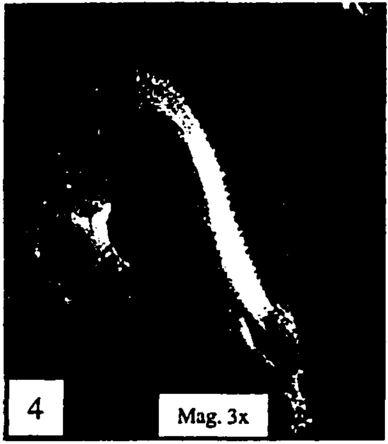
* from Percy 1997

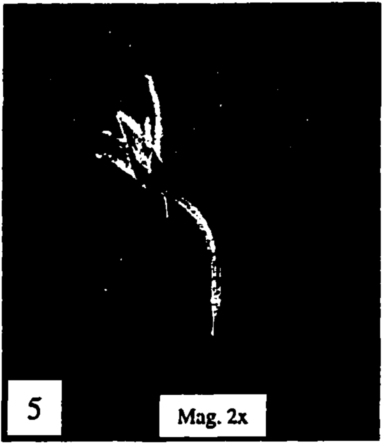
Appendix 2. Germination Scoring System

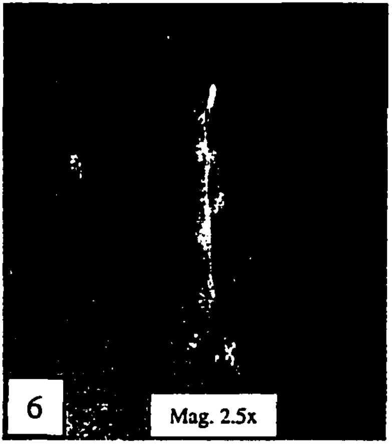


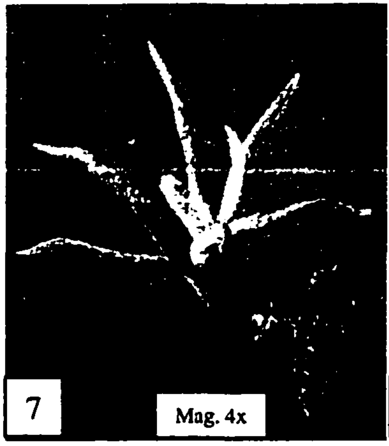


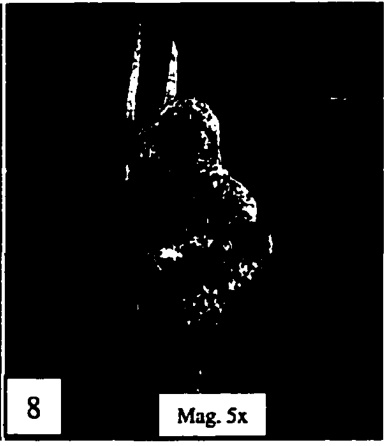


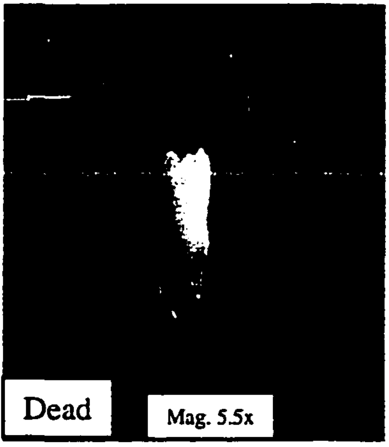






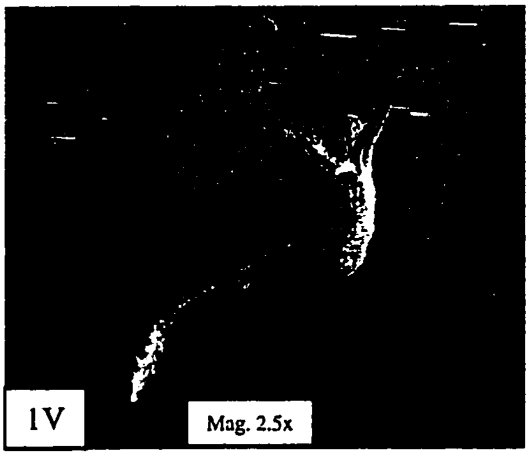


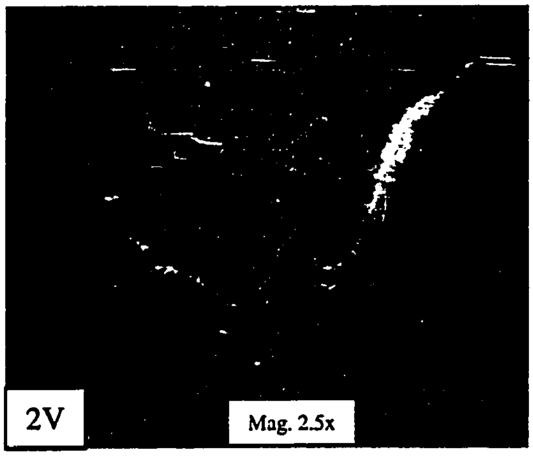


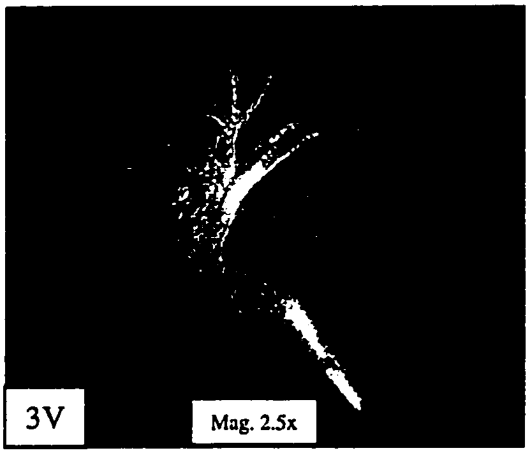


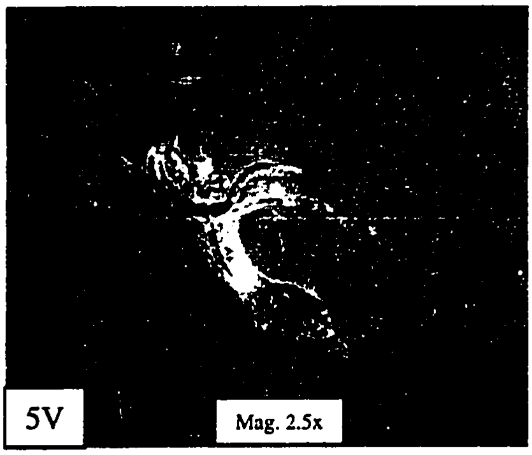
	Normal Elongating				Normal Elongating		
Category	Cotyledons	Hypocotyl	Root	**Category**	Cotyledons	Hypocotyl	Root
1	Yes	Yes	Yes	**5**	Yes	Yes	No
2	No	Yes	Yes	**6**	No	Yes	No
3	Yes	No	Yes	**7**	Yes	No	No
4	No	No	Yes	**8**	No	No	No

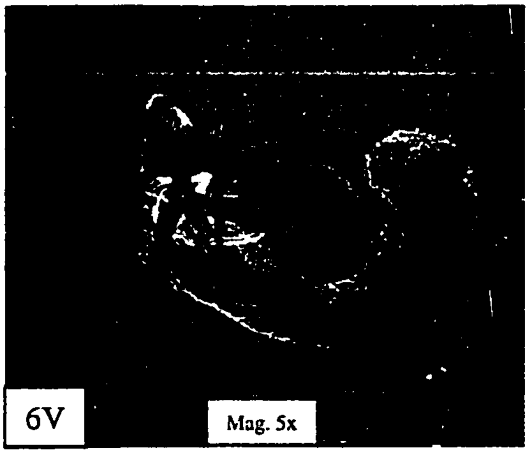
Appendix 3. Germination Scoring System; Vitreous Germinants

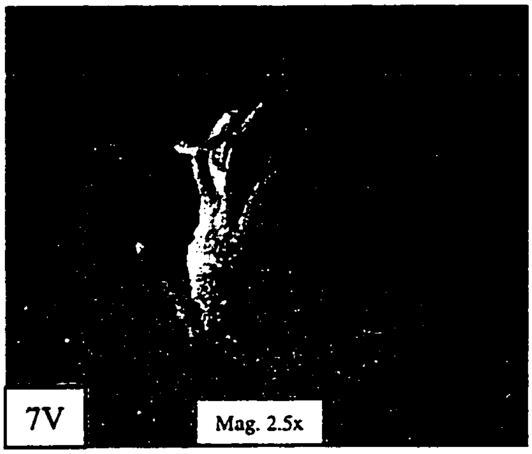












Category	Cotyledons	Hypocotyl	Root	**Category**	Cotyledons	Hypocotyl	Root
1v	vitreous	vitreous	present	**5v**	vitreous	vitreous	absent
2v	absent	vitreous	present	**6v**	absent	vitreous	absent
3v	vitreous	absent	present	**7v**	vitreous	absent	absent

Appendix 4. Average percentage of germinants per quality category

Quality Category	Average
1	4.89
1v	0.24
2	0.15
2v	0.00
3	1.86
3v	0.41
4	11.39
5	1.50
5v	1.97
6	0.15
7	1.24
7v	3.06
8	33.00
Dead	40.09
Combined Categories	
Roots	19.04
Vitreous	5.66
C+H	6.41

1+4+8+Dead+vitreous = 95.03%

Appendix 5. Viability Assay (TTC Test)

Preparation of Stain:

1.) Sorenson's Buffer (Grimstone & Skaer 1972):

Stock A: 9.08 g/L Potassium dihydrogen phosphate

Stock B: 11.88 g/L Disodium hydrogen phosphate

For pH 7.4, use 80.4 ml of Stock B. Bring up to 100 ml with Stock A.

2.) TTC Stain - 1% (w/v) in 0.05 M Sorenson's Buffer (ISTA Rules 1985, Towill & Mazur 1975):

Bring 75 mls of Sorenson's Buffer up to 100 ml with distilled water.

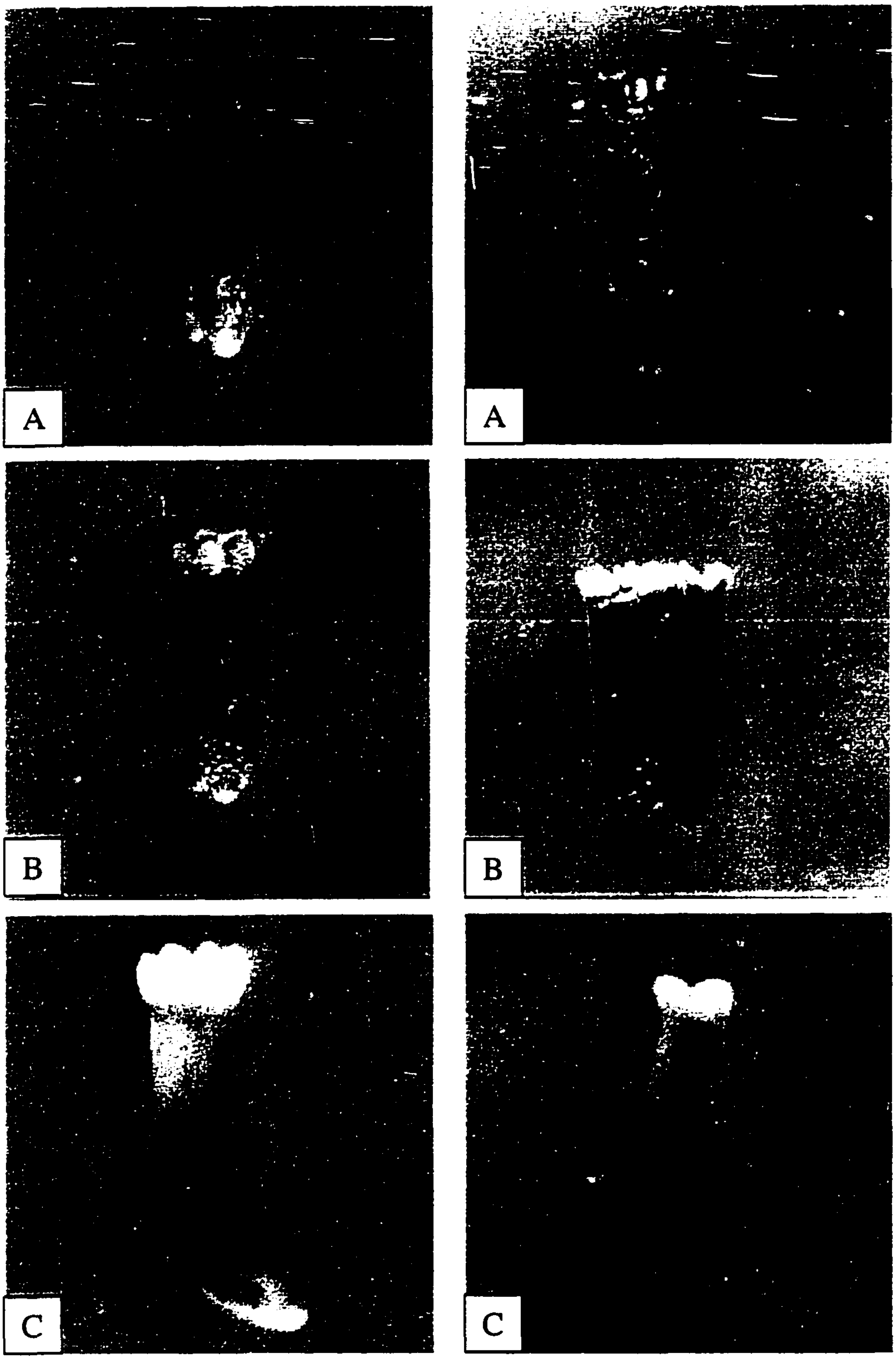
Adjust pH to 7.4.

Add 1 g of TTC (2,3,5-triphenyl, tetrazolium chloride)

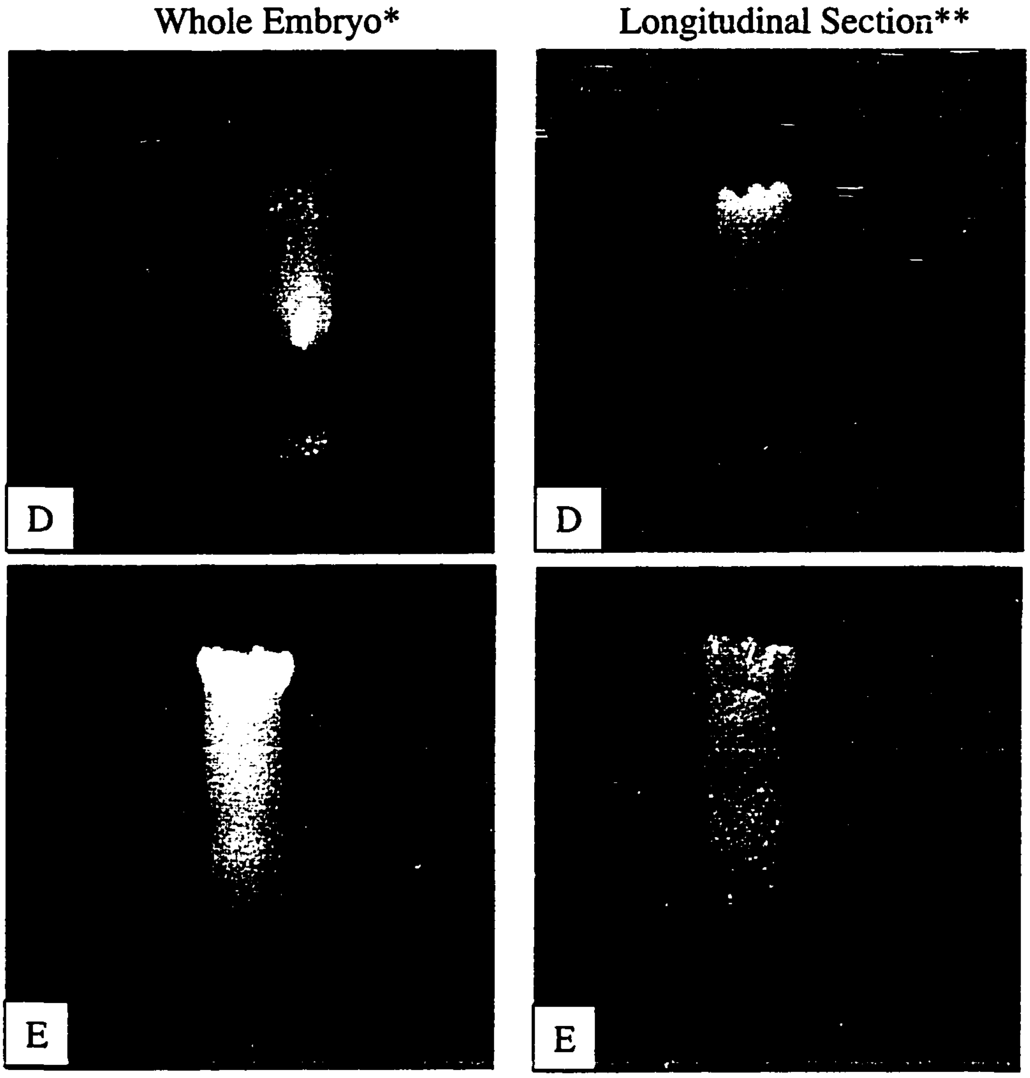
Keep the stain in a foil covered flask in the refrigerator.

Appendix 6.1. TTC Classes A-C

Whole Embryo* Longitudinal Section**



Appendix 6.2. TTC Classes D-E



Class	Description	Score
A	Embryos totally red- Undamaged	8
B	Embryos staining red on over 50% of the embryo- Root and shoot meristems alive	4
C	Embryos staining red on less than 50% of the embryo- Root and shoot meristems alive	2
D	Only root apex staining red- Root meristem alive	1
E	Embryo unstained- Dead	0

* Photograph of the exterior of the embryo. Magnification [13x]

** Photograph of the embryo cut in half longitudinally to show the distribution of the stain in the interior of the embryo. Magnification [13x]

Appendix 7. Number of embryos in each damage category

	TTC Damage Category (Damage Value)							
	A (8)	**B (4)**	**C (2)**	**D (1)**	**E (0)**	**# embryos**	**Score**	**Score/20**
0 hours								
	20	0	0	0	0	20	180	180
0.25 hours								
Direct 5C	11	9	0	0	0	20	124	124
Direct 25C	7	14	0	0	0	21	112	107
Indirect 5C	7	7	3	0	1	18	90	100
Indirect 25C	2	6	3	3	6	20	49	49
0.5 hours								
Direct 5C	13	3	4	0	0	20	124	124
Direct 25C	3	12	5	0	0	20	82	82
Indirect 5C	14	5	2	0	0	21	136	129
Indirect 25C	6	0	4	1	9	20	57	57
1 hours								
Direct 5C	3	6	10	0	0	19	68	72
Direct 25C	0	3	14	2	1	20	42	42
Indirect 5C	12	2	6	0	0	20	116	116
Indirect 25C	0	2	3	11	3	19	25	26
2 hours								
Direct 5C	0	11	9	0	0	20	62	62
Direct 25C	0	1	12	6	1	20	34	34
Indirect 5C	11	4	3	0	2	20	110	110
Indirect 25C	0	0	0	12	8	20	12	12
4 hours								
Direct 5C	1	10	9	0	0	20	66	66
Direct 25C	0	6	12	1	1	20	49	49
Indirect 5C	4	11	3	0	3	21	82	78
Indirect 25C	0	1	0	5	14	20	9	9
8 hours								
Direct 5C	3	6	6	4	0	19	64	67
Direct 25C	0	3	8	6	3	20	34	34
Indirect 5C	1	13	6	0	0	20	72	72
Indirect 25C	0	1	0	7	12	20	11	11
16 hours								
Direct 5C	5	4	6	2	0	17	70	82
Direct 25C	1	8	6	4	0	19	56	59
Indirect 5C	17	2	0	1	0	20	145	145
Indirect 25C	2	3	8	5	2	20	49	49
31 hours								
Direct 5C	1	11	3	5	0	20	63	63
Direct 25C	0	3	12	4	1	20	40	40
Indirect 5C	7	12	0	0	0	19	104	109
Indirect 25C	2	4	6	3	0	15	47	63
64 hours								
Direct 5C	7	7	4	1	0	19	93	98
Direct 25C	0	5	4	8	1	18	36	40
Indirect 5C	7	7	4	1	0	19	93	98
Indirect 25C	0	6	1	7	5	19	33	35

Appendix 8. Average percentage of germinants per quality category

Quality Category	Average
1	9.05
1v	0.92
2	0.00
2v	0.00
3	15.43
3v	0.83
4	13.78
5	8.20
5v	0.00
6	0.00
6v	0.00
7	7.36
7v	4.03
8	22.58
Dead	17.38
Combined Categories	
Vitreous	5.79
Roots	41.76
C+H	17.25

Appendix 9. Average percentage of germinants per quality category for embryos air-dried in the laminar flow hood for 2 hours

Quality Category	Average
1	11.8
1v	3.0
2	3.0
2v	7.5
3	2.2
3v	0.8
4	17.2
5	3.6
5v	3.2
6	0.9
6v	3.4
7	1.7
7v	2.0
8	31.5
Dead	7.7
Combined Categories	
Vitreous	20.0
Roots	45.6
C+H	15.6

Appendix 10. Average percentage of germinants per quality category for embryos desiccated at 5°C over a NaCl solution with a water potential of -10 MPa.

Quality Category	Average
1	56.1
1v	9.6
2	2.7
2v	3.0
3	0.5
3v	0.7
4	1.0
5	9.4
5v	8.4
6	0.5
6v	1.9
7	0.5
7v	0.9
8	3.2
Dead	1.4
Combined Categories	
Vitreous	24.5
Roots	73.8
C+H	65.6