Desiccation and Cryopreservation of Spruce Somatic Embryogenic Tissue and Mature Somatic Embryos

by

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MASTER OF SCIENCE

in the Department of Biology

We accept this as conforming
to the required standard

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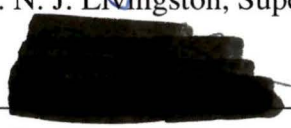

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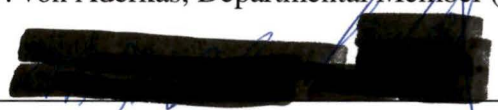

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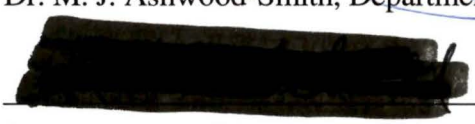

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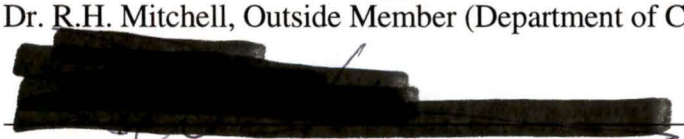

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ABSTRACT

The effects of drying and cryopreservation on the survival of spruce (*Picea glauca* and *Picea glauca* x *engelmanni*) embryogenic cultures were investigated. This work was undertaken with the aim of developing a reliable, well-defined technique for the drying and storage of conifer embryogenic tissue and mature somatic embryos. In this procedure, tissue is dried over salt solutions and then frozen directly in liquid nitrogen. Specific objectives were to: (i) characterize water release from embryogenic cultures using a well-controlled drying system, (ii) determine whether cultured tissue could be desiccated in dry air to low relative water contents (RWC) without causing fatal damage, (iii) determine whether the tissue, once dried to well-defined water contents, could be frozen directly in liquid nitrogen without the use of cryoprotectants and still remain viable after thawing and rehydration, and (iv) determine a convenient method of assessing viability following drying and/or freezing. Two developmental stages were tested: immature somatic embryos (embryogenic tissue) and mature somatic embryos.

My study has shown that TTC (2,3,5-triphenyltetrazolium chloride) stain is a reliable indicator of viability for spruce embryogenic tissue and mature somatic embryos following drying and freezing. However, the vital stain fluorescein diacetate (FDA) is not suitable for use with embryogenic tissue. TTC slightly over-estimates the viability of mature somatic embryos following freezing. It is recommended that more than one test always be used for estimating viability and that any proxy indicator of viability be used in conjunction with regrowth tests.

Embryogenic tissue initially survived drying to very low RWC (< 0.025). There was a decline in desiccation tolerance over time that could not be explained by conventional tissue water relations. The decline in tolerance might have been related to changes in the ratio of small, densely cytoplasmic cells to large vacuolate cells (ratio decreased over time) since the

cultures had recently come out of cryopreservation when the study began. The drying technique employed reliably dried tissue to known RWC and was rigorously tested as an alternative to the conventional multi-step method for cryostorage. However, embryogenic tissue did not survive direct immersion in liquid nitrogen following drying, regardless of the RWC to which it had been pre-dried. It is concluded that drying alone cannot be used as replacement for the steps used to prepare conifer embryogenic tissue for cryopreservation, and that cryoprotectants likely play roles, in addition to their osmotic effect, in the maintenance of cellular integrity.

Mature somatic embryos, however, survived the removal of virtually all free water, and subsequent freezing in liquid nitrogen. Remarkably, no cryoprotectant additives were required. For conifer somatic embryos, this is the first report of embryos cryopreservation without the application of cryoprotectants and a slow-freezing step. In terms of mature conifer somatic embryos, it is the first report of survival following exposure to liquid nitrogen. The RWC of interior and white spruce somatic embryos at bound water was 0.13-0.14 (or 0.28 $gH_2O\ g^{-1}fm$), similar to the average water content of white and interior spruce zygotic embryos excised from stored dried seed (0.325 $gH_2O\ g^{-1}$ fm and 0.365 $gH_2O\ g^{-1}fm$, respectively).

The highest survival after freezing in liquid nitrogen was in those embryos pre-dried to water potentials of -15 to -20 MPa. The RWC at these water potentials was close to bound water values. There was minimal survival after freezing embryos pretreated at higher water potentials, likely due to intracellular ice formation, and there was no survival of embryos after pretreatment over silica gel, probably as a result of severe mechanical disruption within cells.

Examiners:

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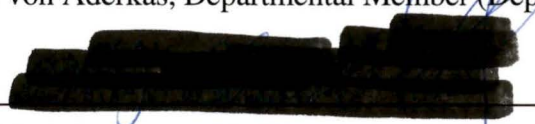

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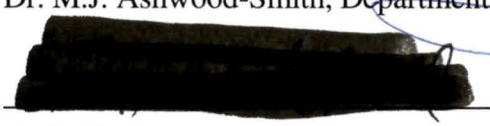

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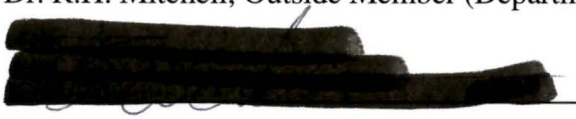

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LIST OF SYMBOLS AND ABBREVIATIONS

ABA	abscisic acid
dmb	water content on dry mass basis (g H_2O g^{-1} dry mass)
C_R	relative electrical conductivity
e_a	vapor pressure (kPa)
e_s	saturated vapor pressure (kPa)
EC	electrical conductivity (dS m^{-1})
DMSO	dimethylsulfoxide
FDA	fluorescein diacetate
fmb	water content on fresh mass basis (g H_2O g^{-1} fresh mass)
LM	Litvay medium
LN_2	liquid nitrogen
M_d	tissue mass after oven drying (g)
M_f	tissue mass after treatment (g)
M_t	tissue mass at full saturation (g)
PEG	polyethylene glycol
PGR	plant growth regulator
R	universal gas constant (8.314 J K^{-1} mol^{-1})
RH	relative humidity
RWC	relative water content
T	temperature (^oC or K)
TTC	2,3,5-triphenyltetrazolium chloride
V_w	partial molal volume of water (18.0 cm^3 mol at 5 ^oC)
2,4-D	2,4-dichlorophenoxyacetic acid
Ψ	water potential (MPa)

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Finally, I would like to dedicate this thesis to my parents, who also worked hard to earn degrees in Biology, and provided me with an environment that encouraged commitment to goals and an appreciation of science.

Chapter 1

INTRODUCTION

Approximately 65 percent of the total land area in British Columbia is forested and between 1994 and 1995, over 75 million cubic metres of wood was harvested. The future of B.C.'s forest industry will be greatly influenced by our ability to shorten rotation cycles, improve tree vigour and fibre quality, to increase tolerance to water and temperature stress, and to increase resistance to pathogen and insect attack. Most economically important forest species are seed-propagated. However, some of the above requirements might be addressed by more extensive use of clonally-derived stock produced through vegetative macro- and micro-propagation. At least some of the traits which cannot be transmitted intact through sexual reproduction can be maintained by clonal propagation.

Micro-propagation encompasses a number of tissue culture techniques that lead to the production of clonal propagules genetically identical to a superior stock plant . One micro-propagation pathway, currently used to regenerate several conifer species including spruce, is somatic embryogenesis. In this process, somatic (asexual) cells are induced to form large numbers of genetically-identical immature embryos (embryogenic tissue). These embryos can be matured, germinated and the somatic seedlings acclimated to greenhouse conditions.

Vegetative micro-propagation through somatic embryogenesis has a number of potential applications, these include;

(i) rapid multiplication of commercially valuable seed obtained from breeding programmes (value-added traits could include increased yield, wood quality, pest and disease tolerance);

(ii) conservation of disease-free stocks of trees which can be used in breeding (i.e. germplasm maintenance);
(iii) facilitation of genetic engineering which would allow transfer of genetic information between vegetative plant cells.

Somatic embryogenesis for conifers has been most successful in spruce (*Picea*) species in terms of culture induction and plantlet development (Tautorus *et al.*, 1991). Many spruce species, including white and interior spruce, are of major economic importance in British Columbia. Approximately 90 million spruce seedlings are planted annually in B.C. Systems for large scale production systems of conifer somatic embryos are currently being developed, and particular emphasis is being placed on spruce species. For example, B.C. Research Inc. and its subsidiary Silvagen Inc. (Vancouver, B.C.), have produced 120,000 interior spruce somatic seedlings for field tests and delivered over half a million propagules to nurseries over the past two years. Additionally, over seven hundred interior spruce lines are being evaluated in province-wide test sites for long-term growth and resistance to weevil damage.

While field tests are being conducted on clonal plantlets, immature cultured tissue can be bulked up and kept for extended periods. However, maintenance of such tissue requires continuous sub-culture to fresh medium. Not only is this costly and time-consuming, but it can lead to genetic mutations and reductions in productivity. An alternative to continuous maintenance is to store cultures in the frozen state through cryopreservation. Immature embryos (embryogenic tissue) of conifers are currently cryostored. However, this requires the use of expensive controlled freezing equipment, a laborious protocol and potentially cytotoxic chemicals such as dimethyl sulfoxide. Frozen storage of mature somatic embryos of conifers in liquid nitrogen has not been reported although it would be very useful for long-term storage of genotypes possessing value-added traits, when large numbers of

embryos are produced and germination times need to be staggered, and ultimately for use in synthetic seed technology.

The purpose of this study was to develop a reliable, quantifiable storage technique for use with conifer embryogenic tissue and mature somatic embryos, specifically, embryogenic cultures of white spruce (*Picea glauca*), and interior spruce, a complex of white and Engelmann spruce (*Picea glauca* x *engelmanni*).

Chapter 2

LITERATURE REVIEW

The following section provides an overview of methods used for initiation, maintenance, and maturation of somatic embryogenic cultures. Additionally, there is a description of the physiology of freezing in plant cells and a review of current methods used to cryostore conifer cultures. The final two sections describe desiccation tolerance and the quantification of plant water status.

2.1 Somatic Embryogenesis in Conifers

In contrast to zygotic embryogenesis, which involves the fusion of two gametes to produce a zygote, somatic embryogenesis is the process whereby somatic (asexual) cells give rise to differentiated bipolar embryos. The first reports of conifer somatic embryogenesis were presented in the same year by two independent groups: Hakman *et al.* (1985) and Chalupa (1985) obtained diploid somatic embryos from cultured zygotic embryos of Norway spruce. The same year, Nagmani and Bonga (1985) initiated embryogenesis from European larch megagametophytes. Since then rapid progress has been made in this field with the objective of using somatic embryogenesis as a technology to propagate superior genotypes for clonal forestry and to produce 'synthetic seed'. This is demonstrated by the increasing number of conifer species for which somatic embryogenesis has been reported (see reviews by Dunstan, 1988; Tautorus *et al.*, 1991; Attree and Fowke, 1993; Gupta and Grob, 1995).

The steps leading to plantlet regeneration can be conveniently categorized into (i) induction, (ii) maintenance, (iii) maturation, and (iv) germination.

2.1.1 Induction

The plant material, or explant, used to initiate embryogenesis differs between species. Generally, juvenile explants are used for conifers. These include various developmental stages of zygotic embryos, either enclosed in, or separated from the female gametophyte, and explants derived from young zygotic seedlings (i.e. cotyledons). The explants are placed onto media containing macro- and microelements, vitamins, amino acids, plant growth regulators (PGRs) and a source of energy, usually sucrose. The type and concentration of PGRs used in the induction medium is important. Usually, both auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), and cytokinins, such as kinetin (KIN) or benzylaminopurine (BAP), are required to initiate embryogenesis. They are generally applied at a ratio of 2:1 auxin to cytokinin. There are some exceptions. For example, embryogenesis was initiated in *Abies normandiana* using cytokinin as the sole PGR (Nørgaard and Krogstrup, 1991). Furthermore, some explants, such as *Larix decidua* megagametophytes, can been induced to form embryogenic cultures without the inclusion of PGRs in the medium (von Aderkas *et al.*, 1987). The culture environment also influences induction success. Various light/dark and temperature regimes are employed but 25 ^{o}C in darkness is generally the most successful.

If growth conditions are favourable for embryogenesis, explants begin to proliferate and a structured white tissue mass forms. This mass, termed embryogenic tissue, is comprised of immature somatic embryos which are constantly undergoing cleavage. The structures are polarized, consisting of an embryonal mass of small meristematic cells, subtended by a long suspensor made up of large, highly vacuolate embryonal tube cells.

2.1.2 Maintenance

Maintenance of embryogenic tissue (immature somatic embryos) involves routine division and transfer of healthy tissue to fresh medium. Subculturing is generally carried out every two to three weeks. The medium used is often the same as that used for induction, but the auxin and cytokinin concentrations are sometimes lowered in equal proportions in the maintenance medium. Depending on the numbers of lines and plates used, the maintenance step can become quite expensive and labour intensive. Furthermore, cultures are prone to inadvertent genetic changes, perhaps resulting from the selective pressures imposed upon the system (Pattanavibool *et al.* 1995; Patel and Berlyn, 1982; Lassner and Orton, 1983). Because of the problems associated with frequent subculture, a means of arresting culture growth at this stage has been sought. The most widely used technique is storage in the frozen state, or cryopreservation. This topic is discussed further in Section 2.2.

2.1.3 Maturation

To produce fully-developed, germinable somatic embryos, embryogenic tissue must be transferred to a medium which promotes development. Such a medium typically consists of the same basal salts used for induction and maintenance, but is supplemented with a different PGR and an osmoticant. Although some species continue to develop on basal medium without exogenously applied PGRs, most species yield greater numbers of higher quality plantlets after treatment with abscisic acid (ABA) (von Arnold and Hakman, 1988; Dunstan *et al.*, 1988). Although its mode of action is not well understood, ABA appears to play a role in promoting the accumulation of storage reserves such as proteins, carbohydrates and fatty acids, in preventing germination, and in suppressing abnormal development (Hakman *et al.*, 1990; Roberts *et al.*, 1990a; Attree *et al.*, 1992). Optimal concentrations of ABA tend to vary between genotypes, and therefore, must be empirically

determined for each clone.

The osmotic stress, sometimes used for promoting maturation, can be imposed by increasing the concentration of compounds such as sucrose and mannitol (Tremblay, 1990; Roberts, 1991), or by adding high molecular weight, non-permeating compounds such as polyethylene glycol (Attree *et al.*, 1991).

2.1.4 Germination

Fully mature somatic embryos that have well-formed cotyledons are isolated and transferred to a medium without PGRs to promote the formation of roots and shoots. The time required for germination ranges from weeks to months depending on the species and procedure used (Lelu *et al.*, 1993). Early reports of somatic embryogenesis show that only a small proportion of embryos were converted to plantlets. More recently, however, it has been reported that an empirically-derived drying treatment, applied to interior and Sitka spruce somatic embryos prior to transfer onto germination media, increases the germination frequency and promotes synchronous root and shoot elongation (Roberts *et al.*, 1990b). It has been suggested that this drying treatment may act as a switch which turns off genes responsible for maturation and activates those required for germination (Kermode and Bewley, 1985). After germination, plantlets are transferred to a soil mix and acclimated to greenhouse conditions.

2.2 Culture Storage

The increased activity in micro-propagation and the problems associated with long-term maintenance of cultures has led to a demand for an effective means of culture storage. Current procedures can be broadly divided into two groups; those that restrict the rate of

growth, and those that completely arrest growth (Withers, 1986; Kartha, 1985). The latter group has received the most attention. Methods used to suspend culture growth all involve processes which bring about a change in the availability of water.

The most commonly used approach has been cryopreservation. This technique is based on 'the arrest of metabolic functions of biological material by lowering the temperature to approach that of liquid nitrogen (LN_2) while maintaining viability' (Kartha, 1985). The ability to recover plant materials after freezing in LN_2 was first recognized by Sun (1958) using pea seedlings. It was not until the early 1970's that successful cryopreservation in LN_2 was achieved with *in vitro* cultured plant tissue. These pioneer studies were carried out with carrot cells and callus of *Populus* x *euramericana* (Nag and Street, 1973; Sakai and Sugawara, 1973).

Since then, cryopreservation as a means of *in vitro* storage has received great attention. The subject has been extensively reviewed (Withers, 1986; 1987; Sakai, 1986; Kartha, 1985, 1987; Chen and Kartha, 1987: Bajaj, 1995). Until recently, there have not been many reports of gymnosperm culture preservation. This may be because it has only been in the last decade, with the first reports of conifer somatic embryogenesis, that the potential of *in vitro*-propagated trees has been recognized, and with this, the need for a reliable storage method has arisen. Successful cryopreservation has been reported for eleven conifer species/hybrids: embryogenic tissue of five *Picea* species (Gupta *et al.*, 1987; Kartha *et al.*, 1988; Find *et al.*, 1993; Klimaszewska *et al.*, 1992; Bercetche *et al.*, 1990), interior spruce (Cyr *et al.*, 1994), two *Pinus* species (Gupta *et al.*, 1987; Lainé *et al.*, 1992), hybrid larch (Klimaszewska *et al.*, 1992), *Abies nordmanniana* (Nørgaard *et al.*, 1993a) and non-embryogenic suspension cells of Douglas fir (Binder and Zaerr, 1980). The protocols used to cryopreserve conifer embryogenic tissue are very similar.

Before reviewing the steps involved in existing methods used to cryopreserve plant cultures, the processes of freezing water and natural freezing in plant tissues will be described.

2.2.1 Freezing water and freezing in plant cells under natural conditions

Although it is generally thought that water freezes at 0 °C, it rarely does. For ice to form, nucleators must be present to act as templates for crystal growth. Dust or glass particles, macromolecules and even ice-nucleating bacteria can act as heterogeneous nucleators. The smaller the nucleus, the lower the temperature that ice forms. Therefore, water will supercool until the temperature is low enough for the largest nucleus present to initiate crystallization (Meryman and Williams, 1985). If there are no heterogeneous nucleators, pure water can supercool to about -40 °C before spontaneous ice nucleation occurs. At this temperature, the randomly arranged water molecules act as homogeneous nucleators. The presence of solutes further lowers the nucleation temperature.

Many trees and shrubs that overwinter in northern temperate, subarctic and alpine regions are able to survive freezing stress. The mechanism of tolerance involves the ability of cells to avoid intracellular ice formation through supercooling. When plant cells are exposed to progressively lower temperatures, ice first begins to form extracellularly, by heterogeneous ice nucleation. The cytoplasm does not contain heterogeneous nuclei and the plasma membrane acts as a barrier against intracellular seeding from the outside. Therefore, the cytoplasm supercools. As the temperature continues to drop, increasing amounts of extracellular water are converted to ice which in turn increases the solute concentration outside the cells. As a result of this increased solute concentration, the water potential decreases outside the cell. The water potential gradient, in combination with the difference in vapour pressure between the liquid water inside the cells and the extracellular frozen

solution (vapour pressure is higher inside than outside), results in a net diffusion of water out of the cells. This efflux of water continues until a thermodynamic equilibrium is established between the inside and outside of the cell.

An important component of current cryopreservation protocols is a slow freezing step that mimics the way that freezing occurs in nature. Its purpose is to facilitate a reduction in the intracellular water content so that the chance of large ice crystal formation within cells is minimized. From the preceding explanation of natural slow freezing in cells, it is evident that successful freeze-preservation of plant cells must involve two stages: first, the removal of a fixed amount of the cellular water, and second, the inactivation of the remaining water.

2.2.2 Existing Protocol for Freezing Conifer Embryogenic Tissue

Cryopreservation procedures can be broken down into the following stages: pretreatment and cryoprotection, freezing, storage, thawing and recovery.

2.2.2.1 Pretreatment and cryoprotection

The freezing tolerance of cells is a function of both cell age and cell cycle stage (Withers, 1985). Generally, cultures are frozen in the early exponential phase of growth. At this stage, cells are denser and less vacuolated, and thus less susceptible to freezing damage (Nag and Street, 1975).

In 1912, Maximov recognized that the recovery of plant tissue after freezing was greatly enhanced by the addition of compounds with cryoprotective qualities. Present-day freezing protocols almost always involve the application of some form of cryoprotection to reduce cellular injury during the freeze-thaw cycle. This cryoprotection may be in the form of an

added chemical or a physical hardening treatment. Cryoprotectants have traditionally been classified on the basis of their ability to cross membranes and their molecular weight (Morris, 1980).

Permeating cryoprotectants, which cross the plasma membrane, include compounds such as dimethyl sulfoxide (DMSO) and glycerol. Although often used in the cryopreservation of animal cells, glycerol alone is seldom effective for protecting plant cells. DMSO has been much more useful (Withers, 1986). The first use of DMSO in freeze-preservation of higher plant cells was by Quatrano (1968) when he froze cultured flax cells to -50 ^{o}C using 10% (v/v) DMSO. The mode of action of cryoprotective compounds such as DMSO is poorly understood. However, it is generally believed that their primary role is to decrease, on a colligative or antifreeze basis, the amount of ice formed at any given temperature. Thus as the internal and external solutions are concentrated when ice is formed, cryoprotectants act colligatively to increase the amount of unfrozen water solvent. As a result, the increase in potentially toxic salt concentrations is not as great and intracellular water loss remains within a tolerable limit (Meryman and Williams, 1985). Unfortunately, not all of the effects of these additives are beneficial. There is evidence that the majority of cryoprotectants exhibit varying degrees of cytotoxicity (Fahy *et al.*, 1990; Kartha *et al.*, 1988; Klimaszewska *et al.*, 1992). The degree of cytotoxicity is apparently a function of concentration, temperature and period of exposure. To reduce these harmful effects, cryoprotectants are applied at the lowest possible concentrations (5-10% v/v for DMSO) at reduced temperature (in an ice bath held at 0 to 4 ^{o}C), and over a short period (usually 1 hour) (Morris, 1980; Withers, 1986).

Another class of protective additives are non-permeating cryoprotectants. This category has historically included sucrose and hexitol sugars, such as sorbitol and mannitol. The group also includes high molecular weight, non-penetrating additives, such as

polyvinylpyrolidone (PVP), dextran and polyethylene glycol (PEG). Both of these groups of cryoprotectants are thought to act mainly through their dehydrative effects (Kartha, 1987). However, in the case of the lower molecular weight additives (the sugars and sugar alcohols), Dumet *et al.* (1993) referred to several recent studies that show that these substances actually enter cells, and therefore might play other protective roles, in addition to osmotic effects.

In cases where use of a single cryoprotectant does not result in high survival, mixtures of cryoprotectants have been very effective. Kartha *et al.* (1988) found that DMSO alone (5-20% v/v), in addition to being cytotoxic, was also ineffective as a cryoprotectant for embryogenic suspensions of white spruce. For optimal recovery, the following pretreatment was carried out; culture of the cells for 24 h in liquid medium + 0.4 M sorbitol, followed by treatment of the cells with a cryoprotectant combination of 0.4 M sorbitol + 5 % DMSO. This protocol has been adopted, with minor modifications, by all others attempting to cryopreserve conifer embryogenic tissue.

2.2.2.2 Freezing

In preparation for freezing, pretreated cells are transferred into suitable cryogenic containers. The most commonly used vessels are pre-sterilized polypropylene ampoules. Cultures are generally frozen slowly, though in a few cases rapid freezing has been successful. Slow freezing methods affect the cells in the same way as *in vivo* freezing. That is, slow freezing before LN_2 immersion prevents lethal ice crystal formation by allowing adequate time for the intracellular water content to be reduced. There are different ways to achieve slow freezing, including cooling at constant rates and stepped cooling. The latter method involves holding the samples at several intermediate sub-zero temperatures for varying lengths of time before transfer to liquid nitrogen (Kartha, 1987).

Some of the most successful attempts at cryopreservation have involved a combination of the two methods -i.e. a sample is cooled at a constant rate to some temperature well-above that of liquid nitrogen, held for a short time, and then directly immersed in LN_2. The rate of slow cooling appears to be important. Conifer embryogenic tissue has been slow-cooled at rates ranging from 0.3 $^oC\ min^{-1}$ (Kartha *et al.*, 1988) to 1 $^oC\ min^{-1}$ (Gupta *et al.*, 1987), to holding temperatures between -30 ^oC and -40 ^oC (i.e. combination of slow freezing methods) before plunging tissue into LN_2. Optimal rates of slow freezing and holding temperatures are empirically derived for each species.

Some very hardy materials can be frozen slowly without the use of cryoprotectants. Winter twigs of *Salix* survived slow freezing to LN_2 temperature. Prefreezing winter willow, poplar and white birch to temperatures of -15 ^oC enabled them to withstand direct immersion in LN_2 (Sakai, 1960; 1965 in Sakai, 1986). Presumably, this was possible because the cells had been sufficiently dehydrated during the slow freezing process.

Recent studies have explored the use of vitrification to eliminate the need for controlled slow-freezing, and thus, the use of expensive controlled-freezing equipment. These techniques have not yet been applied to conifer species. Vitrification, or glass formation, can be achieved by exposure of the specimens to highly concentrated solutions of cryoprotectants. This allows samples to supercool to very low temperatures and then solidify without ice formation. Glass formation occurs during the rapid cooling stage (Towill and Jarret, 1992; Withers, 1987). In one study, multiple bud clusters of asparagus were treated with a cryoprotectant mixture of 30% (w/v) glycerol, 15 % (w/v) ethylene glycol and 15 % (w/v) DMSO in a medium containing 0.4M sucrose before LN_2 immersion (Kohmura *et al.*, 1992). This method apparently increased survival by avoiding the damaging effects of ice formation; however, as discussed earlier, exposure of specimens to high concentrations of cryoprotectants can have deleterious effects.

Consequently, while a simplified method for cryopreservation, glass formation as formed by this route, is limited to a few lines and culture types.

Rapid freezing is generally carried out by immersing the cryoprotectant-treated specimens directly into LN_2. At such rapid cooling rates (several hundred degrees per minute) the water cannot diffuse out to the extracellular space, thus, intracellular ice formation would seem unavoidable. It is speculated that ultra-rapid cooling may prevent the growth of intracellular ice crystals by 'rapidly passing cells through the temperature zone at which lethal ice crystal growth occurs' (Chen and Kartha, 1987; Kartha, 1987). Rapid freezing methods have been successfully used to cryopreserve shoot-tips of a few herbaceous species, most notably *Solanum* spp. (Henshaw *et al.*, 1985), although post-thaw recovery has been low and variable. In general, rapid freezing has been unsuitable for the cryopreservation of cell cultures (Chen and Kartha, 1987).

2.2.2.3 Storage

Samples must be stored at a sufficiently low temperature to avoid damage from ice recrystallization and to maintain arrested metabolism. Temperatures below -100 ^{o}C are recommended and are usually achieved by keeping samples in vessels containing LN_2, which has a liquid phase temperature of -196 ^{o}C and a vapour phase of approximately -150 ^{o}C.

2.2.2.4 Thawing and Recovery

Rapid thawing has been adopted in most cryopreservation protocols. This prevents potentially harmful ice recrystallization which may occur during slow rewarming (Kartha, 1987). Rapid thawing is commonly carried out by removing samples from the LN_2 storage

container and immersing them directly into a circulating 35-40 °C water bath for several minutes (Kartha, 1987; Withers, 1986).

Post-thaw removal of cryoprotectants was conducted in many of the earlier cryopreservation protocols (Nag and Street, 1975). However, more recent evidence suggests that washing leads to rapid deplasmolysis injury and that dilution of cryoprotectants may just as easily take place on nutrient media containing a solidifying gel such as agar. In fact, the transfer of unwashed, LN_2-stored periwinkle cell cultures onto filter paper placed on agar medium increased recovery substantially compared to washed cells (Chen *et al.*, 1984). Direct transfer onto filter paper on medium has also been used successfully with cryopreserved spruce cultures (Klimaszewska *et al.*, 1992; Find *et al.*, 1993).

2.2.3 Cryopreservation of Mature Somatic Embryos

Storage of mature somatic embryos using cryopreservation has been reported for a number of angiosperm species. Somatic embryos of oil palm were frozen in liquid nitrogen after preculture on a 0.75 M sucrose medium followed by an additional dehydration treatment under an air current of a laminar flow or over silica gel (Dumet *et al.*, 1993). Additionally, successful cryopreservation of hydrated carrot and coffee somatic embryos was achieved using a two-step freezing process with sucrose as the only cryoprotectant (Tessereau *et al.*, 1991). Coffee somatic embryos were later cryostored directly in liquid nitrogen after an ABA/sucrose pretreatment followed by desiccation at 75% relative humidity (at 24 °C) for 7 days (Tessereau *et al.*, 1994).

Until very recently, the storage of mature conifer somatic embryos through freeze preservation had not been reported. Attree *et al.*(1995) successfully stored white spruce

somatic embryos at -20 °C for one year following a slow drying treatment. Frozen storage of mature conifer embryos would be useful when large numbers of embryos are produced so that germination could be staggered. Storage at this stage would also provide the potential for germplasm conservation for virtually indefinite periods and facilitate easy delivery of embryos to nurseries.

2.3 Desiccation Tolerance

Every Kingdom of organisms contains species that are able to survive almost total dehydration. In animal species, this desiccation tolerance is often referred to as anhydrobiosis ('life without water') (Crowe, 1971), whereas in studies of plant systems, the terms quiescence, and dormancy are used. However, despite its widespread occurrence and the extensive research that has been conducted in this field, the physiological mechanisms that allow particular organisms to survive extreme desiccation are still not well understood.

Probably the most significant finding over the past two decades of research on desiccation tolerance is that many dehydration tolerant organisms contain high concentrations of specific sugars (Crowe, 1971; Crowe *et al.*, 1984). The disaccharides, particularly trehalose, are thought to play a major role in tolerance. Large concentrations of trehalose are found in dry active baker's yeast, cysts of the brine shrimp, *Artemia*, and in the desert resurrection plant, *Selaginella lepidophylla* (see Crowe *et al.*, 1984 for references). Trehalose seems to be required for these species to survive water stress. Some research has also indicated that if trehalose is introduced into cells that do not normally synthesize it, that they too will survive dehydration (Crowe and Crowe, 1992). The use of trehalose may have practical implications in areas such as food science, health care and agriculture. For example, a group at the Quadrant Research Foundation in Cambridge, United

Kingdom, is conducting extensive research on the use of trehalose as a replacement for freeze-dried storage of foods and pharmaceuticals (Roser, 1991; Roser and Colaco, 1993).

Drying and rehydration processes have been correlated with injury to cellular membrane systems (Crèvecoeur *et al.*, 1976; Simon, 1978; Senaratna *et al.*, 1984; Senaratna and McKersie, 1986). Trehalose appears to be involved in the stabilization of labile structures including membranes. The mode of action of trehalose is still not fully understood; however, three main theories have been proposed.

2.3.1 The Water-Replacement theory

This theory was first suggested by Webb (1965) who found that coliform bacteria cells survived desiccation if they were first suspended in inositol. He theorized that polyols replaced water bound to nucleic acids. Crowe and co-workers took this water replacement theory in another direction and investigated the role of sugars in membrane protection (Crowe *et al.*, 1984). They suggested that trehalose can replace water by hydrogen-bonding to macromolecules such as the hydrophilic polar head groups of membrane phospholipids. Crowe and Crowe (1986) then demonstrated that this disaccharide is capable of completely inhibiting fusion between phospholipid vesicles and lateral phase separations as well as preserving structure and function of the vesicles after rehydration. The efficacy of using other sugars as stabilizers was also tested, but trehalose was found to be the most effective. Reducing sugars, such as glucose are initially effective but their effects soon decline. This is most likely due to a common chemical reaction which occurs between reducing sugars and dry proteins called the Maillard browning reaction. Other non-reducing sugars, such as sucrose, were in some cases able to stabilize membranes, but three times as much sucrose as trehalose was necessary. Different polyols including glycerol, were not at all effective and in fact, may have had a de-stabilizing effect.

2.3.2 Glass Formation theory

In addition to prevention of physical membrane disruption, tolerance to desiccation also requires that deleterious reactions occurring in the dry state are avoided. Glass Formation (vitrification) is another theory that aids in explaining the protective, stabilizing abilities of trehalose. The molecules of vitrified materials are solidified in a random arrangement typical of the liquid state, rather than in an orderly geometric pattern of a crystal. Substances of this type have complex molecular forms for which crystallization is difficult (Mortimer, 1986). The most well-known method for preparing a glass involves cooling a viscous liquid below its thermodynamic freezing point, through a super-cooled (under-cooled) phase which is metastable, and finally below glass transition temperature. Glasses can also be formed by many other routes besides super-cooled solutions (see review by Angell, 1995). For example, supersaturated solutions also form glasses. Highly concentrated solutions of sugars such as trehalose, fructose, sucrose and glucose can, instead of crystallizing, form high temperature aqueous glasses that are stable up to 90°C (Burke, 1986). Because glasses are by definition viscous, cell constituents would be immobilized, slowing deleterious reactions. Also, trehalose glasses are non-hygroscopic because the vapour pressure of water in equilibrium with trehalose is very high and subsequently, the glass loses water to the ambient environment rather than taking it up. This contrasts with the hygroscopic nature of other glasses such as sucrose. Uptake of water by these glasses dissolves the surface layer of sugars in the glass and, because the crystalline state is more stable than the glass state, these dissolved sugars crystallize out of solution (Roser, 1991). This phenomenon, known in food sciences as weeping, does not occur with a trehalose glass.

2.3.3 Chemical stability theory

This hypothesis also relates to the avoidance of destructive chemical reactions. Trehalose is one of the most chemically unreactive and stable of sugars (Roser, 1991). The storage of proteins in reducing sugars at low water content is known to lead to progressive damage due to sugar-amino browning (Maillard) reactions (Eichner and Karel, 1972). Due to its structural stability, it does not easily hydrolyze into its two constitutive reducing glucose molecules. Thus, the Maillard reaction of the amino groups of proteins with reducing sugars does not occur.

2.3.4 Higher Plant Tissues

An explanation for the tolerance of the seeds and pollen of many higher plant species to extreme dehydration may not be so simple. Trehalose is not found in appreciable quantities in these plant structures (Leopold and Vertucci, 1986). Therefore, it is believed that the ability of many seeds to withstand water stress may be linked to the presence of other sugars, as well as to changes in structure and biochemistry that occur during embryogenesis (Bewley and Black, 1994). It has been well-documented that the degree of desiccation tolerance in seeds increases throughout development, peaks at maturity, and then declines through germination (Kermode *et al.*, 1986; Sun and Leopold, 1993; Senaratna and McKersie, 1983; Bewley and Black, 1994). During these stages, many morphological and biochemical transitions occur (see Bewley and Black, 1994; Vertucci and Farrant, 1995 for recent reviews); some of the relevant changes are discussed below.

Three main stages can be recognized in seed development: tissue differentiation, expansion (maturation), and maturation drying (Kermode, 1990). In the early stages of tissue differentiation, overall rates of both metabolism and cell division are high. During this

phase, and early into expansion, the water content increases markedly as cells enlarge through increased vacuolation. At these immature stages, desiccation tolerance in all seed types is low (Berjak *et al.*, 1992; Vertuccci and Farrant, 1995). Following this phase, vacuoles begin to fill with storage reserves including proteins, carbohydrates and lipids resulting in a period of rapid dry weight gain. There is a concomitant decline in the cellular water content as water is displaced to make room for reserves. The degree of vacuolation is reduced and cells become increasingly tolerant to desiccation. Accumulation of storage products and decreased vacuolation may be factors that allow cells, and in particular membranes, to withstand the mechanical stresses imposed upon them by dehydration (Vertucci and Farrant, 1995). Additionally, storage product accumulation is often coupled with changes in membranes of organelles. This may be a phenomenon that allows the seed to escape the repercussions of drying on cellular membrane systems. The final stage in development for many seeds is maturation drying. This phase leads to a state of metabolic quiescence and it is at this point that the seeds of 'orthodox' species can be stored for extended periods (seeds that are sensitive to desiccation and are difficult to store for more than a growing season are called 'recalcitrant'). It is believed that the switch from a developmental to a germinative mode is triggered by the maturation drying phase (Kermode and Bewley, 1985; Kermode, 1990). In many seeds, the ability to withstand dehydration is acquired before the start of the maturation drying process. For example, castor bean seeds become desiccation tolerant approximately mid-way through their development (Kermode and Bewley, 1985). However, tolerance to drying at earlier stages only occurs if seeds are dried slowly. When dehydration treatments are applied closer to the timing of natural drying, seeds are able to withstand rapid desiccation. Therefore, tolerance to drying increases progressively through development and is likely concomitant with morphological changes and the synthesis of various compounds. These include the expression of new proteins, and changes in the carbohydrate and lipid content. Additionally, ABA concentrations increase during the later stages of development.

A number of different classes of proteins are expressed during normal seed development; one prominent group of proteins appears late in embryogenesis and accumulates until maturation. They are called the late embryogenesis abundant (LEA) proteins and are found in the seeds of many desiccation tolerant plant species. Although the mechanism by which LEAs function is not understood, they are thought to play a role in desiccation tolerance because they are hydrophilic and therefore can maintain a water-rich local environment (Bewley and Black, 1994). LEA proteins are encoded by the *lea* genes. In many species, high ABA content coincides with the greatest production of proteins and other storage products; thus, ABA is thought to play a role in the expression of *lea* genes (Bewley and Black, 1994). Since the accumulation of LEAs and other proteins is thought to be related to increased desiccation tolerance, it is not surprising that the addition of ABA confers dehydration tolerance to maturing somatic embryos of many species including alfalfa (Anandarajah and McKersie, 1990b; Senaratna *et al.*, 1989), celery (Kim and Janick, 1991) and white spruce (Attree *et al.*, 1991).

Carbohydrate content also changes in many seeds during development. Generally, the monosaccharide content decreases during maturation and the concentrations of sucrose and oligosaccharides increase (see Vertucci and Farrant 1995 for references). In fact, soluble sugars can make up as much as 14 to 20% of the total dry weight of seeds (Leopold *et al.* 1992). This brings us back to the hypotheses suggested for trehalose-mediated desiccation tolerance. It has been suggested that since trehalose is not present in seeds, other sugars could provide a protective effect for membranes (Leopold and Vertucci, 1986). Koster and Leopold (1988) analysed sucrose and other soluble sugars in three types of seeds (corn, pea and soybean) to determine whether there were any correlations between sugar content and desiccation tolerance. They proposed that sucrose interacted with membrane surfaces to maintain structure (i.e. the water replacement hypothesis). However, based on

information presented earlier in this section, we would expect that sucrose could not fill this role since it is easily hydrolyzed into the reducing sugars glucose and fructose. These sugars would participate in the Maillard browning reactions and threaten viability. The discovery of the presence of oligosaccharides, particularly raffinose and stachyose, in addition to sucrose in these developing seeds may shed some light on this situation. Koster and Leopold also found that sucrose and these larger oligosaccharides were always present when seeds were desiccation tolerant and that tolerance was lost with the disappearance of the oligosaccharides. They proposed that the larger oligosaccharides prevented the sucrose from crystallizing, and in the non-crystalline form, the sucrose interacted with membrane surfaces to replace water and maintain membrane structure.

In addition to the proposed stabilization of membranes by water replacement, there is also some evidence of glass transitions in seeds. Using differential scanning calorimetry, Williams and Leopold (1989) detected glass transitions in corn embryos. When the lipids were extracted from these seeds, a glass transition still appeared in the material. The transition was above 40 ^{o}C in embryos dried to water contents below 12% g H_2O g^{-1} dry mass and fell to below -60 ^{o}C in seeds hydrated above 20%. The water content of stored seeds is generally less than 12%, therefore, the non-lipidic fractions of these seeds would exist in a glassy state even at ambient temperature. Sugars were proposed as the mediators of this non-lipid glassy state since it was found that 20% of the dry mass of the embryo was composed of sucrose (17%) and raffinose (3%), and at these temperatures, these solutes have the tendency to form glasses at low water contents when present at high concentrations. In excised embryos of soybean, glass transitions occurred at water contents below 0.1 g H_2O g^{-1} dry mass, providing more evidence that the capacity of a tissue to be vitrified is associated with a desiccation tolerant state (Bruni and Leopold, 1991). The presence of oligosaccharides in these systems could once again play a role in stabilization by alleviating the possibility of crystallization so that a glassy state is

maintained.

An explanation of desiccation tolerance in seeds unfortunately, is not this simple since there are several examples where these hypotheses fall apart (see Vertucci and Farrant, 1995). In the stages following histo-differentiation, seeds of the recalcitrant mangrove species *Avicennia marina* accumulate high concentrations of soluble sugars, yet they remain intolerant to drying (Farrant *et al.*, 1992). High levels of sugars have been reported in developing and mature seeds of other desiccation intolerant species. Furthermore, the total content of soluble sugars in some desiccation tolerant species can be as low as 1% of the dry mass, an amount which is likely too low for the formation of glasses (Vertucci and Farrant, 1995). Recently, Sun *et al.* (1994) reported that desiccation-tolerant soybean and desiccation-intolerant red oak seeds exhibited similar glass transition behaviour. Thus, the glassy state alone is not a sufficient explanation for desiccation tolerance in orthodox seeds. While it appears that there is a role for sugars in desiccation tolerance of higher plant tissues, this is likely not the only mechanism at work.

2.4 Water Potential and Water Release Curves

2.4.1 Plant Water Status

To study the water relations of cultured tissues, the tissue water status must be quantified. Generally, plant water status is expressed in terms of either water content or the energy status of water within the plant (water potential).

2.4.1.1 Water Content

Measurements based on water mass include absolute and relative water content. Water

content is the mass of water expressed as a function of plant tissue dry weight. Relative water content (RWC) is the amount of water in the plant tissue relative to that when the tissue is fully hydrated (turgid), that is:

$$RWC = (M_f - M_d) / (M_t - M_d); \qquad [2.1]$$

where

M_f = mass at full turgor (g)

M_f = fresh weight (g)

M_d = dry weight (g) after oven-drying at 65 ^oC for 48 hours

RWC may provide a more useful measure of water status than absolute water content since normalization allows easy comparison of data from different sources.

2.4.1.2 Water Potential

Water status can also be expressed in terms of the water potential, that is, the chemical potential of water expressed in terms of energy per unit volume of fluid. For a given species, tissue type or developmental stage, there is a unique relation between water potential and RWC. This will be discussed in the section 2.4.2. The chemical potential of a substance is a measure of the free energy, or more specifically, the free energy per mole (J mol^{-1}) available to do work. Chemical potential is a relative quantity and is expressed as the difference between the potential of a substance in a given state and the potential of the same substance in a standard state (1 atm; 25 ^oC) (Taiz and Zeiger, 1991). Dividing this value by the partial molal volume of water (m^3 mol^{-1}) gives the water potential (J m^{-3}), symbolized as Ψ. Water potential is usually expressed in terms of a force per unit area, or pressure and expressed in Pascals (since J=N•m and 1 MPa = 10^6 Nm^{-2}). Water

potential, like chemical potential, is a relative quantity. It is compared to the standard state of water, defined as pure water at the same pressure and temperature as the sample in question. Pure water held at some reference height is arbitrarily assigned a water potential of zero. Thus, any solution containing dissolved ions, at a height lower than the reference height (i.e. ground water in soils), or held within a matrix has a lower (negative) water potential.

In plant cells, water potential can be expressed as the sum of three components:

$$\Psi = \pi + P + m \qquad [2.2]$$

where π is the osmotic potential and results from dissolved solutes lowering the free energy of the system and by convention, is always negative. Adding solutes can only lower the free energy since solute molecules interact with water molecules and decrease their ability to diffuse. P represents the hydrostatic pressure exerted by water on the cell walls. Because plant cells have rigid cell walls, hydrostatic pressure (turgor pressure) can be large. This pressure component is typically positive, but may also be negative due to tensions arising in the xylem or apoplast. The symbol m represents the matric potential, water's adhesion to non-dissolved structures such as cell walls and membranes. Adhesion can only decrease the free energy, therefore, it is always negative. In living cells, the matric component is usually negligible except in relatively dry materials such as seeds.

2.4.1.3 Water Movement

Water movement can be accounted for by one of two physical processes: either bulk (mass) flow or diffusion. Bulk flow is pressure-driven; it occurs when an external force, such as pressure or gravity, is applied. In most cases, water moves in or out of cells by diffusion,

and not by bulk flow. Diffusion is the spontaneous transport of particles or molecules in space and is directly proportional to the gradient of concentration. The directional movement is based on the random thermal motion of individual molecules (Brownian molecular movement). The diffusion of water through a selectively permeable membrane, in response to a gradient in water potential, is known as osmosis. Water will move from regions of high to low water potential until thermodynamic equilibrium is reached.

2.4.2 Water-Release Curves

Valuable information can be derived from plots of water potential *vs.* RWC. These water-release (pressure-volume) curves can be generated in a number of ways. For rigid plant tissues such as whole shoots or leaves, pressure chambers are often used (Scholander *et al.*, 1965); however, this method is not appropriate for use with small cultured samples. Therefore, I used a modified version of a technique first described by Livingston and de Jong (1988) in their study of monocot leaf tissue. In their procedure, a series of unsaturated salt solutions were prepared with molalities corresponding to water potentials ranging from -0.1 to -5.0 MPa. Leaf disc samples were suspended above the solutions in sealed test tubes. The discs were then left to equilibrate for 12 hours. At equilibrium, the Ψ of the tissue sample is the same as the Ψ of the salt solution. The discs were weighed, dried and re-weighed to allow calculation of RWC. This technique allows rapid production of water-release curves; over 40 curves were produced in 2 days compared to only 10 plots using the pressure chamber method over the same period. The unsaturated salt solution technique has since been modified to allow generation of curves for entire conifer needles (Livingston and de Jong, 1990), as well as for embryogenic cultures and seedlings of European larch (Livingston *et al.* 1992).

Analysis of these plots, and plots of $1/\Psi$ vs. RWC, yields a number of useful water

relations parameters. The model that is the foundation of this analysis, as well as analyses of more conventionally obtained plots, assumes that the cell behaves as an osmometer. A living cell can be thought of as an osmotic system in that it possesses: (i) a membrane separating two or more volumes of solutions that allows water to move readily across it, but restricts the movement of certain solutes; and (ii) a means of allowing pressure to build up in at least one of the volumes. In the osmometer this pressure is typically exerted by raising a solution in a tube against gravity. In a cell, the rigidity of the cell wall is responsible for the increased pressure. The Boyle-Van't Hoff equation provides a model of the relation between the volume of water in the cell and the various components of water potential (Nobel, 1969). Water relations parameters derived from these analyses include: bulk elastic modulus (ε); osmotic potential at full turgor and at zero turgor ($\Psi_{\pi t}$,Ψ_o); RWC at zero turgor (RWC_o); and bound water (A). The concept of bound water is somewhat contentious. For this study, bound water values were derived from the analysis of water release curves, as outlined below.

At high RWC, there are large changes in water content for a given change in water potential. This results in an initial curvilinear relationship between RWC and $1/\Psi$. Below the turgor loss point, $\Psi=\pi$, giving rise to a linear relationship. Extrapolation of the linear portion of the curve to the y-axis (at $1/\Psi=0$, $\Psi=\infty$) provides an estimate of the bound water in the sample. Thus, in our study, bound water is defined as water physically or chemically held by surfaces and removable only with the exertion of very large (infinite) water potentials.

2.5 Conclusions

Current cryopreservation techniques have proven to be a satisfactory means of long-term storage for a number of woody plant cultures. However, while these methods eliminate the

problem of continuous maintenance, they are far from ideal. Each species still requires its own labour-intensive protocol, especially in the pretreatment and slow freezing stages. All procedures require the use of potentially cytotoxic chemicals for cryoprotection and expensive controlled-freezing equipment. Finally, cryopreservation is still restricted to a limited number of species, lines and developmental stages.

These existing protocols involve several preparatory steps which lower the amount of intracellular water. This reduction in water content reduces the possibility of intracellular ice formation. Some of the limitations above may be overcome by lowering the cell water content in a different manner. If tissue is desiccation tolerant and can be dried to a well-defined low water content, then it should be possible to immerse samples directly in liquid nitrogen. This would eliminate the need for the pretreatment, the slow-cooling step and perhaps the addition of cryoprotectants.

Chapter 3

DESICCATION AND FREEZING OF SOMATIC EMBRYOGENIC TISSUE

3.1 Introduction

The current method used to cryopreserve conifer embryogenic tissue, described in Section 2.2.2, involves preliminary dehydrative steps that reduce the possibility of lethal ice formation during freezing. These include a pretreatment step, the addition of cryoprotectants and a slow-freezing step. This chapter describes work conducted to develop a modified storage protocol that would eliminate this multi-step procedure. The method employs a simple dehydration technique based upon well-established water relations theory.

The first objective of this study was to establish the appropriate criteria for determining tissue viability after drying and freezing treatments. The methods most often used for assessing viability of plant tissue following a stress treatment are regrowth, triphenyltetrazolium chloride reduction, vital staining (e.g. neutral red; iodine-potassium iodine; fluorescent dyes such as fluorescein diacetate), protoplasmic streaming, plasmolysis and leakage of ions or organic material (Ishikawa *et al.*, 1995; Calkins and Swanson, 1990). Although regrowth of the tissue after a given treatment is the most sensitive test, this is time-consuming and requires completely sterile growing conditions. This need for aseptic conditions proved to be especially problematic in my study with the hundreds of vials, solutions and tools required for each experiment. Two staining techniques were explored and compared to regrowth tests. Additionally, I wanted to determine whether there is a relation between electrolyte leakage measurements and damage to embryogenic tissue after drying treatments. The literature suggests that injury at low hydration is related

to damage to cell membranes (Crevecouer *et al.*, 1976; Simon, 1978; Crowe and Crowe, 1986; Senaratna and McKersie, 1986). Although the precise mechanism by which dehydration stress injures membranes has not been clearly defined, it appears that membrane injury due to drying always results in increased permeability. It seemed therefore appropriate to try and relate the effects of drying to the loss of cytoplasmic solutes through the cell membrane.

A second objective was to was to characterize water release from spruce embryogenic tissue. This involved dehydration of tissue in a controlled atmosphere, quantification of tissue water content at equilibrium with a given atmosphere, and the generation of tissue water release curves. This would allow calculation of bound water values and other water relations parameters for tissue and ensure that drying protocols would be repeatable.

A third objective was to test the hypothesis that embryogenic tissue could be desiccated in dry air to bound water without causing fatal damage. After generating water release curves for the embryogenic tissue, I was able to consistently dry tissue samples to a series of specified RWCs, ranging from 100% RWC down to bound water. Following rehydration by diffusion, samples were assessed for viability.

Finally, I wanted to investigate the hypothesis that dried tissue could be directly frozen in liquid nitrogen. The premise for this hypothesis was that if tissue could first be non-fatally desiccated in dry air to low RWC, it should then be possible to immerse it directly into liquid nitrogen. This would both simplify the freeze-storage procedure, by cutting down on the number of steps used, as well as give the cryopreservation protocol a more quantitative foundation.

3.2 Materials and Methods

3.2.1 Plant Material

White spruce (*Picea glauca*) somatic embryogenic tissue derived from zygotic embryos (Park *et al.*, 1993) was provided by Dr. J.M. Bonga, Canadian Forest Service, Fredericton, New Brunswick. The line used (WS10) was selected on the basis of its vigorous growth and high embryo yield. The tissue was maintained on modified Litvay's medium (Litvay *et al.*, 1981) containing half-strength Litvay's salts and vitamins, 29.2 mM sucrose, 1 g/L casein hydrolysate, 0.55 mM inositol, 10 μM 2,4-D, 5 μM kinetin and 0.4% (w/v) Gelrite gellan gum (Tremblay, 1990). Tissue was incubated in the dark at 24 ^{o}C and sub-cultured weekly prior to treatments.

3.2.2 Generation of Water Release Curves

The drying technique used to characterize water release in embryogenic tissue followed the procedure described by Livingston *et al.* (1992). Briefly, the method involves drying cultured tissue to known RWC by suspending samples over unsaturated salt solutions of known water potential. Once the samples are sealed in this environment, they slowly come to vapour equilibrium with the surrounding air, which is in equilibrium with the salt solution. If the salt solution has a lower Ψ than the tissue, there is a net movement of water out of the tissue and it dries (Fig. 3.1). By plotting RWC versus time for tissue suspended over water and over salt solutions, it was determined that for my particular system time to equilibration takes no more than 6 days (data not shown). Samples are placed in modified 0.5-mL Eppendorf microcentrifuge tubes and hung several millimetres above a salt solution of known molality. The salt solution (2 mL), Eppendorf and sample are all placed in a 10-mL culture tube sealed with a rubber septum stopper. The inner wall

of the culture tube is lined with filter paper. This effectively increases the surface area of the salt solution in contact with the air. To maintain a constant Ψ, eliminate temperature gradients, and decrease respiration during the drying treatment, the sealed culture tubes are held in a well-stirred water bath at 5 ^{o}C.

There were two modifications to the referenced procedure. First, the collars, fashioned from tygon tubing and fitted around the bottom of Eppendorfs, were no longer used since it was discovered that they take up small amounts of water thus confounding tissue weight measurements. Second, Eppendorfs containing samples to be cryopreserved were further modified to allow them to fit into cryogenic ampoules. The lip around the top of the microcentrifuge tube was removed, except directly above the connecting tab.

Twenty-three NaCl solutions were prepared with molalities that ranged from 0.013 to 3.99 mol kg^{-1}. These concentrations provided a corresponding range of Ψs from -0.05 to -20 MPa (Appendix I). In addition, several samples were suspended above deionized distilled water, which has a Ψ of 0 MPa. Water potentials were calculated using the formula and coefficients described by Lang (1967). At 5 ^{o}C, the relative humidity (RH) of the air in equilibrium with these solutions ranged from 99.9 to 85.8%, calculated according to the following equation (Nobel, 1991):

$$\Psi = -(RT/V_w)\ln (RH(\%)/100) \qquad [3.1]$$

where

Ψ = water potential (MPa)

R = the universal gas constant (8.314 $JK^{-1}mol^{-1}$)

T = absolute temperature (K)

V_w = the partial molal volume of water (18.0 cm^3mol at 5 ^{o}C)

Relative water content of samples was determined by weighing tissue on a precision five-digit balance (Mettler AE 240) before drying (M_t), after the drying treatment (M_f), and then after oven-drying at 65 °C for 24 hours (M_d). RWC was calculated according to Equation 2.1.

Water release curves were obtained by plotting paired values of tissue RWC at equilibrium with Ψ. At least four replicates were used for each Ψ. Over the duration of the study three water release curves were generated in which all all twenty-four solutions were used. Additional curves were plotted using a limited number of Ψs (five solutions) during drying and freezing experiments as a convenient means of determining whether the relation between RWC and Ψ had changed. The five solutions, 0, -5, -10, -15, and -20 MPa, were chosen after examination of the complete curves revealed that these five points afforded reasonable representation of water release from the tissue.

3.2.3 Viability Testing

(i) Fluorescein diacetate (FDA) staining

FDA stain was tested using the protocol described by Widholm (1972). When applied to live cells, esterases in the cells cleave the acetates from the FDA, leaving fluorescein. When excited at the correct wavelength (475 nm), the remaining fluorescein fluoresces at 530 nm in viable cells. Stock solutions were prepared by dissolving FDA in acetone (0.5% w/v), and stored frozen. Stocks were diluted to a concentration of 0.01 % w/v with distilled water. This diluted solution was usable only for a few hours. The stain was applied to treated, rehydrated samples on a microscopic slide and left for 5 to 10 minutes to allow the stain to diffuse into the cells. Specimens were then viewed using a compound light microscope (Zeiss Axioplan, Germany) fitted with an attachable fluorescence

illuminator (HBO 50W high pressure mercury discharge lamp). The microscope was equipped with a filter system consisting first of an exciter filter (BP 450-490) that exposed the specimen to as much light as possible in the active (blue) band. After the UV irradiated the fluorescing material, the light passed through a second barrier filter (LP 520), opaque to all light except the fluorescent frequency. This filter separated the emitted light from the unabsorbed exciting light and prevented prolonged exposure of the eye to UV. Several minutes after irradiation, individual embryonal masses were examined by switching between Köhler illumination and fluorescence. Immature embryos were recorded as alive if the majority of the cells in the embryonal heads fluoresced.

(ii) 2,3,5-triphenyltetrazolium chloride (TTC) assays

When this straw-coloured stain is imbibed into living tissues, dehydrogenase enzymes in the mitochondria reduce the TTC to a stable, non-diffusable triphenyl formazan which stains red. Dead cells or tissues do not reduce the solution and remain colourless. The stained product of this reaction can be quantified colorimetrically by extraction with an organic solvent, but in this study, the assessment was made visually. A 0.07% (w/v) TTC solution was prepared in 0.05 M Sørensen's phosphate buffer (pH 7.4). Treated samples were incubated in TTC solution (approximately 0.03 mL of stain/mg tissue) for 18-20 h at room temperature in the dark. This is important since TTC is light-sensitive and exposure to light will cause reduction of the tetrazolium solution, regardless of whether the tissue is viable. After incubation the samples were scored as either alive or dead based on the appearance of the dark pink stain throughout the tissue. A small number of samples stained only pale pink or irregularly and these were scored as non-viable (tissue was not viewed microscopically since the reduction reaction proceeds when the stain is exposed to light.).

(iii) Conductivity measurements

Electrolyte leakage was measured using a conductivity meter. Shell vials (8 dram) were

filled with 9 mL of distilled de-ionized water and the conductivity measured (EC_{water}). Treated rehydrated samples were placed in the vials (one sample/vial), and sealed with aluminum foil. The samples were then agitated on a Gyratory shaker at 100 rpm. After 2 hours, electrical conductivity was measured ($EC_{treated}$). All vials were then resealed and placed in a 100 ^{o}C oven for 2 hours after which they were cooled and agitated for 19 hours. Conductivity was again measured (EC_{killed}). The relative conductivity (C_R) was calculated according to Colombo *et al.* (1984):

$$C_R = EC_{(treated)} - EC_{(water)} / EC_{(killed)} - EC_{(water)} \quad [3.2]$$

3.2.4 Viability after Drying

Samples were suspended over unsaturated salt solutions with water potentials of 0, -5, -10, -15, and -20 MPa using the methodology described in Section 3.2.2. Ten samples were used for each Ψ and control samples were taken from culture plates immediately prior to viability tests.

After equilibration, samples to be tested for viability were suspended for 24 hours in vials containing distilled water. Tissue was rehydrated by diffusion to avoid the possibility of imbibitional damage which can occur if samples are rehydrated by direct immersion in water. This was especially important before measuring conductivity since imbibitional damage would lead to increased electrolyte leakage and confound results.

This procedure was conducted five times using the FDA stain test for viability. The same experiment was repeated twelve times using TTC as the viability test and twice using electrolyte leakage measurements.

3.2.5 Viability after Drying and Freezing

For pre-drying before freezing, samples were suspended over unsaturated salt solutions corresponding to water potentials of 0, -5, -10, -15, and -20 MPa using the methodology described in Section 3.2.2. After the drying treatment, the Eppendorf and sample were removed from the culture tube and immediately placed into 2-mL polypropylene cryovials (Nalge Co.). Cryovials were clipped into 5-sample cryocanes (Nalge Co.), immersed directly into a LN_2-filled 10-L dewar flask (Taylor-Wharton) and held for 48 hours.

Following storage, samples were removed from liquid nitrogen, a few at a time, and thawed rapidly by plunging the canes into a water bath held at 35 ^{o}C. Vials remained in the bath for 5-10 minutes or until the ice pellet thawed.

Before conducting viability tests, samples were suspended over distilled water for 24 hours to allow rehydration by diffusion. Ten samples were used for each Ψ and control samples were taken from culture plates immediately prior to viability tests. This experiment was repeated five times using FDA and twelve times using TTC for the assessment of viability.

3.3 Results

3.3.1 Water Release Curves

A typical water release curve for embryogenic tissue is given in Figure 3.2a. At high water contents, there are relatively large changes in RWC for small changes in water potential. For example, between 0 MPa and -5 MPa, tissue dried from a RWC of 1.0 to approximately 0.1. At lower RWC very large changes in water potential are required to remove additional water. The relation between $1/\Psi$ and RWC (used to determine the

bound water fraction) is given in Figure 3.2b. The value is estimated from the intercept of the linear portion of the curve (i.e. at $1/\Psi=0$, $\Psi=\infty$). Bound water fractions calculated for spruce embryogenic tissue were very small and ranged from 0.00 to 0.026 RWC. Water relations parameters for embryogenic tissue were determined following the method described in Livingston *et al.* (1992) and Dumont-Beboux *et al.* (1996) and are given in Table 3.1.

3.3.2 Viability Assays

Viability assessments based on FDA staining indicated that treated samples survived drying over the entire range of water potentials tested. Cells of the embryonal mass, and suspensor cells immediately adjacent to the masses, exhibited bright green fluorescence when viewed using fluorescence microscopy. The greater the distance between the suspensor cells and the heads, the dimmer and more sporadic the fluorescence. Samples that were dried and frozen also stained positive.

Further experimentation revealed that FDA gives false positives. The control samples always came directly from the tissue culture medium, and staining of these samples was compared to the treated tissue. A negative control had not been used. When samples were removed from the medium, killed by autoclaving for twenty minutes or by exposure to 3% formaldehyde, and then treated with FDA, they also fluoresced brightly. Additional tests using embryogenic lines of tobacco and alfalfa supported these findings. Samples of these tissues that were killed by autoclaving or exposure to formaldehyde also fluoresced when FDA was applied.

The stain TTC yielded more reliable results. In living tissues, embryonal masses and portions of the suspensor region stained a deep red to purple colour. Dead tissues were

easily identified since they remained white. Negative controls, produced by heat killing or exposure to 3% formaldehyde, did not reduce the stain and remained white.

Survival of tissue after drying to water potentials of 0, -5, -10, -15, and -20 MPa was compared using TTC, FDA and regrowth as viability tests. Samples dried over -15 and -20 MPa solutions did not stain pink with TTC nor did they re-initiate growth when placed back into culture. They did however, fluoresce when FDA was applied, confirming that this stain was not a useful method for determining viabilty after drying treatments on these types of tissues. All subsequent viability staining was performed using TTC.

There was an inversely proportional relationship between leakage of cytoplasmic solutes from dried embryogenic tissue and TTC viability testing (Fig. 3.3). Conductivity measurements revealed a constant increase in relative conductivity from approximately 0.3 at -5 MPa, which then plateaued at about 0.72 after tissue was dried to -15 and -20 MPa. These results were consistent with survival tests run concurrently, 60% of the samples survived drying over -5 MPa, and there was no survival at lower water contents (Section 3.3.3 describes a decrease in desiccation tolerance observed prior to electrolyte leakage measurements).

3.3.3 Viability after Drying

In initial viability (TTC) tests, samples equilibrated to Ψs of 0, -5 and -10 MPa all survived (Fig. 3.4a). There was a marked decrease in tissue survival at Ψs of -15 and -20 MPa. Notably, this decrease in survival also corresponds to the point at which virtually all free water was removed from the cells (i.e. at or below bound water).

Subsequent repetitions of this experiment revealed a progressive decrease in the desiccation

tolerance of the tissue over time (Fig. 3.5) so that in Run 4, only 20% of the samples survived drying to a Ψ of -10 MPa (Fig. 3.4b). From there, survival became very erratic. After Run 8, only those samples that were suspended over water consistently survived (Fig. 3.4c,d). Frequent sub-culturing did not improve drying tolerance, although the tissue continued to look very white and healthy in culture. Microscopic examination of the tissue did not reveal any changes in the embryogenicity of the tissue; there were still many well-formed immature embryos present. However, attempts to mature the embryos by transfer to media supplemented with ABA were unsuccessful. Interestingly, water release curves generated concurrently with viability runs were virtually identical to water release curves generated the year before and the water relations parameters derived from these curves did not change (Table 3.1).

To determine whether there was any relation between the decreased drying tolerance and failed maturation, three embryogenic lines of interior spruce (*Picea glauca-engelmannii* complex, BC Research Inc.) and one line of white spruce (CFS, Fredricton), all exhibiting high embryo productivity, were tested for viability using the same experimental protocol as above. No relationship between drying tolerance and embryo productivity was observed. The newly obtained lines did not tolerate any drying and only survived when suspended over water. However, water release curves plotted for the three lines (5 solutions used) fell on curves produced for the original line using all 24 solutions (data not shown).

3.3.4 Viability after Drying and Freezing

Cryopreservation was attempted on embryogenic tissue from early runs which survived drying. This tissue did not survive freezing. Even those samples that survived drying to a Ψ of -10 MPa in the early desiccation runs did not survive direct immersion in liquid nitrogen.

3.4 Discussion

3.4.1 Viability Assays

My results indicate that TTC staining is a reliable indicator of tissue viability in spruce somatic embryogenic tissue, but that FDA staining should not be used. When FDA was applied to tissue that had been previously subjected to lethal heat, drying and freezing stresses, embryonal heads fluoresced brightly, falsely indicating that the cells were viable. This result contrasts with the findings of Kristensen *et al.* (1994) who reported that FDA could be used to monitor the recovery of embryogenic suspension cultures of sitka spruce after conventional cryopreservation. The use of a negative control, however, was not reported. Widholm (1972) also reported that FDA was an appropriate stain for detecting viable cells of cultured tobacco, tomato, carrot, rice, and soybean following various treatments, including heat-killing at 60 °C for 10 minutes. This result conflicts with what was found in my study using embryogenic cultures of tobacco (data not shown), but may be attributable to differences in the cell cultures used. The tobacco cultures used in the Widholm study were non-embryogenic, uniform suspension cell cultures and would not have contained clumps of tissue made up of a hetreogeneous mix of cell types, as did the embryogenic tobacco calli used in my study. The presence of numerous distinct cell populations within the cultures may have resulted in several different responses to the stain. Additionally, it has been noted that the use of fluorescent dyes for testing viability can be complicated by the presence of endogenous, fluorescent compounds (Calkins and Swanson, 1990).

Ishikawa *et al.* (1995) conducted a comprehensive study that compared the use of several viability tests, including the use of FDA and TTC, for assessing the effects of ABA on

bromegrass cells subjected to various physiological stresses. They concluded that TTC was the most convenient method for assessing viability. Interestingly, their results indicated that TTC somewhat overestimated the freeze tolerance of the tissues. I also observed this phenomenon in a separate study (see Section 4.3.2), when regrowth and TTC were compared for mature somatic embryos after freezing.

Leakage of cytoplasmic solutes from dried embryogenic tissue correlated well with both regrowth and TTC viabilty testing. These findings indicate that the primary site of desiccation injury was at the level of the cell membrane.

3.4.2 Viability after Drying

Initial viability tests demonstrated that white spruce embryogenic tissue could be dried to water potentials of -10 MPa and remain viable following rehydration. When all free water was removed, tissue was not viable. These results suggest that once most of the free water has been extracted from tissue, additional removal of very small amounts of water can be critical to survival. Electrolyte leakage measurements suggested that the injuries incurred at low RWC were primarily due to damage to cell membranes.

It is difficult to account for the gradual decline in tolerance to desiccation. There were no changes in the tissue macromorphology, and there were no conspicuous variations detected when samples of the tissue were viewed microscopically. Additionally, there were no distinct changes in the water relations of the tissues. The importance of this latter finding is discussed in more detail below. There was, however, a correspondence between the decline in desiccation tolerance and the ability to mature this embryogenic line. At the start of the study, it consistently produced large numbers of mature embryos, yet after one year, maturation was extremely poor when samples were transferred to ABA-containing

medium. Declines in productivity are not unusual in somatic embryogenesis research. For example, Roberts *et al.* (1993) reported diminished maturation potential in interior spruce embryogenic lines. Of the 72 genotypes evaluated, most showed a decline in their ability to produce mature embryos after one year in culture, with only a few genotypes producing mature embryos after three years. In my study, the fact that other high-embryo producing lines were not desiccation tolerant would suggest that the declines in desiccation tolerance of the immature tissue and in maturation potential were not directly related even though they occurred at approximately the same time.

Earlier work done in our laboratory suggested that there is a correspondence between water relations parameters and desiccation tolerance in European larch, western larch, white spruce and loblolly pine (Livingston *et al.* 1992; Dumont-Béboux *et al.* 1996). My results strongly suggest that no such relationship exists, at least in spruce. In the previous work, water relations parameters, including osmotic potential at full turgor, relative water content at turgor loss point, bound water and elastic modulus, were calculated from water release curves for several developmental stages of somatic tissue. It was found that for a given developmental stage, water relations parameters were very conservative across species, but that the parameters changed for different stages of development. It was suggested that characterization of these parameters at various stages of development would be good predictors of the effects of drying on embryo growth and development. My results, however, indicate that this is not necessarily the case. Nonetheless, water release curves do provide valuable information; paired values of tissue equilibrium water content with a series of carefully controlled water potentials. These data can be used to standardize and develop more consistent drying protocols. However, derivation and comparison of these particular water relations parameters may not be as useful, at least for this developmental stage, since the results of the present study suggest that parameters can remain similar while the effects of drying on tissue growth differ markedly.

One very intriguing aspect of my results is the fact that the embryogenic tissue initially survived considerable desiccation. It has been well documented that the ability of zygotic embryos of both recalcitrant and orthodox species to withstand dehydration stress improves progressively as the embryo develops, and then declines at germination (Berjak *et al.*, 1992; Finch-Savage, 1992b; Kermode and Bewley, 1985; Kermode, 1990; Sun and Leopold, 1993; Tompsett and Pritchard, 1993). The early stages of zygotic embryo development (histodifferentiation) would be analogous to the level of differentiation in somatic embryogenic tissue. According to the literature, embryos at this stage do not tolerate drying (Kermode *et al.*, 1986; Kermode, 1990). Although there is little information on just how much water is required to maintain cellular integrity during histodifferentiation, it has been proposed that a water potential of at least -1.6 MPa is necessary (Vertucci and Farrant, 1995), a value much higher than the -10 MPa at which the white spruce tissue initially survived. Although there are data that suggest that the transition from desiccation intolerance to tolerance can be prematurely induced in developing embryos (Kermode, 1985, 1990), studies indicate that this early transition would not be possible in tissues as immature as those used in my study. Thus the evidence suggests that these very immature somatic embryos would not survive drying to bound water. It is possible that the observed tolerance was unique to the line (WS10) first selected for testing.

The fact that the decline in tolerance was gradual would rule out the possibility of an abrupt biological change, for example, in protein synthesis. A progressive decline would however, support the idea that the change was related to serial sub-culture of the tissue. That is, with each transfer, tissues tolerant to drying were selectively excluded for reasons likely related to tissue position or appearance (i.e. only the newest, whitest tissue is transferred). This does not explain the initial stress tolerance. This however, might be

related to the fact that the WS10 line had only recently come out of cryopreservation when experiments began. The other spruce lines tested had been maintained in culture for one to two years. It has generally been observed that when conifer embryogenic cultures are cryopreserved using standard multi-step protocols, only small, densely cytoplasmic cells located in the embryonal mass survive, and the long, vacuolate cells of the suspensor perish and must regrow in culture (Gupta *et al.*, 1987; Kartha *et al.*, 1988; Klimaszewska *et al.*, 1992; Lainé *et al.*,1992; Find *et al.*, 1993; Nørgaard *et al.* 1993). Cultures comprised of small, densely cytoplasmic cells, with a reduced degree of vacuolation, would also be the best candidates for withstanding the mechanical stresses imposed by dehydration. Although there have been reports that normal growth of cultures resumes within several weeks following thawing (Gupta *et al.*, 1987; Lainé *et al.*, 1992; Cyr *et al.*, 1994.), other studies involving large numbers of embryogenic lines have indicated that substantial genotype-dependent variation exists in the length of time required for cultures to re-establish their original morphology (Nørgaard *et al.*, 1993a; 1993b). In these reports, growth rates of embryogenic tissue after cryopreservation were much lower than those of untreated controls over a two month observation period.

The mortality of larger cells in cultures may occur as a result of pretreatments using sorbitol and DMSO, as has been reported for embryogenic tissue of *Picea glauca* (Kartha *et al.*, 1988), *Pinus caribaea*, (Laine *et al.*, 1992) *Larix* x *europlepis* and *Picea mariana* (Klimaszewska *et al.*, 1992), rather than being due to the actual freezing process. The sorbitol treatment has dehydrative and physiological effects on the tissue, somewhat similar to cold-hardening, including reductions in cellular water content, substantial reductions in cell size, and conversion of large vacuoles into many smaller ones (Withers 1985,1986). The physiological effects of the "hardening" pretreatment could have affected the drying tolerance of the cells.

Thus, it is possible that because the WS10 line went through the cryopreservation process, the cultures contained a high ratio of small densely cytoplasmic cells to large vacuolate cells at the start of this study, and this 'cryoselection' process gave these cultures an advantage over the other 'older' spruce cultures cultures when they were subjected to dehydration stress. The decreased ratio of larger cells to small cells may have been a result of the freezing process itself, or due to the preculture in sorbitol and DMSO. Long-term maintenance of the WS10 tissue through routine sub-culture would have resulted in cultures of mixed cell types and possibly, the loss of desiccation tolerance.

3.4.3 Viability after Drying and Freezing

The inability to freeze the WS10 embryogenic tissue after drying during its desiccation-tolerant phase is also hard to explain. These results were unexpected since it was believed that if the tissue was dry enough, direct freezing would cause no further damage since there would not be enough water remaining in the tissue to cause lethal ice crystal formation during the freezing or the thawing phases. It can only be concluded that the drying treatment imposed upon the tissue did not sufficiently mimic the multi-step system used to prepare tissue for the conventional cryopreservation procedure. The surrounding medium in which the cultures are frozen and cryoprotective additives used could possibly play a role. As described earlier (Section 2.2.2.1), DMSO is thought to act mainly by decreasing, on an anti-freeze or colligative basis, the amount of water frozen at any given temperature. The sorbitol is believed to behave primarily as an osmoticant, reducing the amount of cellular water and consequently, the possibility of intracellular ice crystals during freezing. The slow-freezing step employed after the pretreatment and addition of the cryoprotectives is also thought to have a dehydrative effect. If these hypotheses are true, then one would expect that, given the fact that the tissues were able to survive drying to bound water, immersion in liquid nitrogen would be possible. However, this study shows that

cryoprotection is not conferred by dehydration alone. Although the complete mechanism of action of cryoprotectants is still not fully understood, it has been proposed that they might, in addition to their colligative action, play some role in maintenance of cellular integrity. Nuclear magnetic resonance data from the freezing of periwinkle cells treated with DMSO support this hypothesis (Chen *et al.* 1984). The results from this work suggested that a minimal amount of unfrozen water is essential for cells to survive freezing, and that a major function of DMSO as a cryoprotectant may be to preclude this water loss so that disorganization of cellular structures is avoided. It has also been proposed that large molecular weight compounds (e.g. sugars, sugar alcohols) may intussucept into membranes and possibly prevent denaturation when solutions become concentrated during freezing, alter membrane permeability, increase viscosity, or delay ice nucleation (see review by Finkle *et al.* 1985).

The presence of sorbitol, DMSO or a mixture of the two compounds in a medium surrounding the tissue during freezing may be a requirement for the successful cryopreservation of immature spruce embryos, although this is not the case for mature spruce embryos, as will be discussed in the next chapter. If it were possible to confer desiccation tolerance to embryogenic tissue through cryoselection, storage in the dry state may be used in conjunction with, or as an alternative to, conventional cryopreservation protocols.

3.5 Conclusions

This work was conducted to simplify the multi-step protocol currently used to cryopreserve conifer embryogenic tissue, and was based on the assumption that the pretreatment, the addition of cryoprotectants and the slow-freezing procedure were all processes that facilitated the reduction of intracellular water so that lethal ice crystal formation during

freezing was avoided. The simplified technique used proved to be a reliable means for drying embryogenic tissue to known water contents, and the procedure was rigorously tested as an alternative to the multi-step method for cryostorage. Unfortunately, the steps used to prepare conifer embryogenic tissue for cryopreservation cannot be replaced by drying alone. It is likely that cryoprotective additives play an additional role in the maintenance of cellular integrity.

TTC staining was determined to be an effective, convenient indicator of viability for white spruce embryogenic tissue. This staining protocol is now being successfully used in a commercial setting for quick viability assessment after drying conifer somatic embryos. FDA was not an appropriate stain for assessing viability of these types of tissues following drying and freezing treatments. The literature suggests that for different species, culture and cell types, only certain viability tests will be optimal. My findings illustrate the importance of use of negative controls with these stain tests, and of testing more than one method for assessing viability.

Initial results indicated that embryogenic could be dried to bound water and survive following rehydration. This initial tolerance and subsequent loss cannot be explained, but was not related to changes in tissue water relations. It is conceivable that because the line went through the cryopreservation process, the cultures contained a high ratio of small densely cytoplasmic cells to large vacuolate cells at the start of this study, thus allowing them to withstand dehydration stress. The decreased ratio of larger cells to small cells may have been a result of the freezing process itself, or due to the preculture in sorbitol and DMSO. It would be very interesting and fairly easy to test this cryoselection theory. If it is possible to maintain cultures in a desiccation tolerant state, dry storage could be used as an alternative to cryopreservation protocols.

Figure 3.1. Schematic diagram of tissue sample suspended above a salt solution of known water potential (Ψ)in a sealed culture vial. Although not shown, the inner wall of the vial is lined with filter paper to increase the surface area of the the salt solution in contact with the air. At vapour equilibration, the Ψsolution=Ψair=Ψtissue.

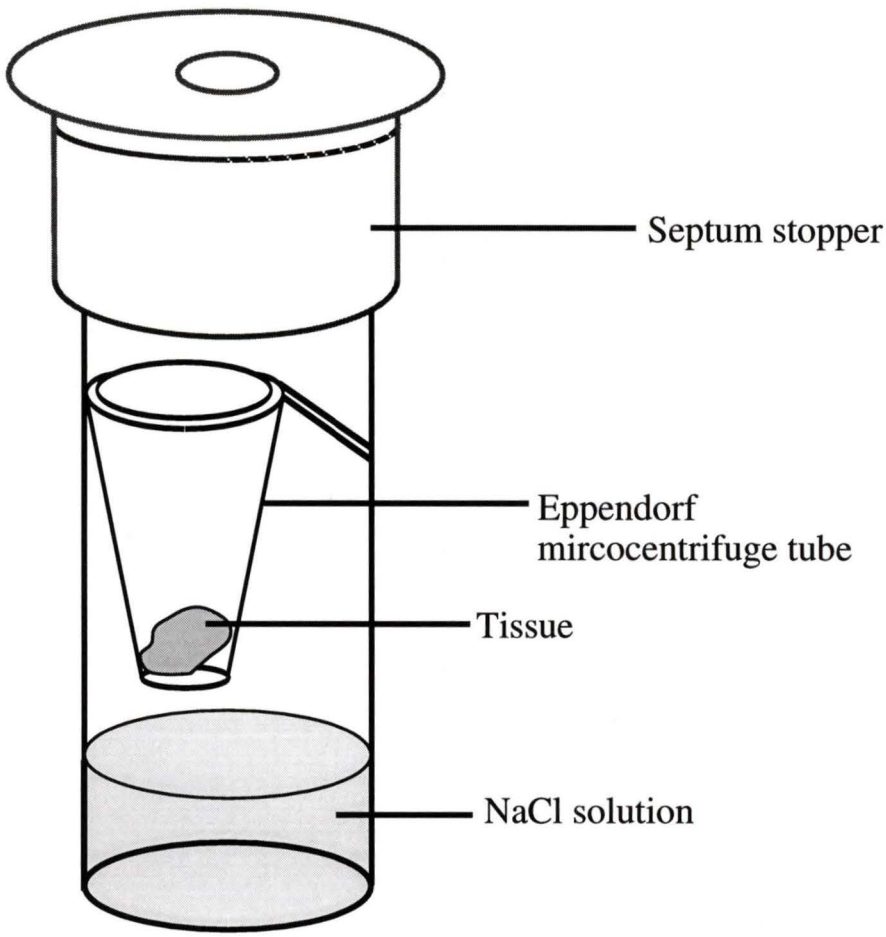
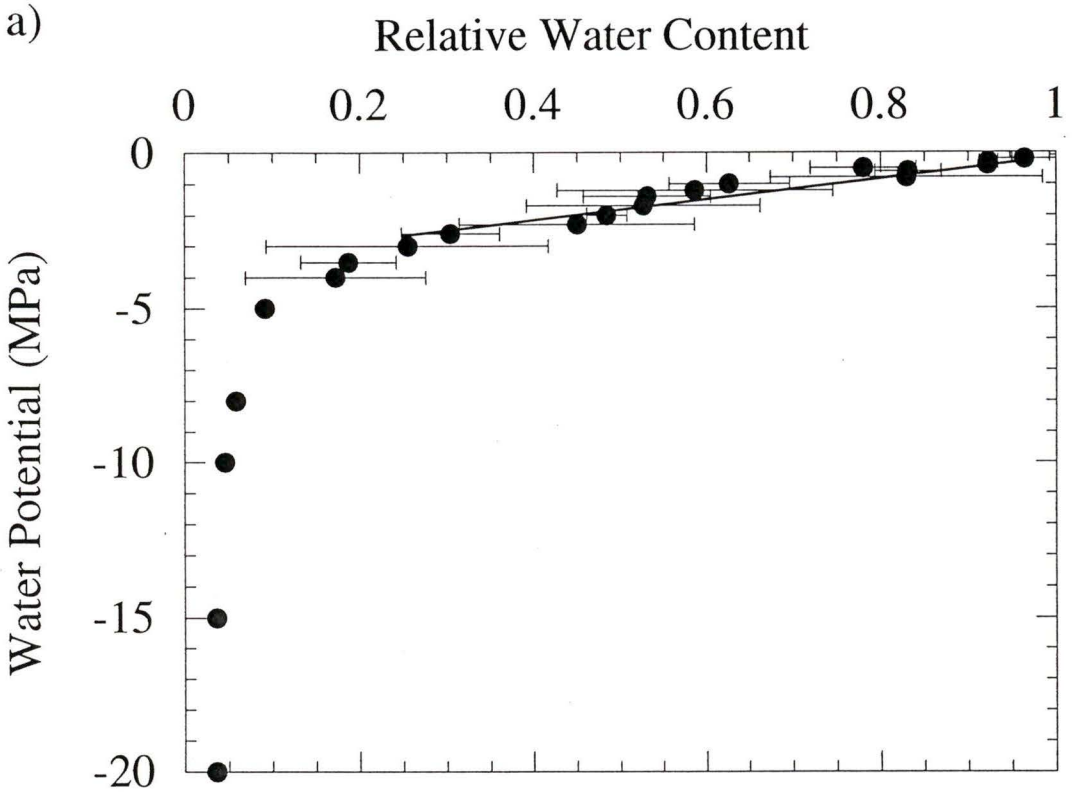

Septum stopper
Eppendorf
mircocentrifuge tube
Tissue
NaCl solution

Figure 3.2a. The relationship between water potential (Ψ) and relative water content (RWC) for white spruce embryogenic tissue. Each point is the mean ($\pm$ SD) of four replicates. The equation of the linear portion of the curve is $\Psi = -2.74 + 2.63$ RWC, $R^2 = 0.94$ (n=13).

Figure 3.2.b. The relationship between the reciprocal of water potential ($1/\Psi$) and RWC for white spruce embryogenic tissue. The linear portion of the curve (at $1/\Psi=0$) gives and estimate of bound water. The equation of the linear portion of RWC versus $1/\Psi$ is $1/\Psi = 0.0006 - 1.033$ RWC, $R^2=0.94$ (n=15).

a)


Relative Water Content
0
0.2
0.4
0.6
0.8
1
Water Potential (MPa)
0
-5
-10
-15
-20

b)

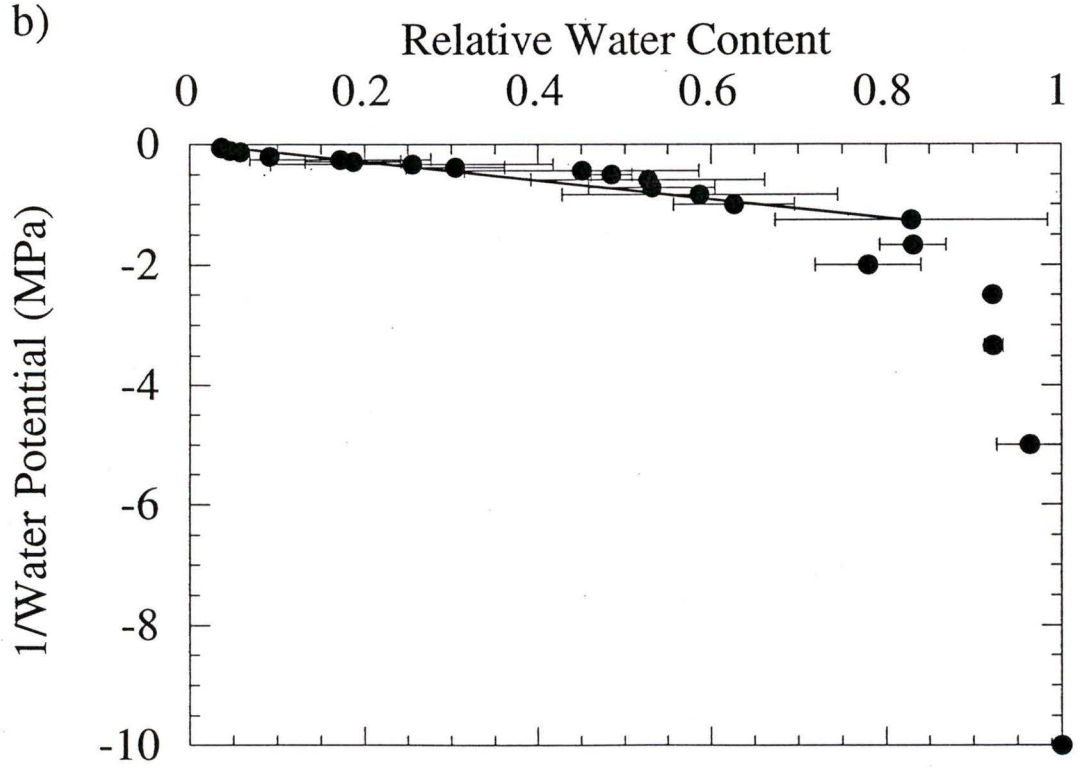

Relative Water Content
0
0.2
0.4
0.6
0.8
1
1/Water Potential (MPa)
0
-2
-4
-6
-8
-10

Figure 3.3. Relationship between relative conductivity (C_R), water potential (Ψ) , and relative water content (RWC) for white spruce embryogenic tissue. Each C_R data point is the mean (± SD) of ten replicates and each RWC data point is the mean (± SD) of forty-eight replicates. In the controls (squares), the conductivity was measured on samples taken directly from culture plates. The RWC of these samples was 1.0.

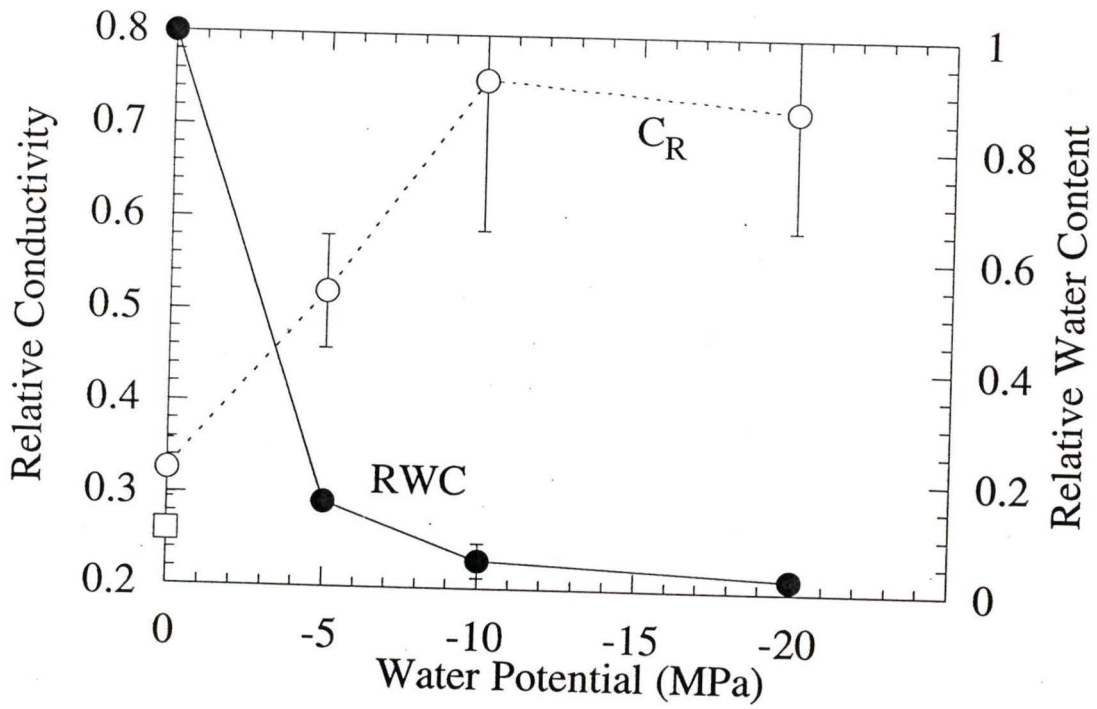
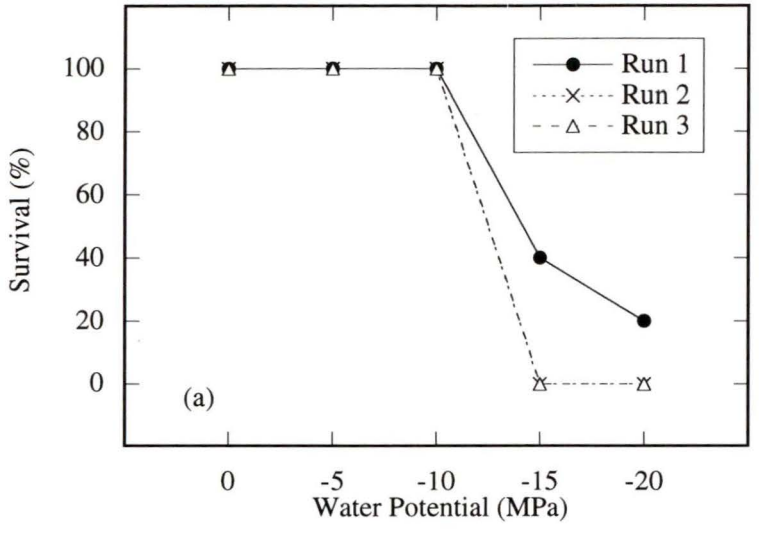
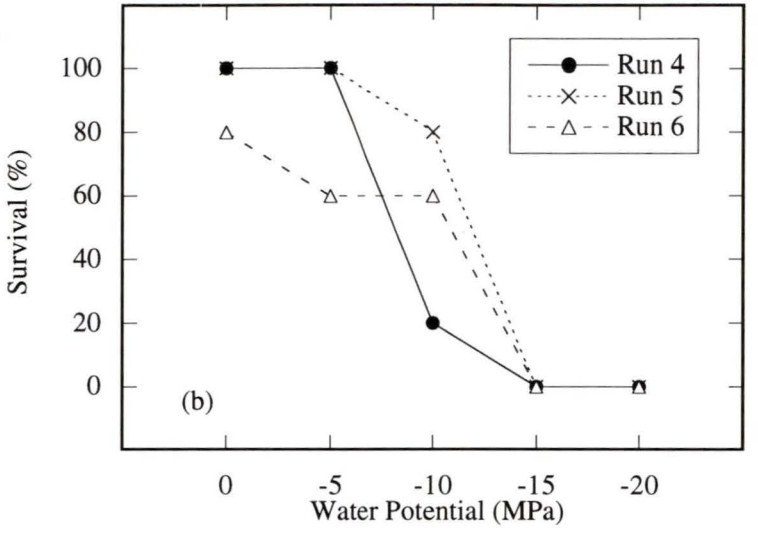
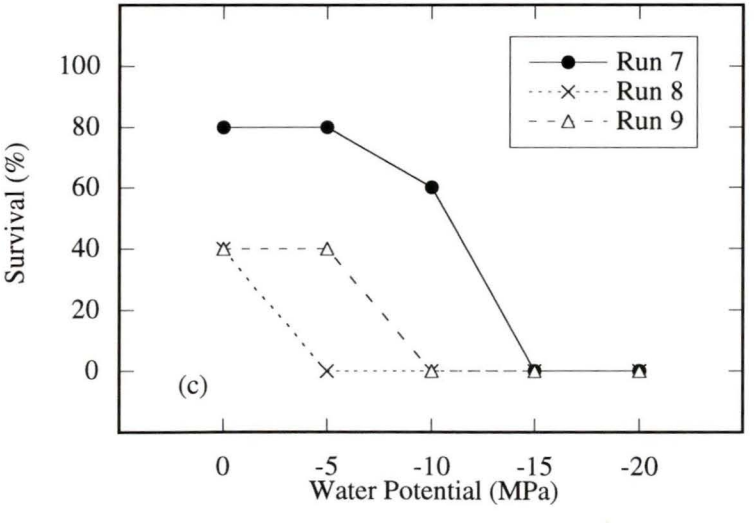

0.8
0.7
0.6
0.5
0.4
0.3
0.2
Relative Conductivity
1
0.8
0.6
0.4
0.2
0
Relative Water Content
C_R
RWC
0
-5
-10
-15
-20
Water Potential (MPa)

Figure 3.4. Viability of white spruce embryogenic tissue after drying treatment as based on TTC staining. (a) Runs 1-3 (day 0-66), (b) Runs 4-6 (day 81-122), (c) Runs 7-9 (day 148-180), (d) Runs 10-12 (day 296-345). Each data point represents the mean of ten samples. Samples suspended over water (0 MPa) were held for the duration of the drying treatment and then tested with TTC. Control samples were removed directly from culture medium and stained. In all cases, controls gave 100% survival.


Survival (%)
100
80
60
40
20
0
Run 1
Run 2
Run 3
(a)
0
-5
-10
-15
-20
Water Potential (MPa)


Survival (%)
100
80
60
40
20
0
Run 4
Run 5
Run 6
(b)
0
-5
-10
-15
-20
Water Potential (MPa)


Survival (%)
100
80
60
40
20
0
Run 7
Run 8
Run 9
(c)
0
-5
-10
-15
-20
Water Potential (MPa)

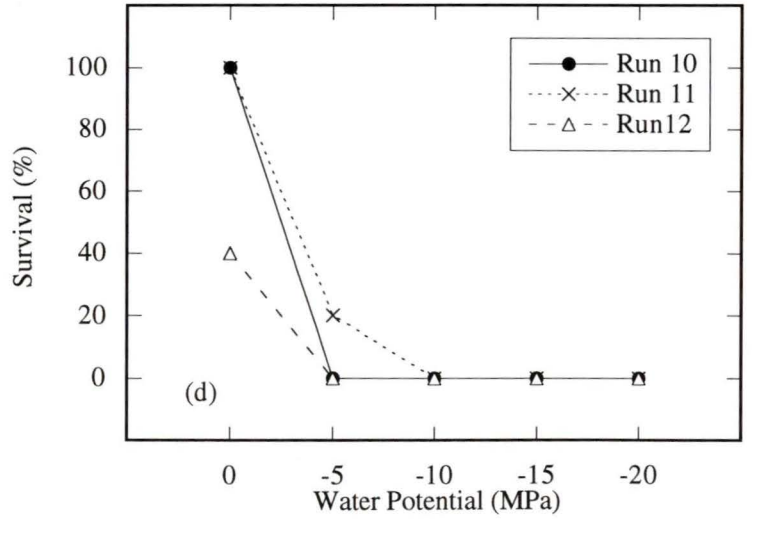

Survival (%)
100
80
60
40
20
0
Run 10
Run 11
Run12
(d)
0
-5
-10
-15
-20
Water Potential (MPa)

Figure 3.5. The relation between survival of white spruce embryogenic tissue after drying to -5 MPa, -10 MPa and -20 MPa and time, as based on TTC staining. Each data point is the mean of four replicates.

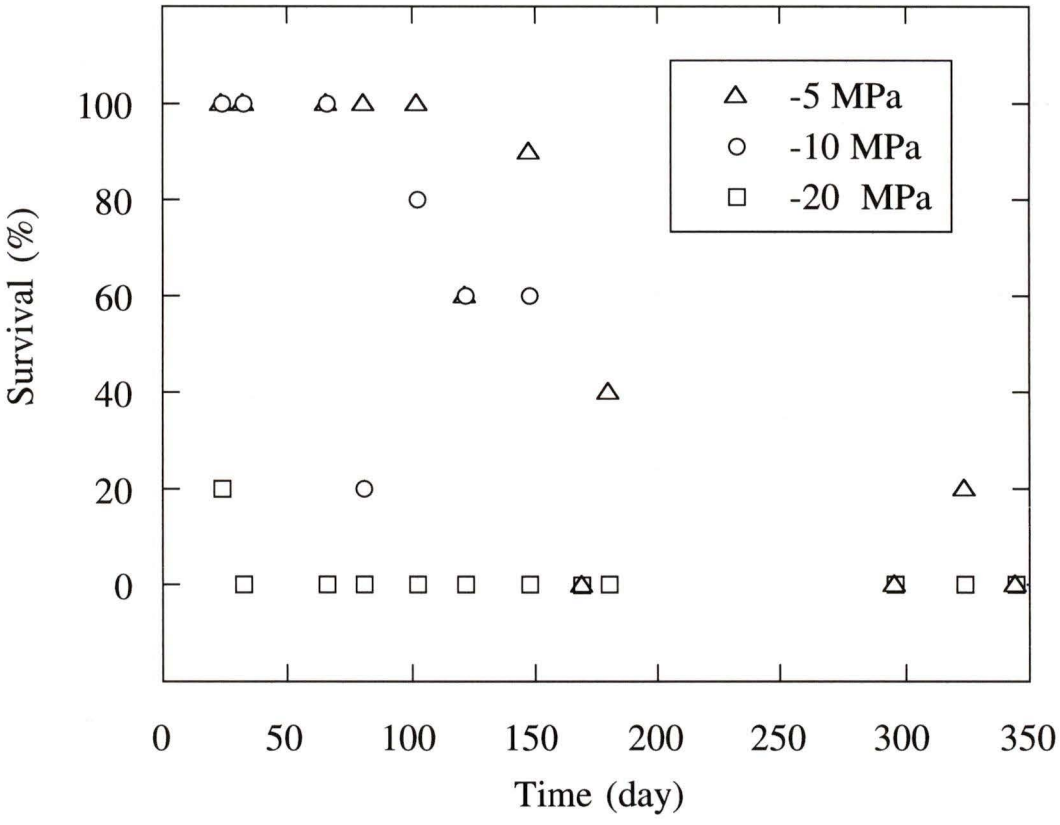

Survival (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
350
Time (day)
-5 MPa
-10 MPa
-20 MPa

Table 3.1. Water relations parameters of white spruce embryogenic tissue: bound water (A), elastic modulus (ε), relative water content at zero turgor (RWC_o) and osmotic potential at full turgor ($\Psi\pi t$).

Table 3.1

RUN (day)	A	ε (MPa)	RWC_O	$\psi\pi t$ (MPa)
60	0.026	3.84	0.83	-0.96
390	0.000	4.76	0.85	-0.97
460	0.023	3.51	0.74	-0.86
Mean	0.016	4.02	0.83	-0.93
SD	0.014	0.66	0.06	0.06

Chapter 4

DESICCATION AND CRYOPRESERVATION OF MATURE SOMATIC EMBRYOS

4.1 Introduction

Various drying treatments when applied to maturing somatic embryos promote rapid and synchronous germination and enhance post-germinative vigour. In conifer somatic embryos, such treatments include the application of osmoticants such as polyethylene glycol, and partial drying (Attree *et al.*, 1991; 1995, Roberts *et al.*, 1990b; 1991, Beardmore and Charest, 1995a, Lelu *et al.*, 1995). Drying treatments, applied during the latter stages of somatic embryo maturation, are thought to mimic the natural drying process that occurs in zygotic embryos of orthodox seeds, sending a signal for the arrest of development. Recently, research has been directed towards the use of drying treatments for the storage of mature conifer somatic embryos. Much of this work has stemmed from the growing interest in the production of artificial seeds (Redenbaugh 1993; Compton *et al.*, 1995). A fundamental problem with current drying protocols is that they lack a quantitative basis. This chapter describes work conducted to devise a reliable, quantifiable technique for drying mature somatic embryos that could be used as a basis for drying and storage protocols for large scale operations.

The first objective of this study was to characterize water release from mature somatic embryos by equilibrating embryos over a series of solutions with known Ψ, determining the equilibrium RWC at each Ψ, and then generating water release curves. This process entails destructive sampling. As outlined in section 3.2.2, a number of water relations parameters can be derived from these curves. This drying profile can then be used to predict the equilibrium RWC for other embryos dried over solutions of known water

potential. The second objective of this research was to determine the Ψ (and RWC) to which mature somatic embryos can be dried and still remain viable after rehydration. A specific goal was to determine whether mature embryos could be dried to remove all but bound water without causing mortality. The final objective was to determine whether dried embryos would survive freezing by direct immersion in liquid nitrogen.

4.2 Materials and Methods

4.2.1 Plant Material

Mature somatic embryos of white spruce (*Picea glauca* (Moench) Voss.) and interior spruce (*Picea glauca-engelmannii* complex) were used in this study.

White spruce embryogenic tissue was initiated and maintained as described in Section 3.2.1. To promote somatic embryo development, embrogenic tissue was transferred to half-strength Litvay's medium (LM) (Litvay *et al.*, 1981) modified according to Tremblay (1990), supplemented with 40 µM ABA, 175.3 mM sucrose, and solidified with 0.4% (w/v) Gelrite gellan gum. The cultures were incubated at 25 °C in low light (40 µmol m^{-2} s^{-1}, 16 hour photoperiod).

Interior spruce represents a mixture of two closely related species (*Picea glauca* (Moench) Voss. and *Picea engelmannii* Parry) that hybridize with one another in the regions of interior British Columbia where their ranges overlap. Interior spruce embryogenic cultures were provided courtesy of Dr. D. Cyr, B.C. Research Inc., Vancouver, British Columbia. The cultures were initiated from mature zygotic embryos as described by Webb *et al.* (1989). Culture maintenance and maturation followed the procedure described by Cyr *et al.* (1994).

For regrowth tests after drying and freezing treatments, mature somatic embryos were transferred to modified half-strength GMD medium (Mohammed *et al.*, 1986) containing 58.4 mM sucrose and 0.6% (w/v) Difco Bacto agar. Germination was carried out under the same light and temperature conditions as those used in maturation.

4.2.2 Generation of Water Release Curves

The drying procedure used to generate water release curves followed the methodology described in Section 3.2.2 with the following modifications. Instead of placing the tissue on the inside wall of the microcentrifuge tubes, the cut bottom of the tubes was heated and a nylon mesh disc (NGG 52, 335 μm opening, 46% porosity) was fused across the cut opening. Three to five embryos were placed on the mesh inside each Eppendorf tube. Care was taken to ensure that they did not touch one another.

Relative water contents were calculated as described in Section 3.2.2, using Equation 2.1, except that M_t (hydrated mass) was determined prior to drying, by rehydrating the embryos over distilled water for 6 days. This was done to obtain an accurate value for water content at full turgor and was not necessary for embryogenic tissue since initial experiments established that immature tissue was fully hydrated when on the tissue culture medium. Three water release curves using twenty-four salt solutions were generated for white spruce (Dumont-Béboux *et al.*, 1995), and two for interior spruce. Additional water release curves, using water potentials of 0, -5, -10, -15, -20 were completed each time drying and freezing viability tests were conducted to determine whether the water relations of the tissue had changed over the course of the study.

4.2.3 Viability Assays

Viability after drying and freezing treatments was assessed following rehydration by regrowing samples on germination medium. Additionally, TTC staining and electrolyte leakage were investigated as a simple and less time-consuming alternative.

For TTC stain tests, treated samples were incubated in 0.2 mL TTC solution (1.0% w/v in 0.05M Sørensen's buffer, pH 7.4) for 20 hours in the dark. After incubation, the samples were scored as alive if the entire embryo stained a deep pink colour. If samples remained white, or were pale or irregularly stained, they were scored as dead. Electrolyte leakage was measured using a conductivity meter, as described in Section 3.2.2.

4.2.4 Viability after Drying

Embryos were dried to water potentials of 0, -5, -10, -15, and -20 MPa. A sixth drying level, silica gel (Ψ = approximately -540 MPa, RH at 5 ^{o}C = approximately 2%), was also tested. To determine the Ψ of air in equilibrium with silica gel, air was pumped through a silica gel column and its vapour pressure (e_a) determined using an infrared gas analyzer (Li-6260, LI-COR Inc., Lincoln, Nebraska, USA). The column temperature was measured with a fine wire thermocouple. Relative humidity was calculated as $e_a/e_{s(T)}$ where e_s is the saturated vapour pressure at a given temperature (T). Values of RH were converted to Ψ using Equation 3.1.

The drying treatment was conducted using the same methodology as used for the generation of water release curves (Section 4.2.2, and 3.2.2). Large numbers of mature somatic embryos were not available so sample sizes and repetitions were lower than those in the experiments conducted using embryogenic tissue. One embryo was placed in each

Eppendorf microcentrifuge tube and five to ten tubes used for each Ψ (five replicates for TTC and electrolyte leakage experiments, ten for regrowth). After six days, samples were transferred to vials containing distilled water and left to rehydrate by diffusion for 24 hours. Viability was then assessed by comparison to control samples that were taken directly from culture plates and stained with TTC. Experiments for testing viability after drying were repeated twice using white spruce mature somatic embryos and TTC staining as the viability assay. Experiments were repeated eight times using interior spruce somatic embryos; three times using TTC staining, three times using regrowth and twice using electrolyte leakage to assess viability.

4.2.5 Viability after Drying and Freezing

Dried samples were removed from culture vials following a 6 day drying treatment and transferred to 2-mL polypropylene cryovials (Nalgene, Nalge Co., Rochester, New York). Freezing, thawing and rehydration were carried out as outlined in Section 3.2.5.

Two freezing experiments were conducted on white spruce somatic embryos. TTC was used to assess viability after treatment. Five experiments were conducted on interior spruce. In three runs, viability was assessed with TTC and in two runs, viability was assessed using regrowth.

4.3 Results

4.3.1 Water Release Curves

Representative water release curves showing Ψ *vs.* RWC and 1/Ψ *vs.* RWC for interior spruce are given in Figure 4.1. Water relations parameters for white spruce somatic

embryos are reported in Dumont-Béboux *et al.* (1996). Interior and white spruce embryos have similar water release curves and water relations parameters. Mean values of bound water (A), elastic modulus (ε), relative water content at zero turgor (RWC_o) and osmotic potential at full turgor ($\Psi_{\pi t}$) for interior spruce were 0.13, 3.6, 0.66, and 0.78, respectively, and for white spruce, 0.14, 3.4, 0.60, and 0.65, respectively.

4.3.2 Viability Assays

Stain tests using TTC proved to be the quickest method for assessing viability and overall, there was a good correspondence between regrowth and staining. However, the viability results after freezing of embryos, particularly those at higher water contents, might have been slightly over-estimated by TTC. For example, TTC stain tests indicated that some of the interior spruce embryos dried over water or -5 MPa survived freezing (see Section 4.3.3). Regrowth tests on similarly-treated embryos demonstrated that of the very few that did grow, only the hypocotyls were capable of greening and elongation, neither the roots nor the cotyledons expanded.

Electrolyte leakage measurements were also good indicators of tissue viability after drying. While there were large changes in RWC when samples were dried over salt solutions of 0 to -20 MPa (from 1.0 to 0.15, respectively), relative conductivity remained relatively constant over all the salt solutions tested (Fig. 4.2a), as did viability. There was a significant increase ($P=0.0001$, Appendix II) in conductivity when additional (bound) water was removed from embryos by drying over silica gel. This was associated with an abrupt decline in viability (TTC, regrowth) (see Section 4.3.3).

4.3.3 Viability After Drying

There was high survival, as determined by TTC staining, of white and interior spruce mature somatic embryos dried using salt solutions (Fig. 4.2b). Even samples that had virtually all free water removed by drying over -20 MPa solutions (Fig. 4.1) were viable. However, TTC staining indicated that there was no survival when embryos were dried over silica gel to a RWC of approximately 0.02.

Regrowth experiments conducted with mature interior spruce embryos confirmed the initial findings of the stain tests. All embryos dried over salt solutions re-initiated growth when rehydrated (Fig. 4.3). Those embryos dried over silica gel did not elongate, and did not produce chlorophyll (Fig 4.3f). The primary focus of this study was on viability following drying and freezing, however, the percentage of germinants exhibiting root, hypocotyl, and cotyledon elongation, was also determined after 21 days on germination medium (Figure 4.2c). These results indicated that drying affected regrowth of different regions of the embryos with varying degrees of severity, with the root region being most sensitive, followed by the cotyledons and then the hypocotyl.

To determine whether the lack of survival of embryos following drying with silica gel was related to the rate at which the water was removed from the tissues (i.e. higher rate of water loss using silica gel than with solutions of -20 MPa), a simple experiment was carried out in which samples were dried in two stages. Firstly, samples were dried to -20 MPa over 6 days, after which they were transferred to vials containing silica gel for another 6 days. Survival following this treatment was compared to that of embryos suspended over solutions of -20 MPa for 12 days. Samples transferred to silica gel were not viable whereas all embryos dried to a water potential of -20 MPa survived.

4.3.4 Viability After Drying and Freezing

Very similar trends in survival after freezing were observed between white and interior spruce (Figure 4.4a). Survival was low at the higher water contents, and then increased dramatically once embryos were dried to below -5 MPa (RWC approximately 0.30). Staining showed that the highest survival following freezing was obtained by those embryos that were dried to between -10 and -20 MPa, and indicated that, over the whole range of salt solutions used, there was always some survival, regardless of the drying treatment. Embryos dried over silica gel, then frozen, either remained white after TTC incubation, or stained very lightly and irregularly, and were therefore scored as dead.

As mentioned in Section 4.3.2, TTC staining appeared to slightly over-estimate survival after freezing. Regeneration experiments indicated that not all embryos that were dried and frozen were actually capable of greening and elongation. Control embryos (taken directly from the culture dish) and the majority of those pre-treated by suspension over 0 MPa, -5 MPa and silica gel did not grow in culture. In general, these embryos became necrotic after 21 days on germination medium (Fig. 4.5a,b,f). A small number of these samples did show some slight greening and swelling in the hypocotyl region (Figure 4.4b). Regrowth tests showed that re-initiation of growth after freezing was best in those embryos pre-dried to Ψs of -10 MPa, -15 MPa and -20 MPa (Fig. 4.5c,d,e), particularly for the latter two salt solutions (Figs. 4.4b). These results were consistent with those obtained by staining. As was observed in the experiments where embryos were subjected to drying alone, roots, followed by cotyledons and hypocotyls, were the most sensitive drying and freezing treatments. Further, initiation of roots was generally lower for embryos that were dried and frozen than for those that had only been dried (Figs. 4.2c, 4.4b).

4.4 Discussion

4.4.1 Water Release Curves

White and interior spruce somatic embryos have very similar water relations parameters. This similarity is not unexpected, given the relationship between white and interior spruce, and because other studies have shown that water release is remarkably conservative among other pinaceous species such as loblolly pine (bound water approximately 0.12) and western larch (0.07) for a given developmental stage (Dumont-Béboux *et al.*, 1996). The bound water fraction increased with development, the values for mature embryos were more than six times higher than in embryogenic tissue (Chapter 3). Again, this is in agreement with results for immature and mature somatic embryos of other coniferous species (Livingston *et al.*, 1992, Dumont-Beboux *et al.*, 1996). The increased amount of bound water is likely related to the many physiological and biochemical changes which occur during embryo development such as accumulation of proteins, carbohydrates and lipids, all of which can form putative water-binding sites.

Most studies on the desiccation of conifer somatic embryos have lacked accurate control and quantification of embryo water status. Characterization of water release from somatic embryos provides valuable data that can be used to standardize and develop more consistent drying protocols (see Section 4.4.3).

4.4.2 Viability Assays

Tests using TTC stain proved to be a convenient method for assessing viability and overall, results from these tests were consistent with those obtained from the regrowth tests. Results from TTC tests for drying alone corresponded well with the results of regeneration

experiments. However, there was a discrepancy between TTC staining and regrowth after freezing of embryos at higher water contents. This suggests that viability assessments using stain tests may not necessarily be good indicators of growth potential. Ishikawa *et al.* (1995) also found that TTC tended to slightly over-estimate freezing tolerance, but that the method was reliable enough to distinguish various levels of freezing tolerance in bromegrass suspension cultured cells. They postulated that the slight discrepancy between TTC and regrowth may have been due to an excess number of electron donors in samples containing a mixture of both intact and injured cells that would cause the TTC reduction to proceed. Therefore, the over-estimation would occur only in samples that were on the borderline of being viable. They found that washing the cells after thawing alleviated the problem, possibly because excess electron donors were removed.

Measurement of electrolyte leakage was also an appropriate method for detecting desiccation injury in mature somatic embryos. However, the protocol was not as convenient to use as the TTC staining method. The significant increase in relative conductivity after embryos were dried over silica gel was consistent with regrowth tests, where none of the embryos germinated or even produced chlorophyll, and suggests that drying damage was associated with injury to cellular membranes.

4.4.3 Viability After Drying

Various drying treatments have been applied to somatic embryos of both angiosperm (Anandarajah and McKersie 1990a;b; Senaratna *et al.*, 1989;1990; Saranga *et al.*, 1992; Kim and Janick 1991; Parrot *et al.*, 1988; Gray *et al.*, 1987; Gray 1989; Florin *et al.*, 1993; Lecouteux *et al.*, 1993; Etienne *et al.*, 1993) and conifer species (Attree *et al.*, 1991; 1992; 1995; Roberts *et al.*, 1990b; 1991; Beardmore and Charest, 1995a; Lelu *et al.*, 1995). Generally, research on improving the desiccation tolerance of angiosperm somatic embryos

has been conducted with a view to finding ways to preserve somatic embryos in the dry state, and has been carried out particularly in response to the need for dry somatic embryos in synthetic (artificial) seed technology. In conifers, the entire somatic embryogenesis process, from initiation to plantlet establishment, has been much more difficult to optimize and until very recently, most of the focus on drying has been geared towards the use of dehydrative treatments for improving (i) the quality of mature conifer somatic embryos (increased storage reserve accumulation), (ii) the synchronization of germination, and (iii) the vigour of the germinants. In some conifer species, techniques used to produce mature somatic embryos for some conifer species have been much improved over recent years and within the past two years, research has been extended to look at drying as a means of long-term storage (Lelu *et al.*, 1995; Attree *et al.*, 1995; Beardmore and Charest, 1995a), with the eventual goal being the production of artificial seed.

Methods that have been used to bring about and to quantify reductions in somatic embryo water content vary considerably, making comparison of different protocols difficult. In my study, RWC was used to express water content. This expression gives a measure of the amount of water remaining after drying relative to the maximum amount of water that tissues can hold when saturated. RWC provides a more versatile measure of water status than absolute water content because normalization allows easier comparison of data from different sources. Reports on drying of somatic embryos have typically presented water content either on a dry mass (dmb, g H_2O g^{-1} dry mass) or on a fresh mass basis (fmb, g H_2O g^{-1} fresh mass). In some studies, the basis for the water content calculation is not even given. To facilitate comparison of published work with my data, I have converted some of the RWCs measured in my study to water content on fmb and dmb (see Appendix III) since it is not possible to transpose water contents expressed on these bases into RWC without knowledge of the water content at full hydration.

Beginning with a well-controlled, closed drying system and a profile of water release for spruce somatic embryos, this part of my study focused on identifying the lowest water content to which somatic embryos could be desiccated and still survive following rehydration. The results suggest that spruce somatic embryos can tolerate drying to bound water. The only drying treatment that the embryos would not tolerate was that using silica gel. The RWC of samples following the silica gel treatment was approximately 0.02 (about 0.075g H_2O g^{-1} dm, 0.069 g H_2O g^{-1} fm), well-below the bound water fraction (RWC = 0.13-0.14, or approximately 0.39g H_2O g^{-1} dm, 0.28 g H_2O g^{-1} fm) determined using the water release curves. Drying with silica gel likely removed a large proportion of bound water, therefore it is not overly surprising to find that the silica gel treatment imposed so much strain on cellular components that the embryos were fatally injured.

It has been suggested that the rate rather than the degree of drying affects survival of somatic embryos (Senaratna *et al.*, 1989; Blackman *et al.*, 1992; Florin *et al.*, 1993). My results following progressive transfers from -20 MPa to silica gel suggest that it is the actual water content, rather than the rate at which water is removed from the tissues that determines survival. Further work could be done in this area to determine whether there is any benefit to slowing down the rate of drying beyond the bound water fraction.

Although the quality of germinants was slightly higher for those embryos treated at higher water potentials, long-term storage of the embryos after drying would likely be more feasible for embryos dried to lower water contents. Effective long-term preservation requires that there is as little physiological activity as possible. Activity would be reduced at low water contents since water is inextricably linked to so many cellullar functions: it acts as a substrate or solvent for many reactions, it is the medium through which solutes diffuse in plant cells, it facilitates intramolecular motions of proteins essential for catalytic activity and its interaction with phospholipid structure may mean it plays a role in

membrane permeability (Leopold and Vertucci, 1989). Deteriorative reactions would also be reduced at low water contents. Embryos dried to water potentials of -15 and -20 MPa had RWCs very close to calculated bound water values. Orthodox seeds are typically stored at a water content close to bound water (Leopold and Vertucci, 1989). Mature zygotic embryos excised from stored dry seed of interior and white spruce have water contents of approximately 0.365 g H_2O g^{-1} and 0.325 g H_2O g^{-1}, respectively, on a fmb (Roberts *et al.*, 1990b; Atree *et al.*, 1991). The corresponding water contents (fmb) for somatic embryos dried at water potentials to -15 and -20 MPa were approximately 0.33 to 0.28 g H_2O g^{-1}fm, respectively. Thus, somatic embryos can be dried to water contents similar to those suitable for mature seed storage and still remain viable (Note: the water content commonly quoted for intact dry spruce seeds, 0.04 to 0.08 g H2O g^{-1} fm, is lower than that of the excised dry zygotic embryo because the mass of the dry seed coat is included in measurements of tissue mass). The effects of long-term storage in the dry state was not investigated in this study since freezing was carried out after drying. The longest period that these embryos were stored was for two weeks at 5 °C, and no loss in viability was observed. Further studies to look at the effects of longer storage times after using this drying technique may be beneficial to research on synthetic seed.

Little research has been conducted on drying of conifer somatic embryos for storage purposes. Lelu *et al.* (1995) reported the use of a sucrose/cold pretreatment and saturated salt solutions to dry hybrid larch somatic embryos to water contents approaching those of dry zygotic embryos excised from stored seed. At a relative humidity of 59% (at 4 °C) somatic embryos were dried to approximately 0.30 g H_2O g^{-1} dm (0.23 gH_2O g^{-1} fm) which was not disimilar to values obtained for dry zygotic embryos of 0.20 gH_2O g^{-1} dm (0.17 gH_2O g^{-1} fm). The system used to dry the embryos was adopted from a multi-well plate method, originally described by Roberts *et al.* (1990b; 1991) for improving germination frequency and synchrony in interior spruce somatic embryos. In these reports

mature embryos of interior and sitka spruce were placed in half the wells of a twelve-well culture plate and the remaining wells filled to the three quarter level with distilled water. Plates were sealed with Parafilm and stored at room temperature. However, the plates could not have been completely sealed since Parafilm is not impermeable to water vapour. This can easily be observed by wrapping a medium-containing petri plate (without tissue) with Parafilm and leaving it in an incubation room for a week, the level of the medium goes down. Because the multi-well plates are not closed, their ability to dry tissue is strongly influenced by the ambient atmosphere. That is, water loss is due to the gradient between the inside and the relatively dry atmosphere outside the vessels. In the hybrid larch study, saturated salt solutions were used in the multi-well plates in place of distilled water to generate a RH of 59%, and experiments were conducted in a refrigerator. Even in a perfectly sealed system, there would still be substantial variation in the RH because of fluctuations in the fridge temperature. However, in an unsealed system, there will be even greater variation in RH, and thus in the water contents of the embryos, because of large gradients in vapour pressure between the inside and the outside of the container. The use of poorly controlled, improperly sealed systems for drying conifer somatic embryos on a small scale will severely hamper research to scale up the production process, since it will be very difficult to reproduce the conditions that existed in small scale experiments. It would be more efficient to design a standardized and repeatable system that could be adapted for large scale production of embryos.

Studies on dry storage of angiosperm species provide some insight as to the mechanisms that allow somatic embryos to tolerate desiccation. It appears that increased levels of endogenous ABA may play a key role in the induction of desiccation tolerance. Desiccation tolerance has been conferred by the exogenous application of ABA to maturing somatic embryos (Kim and Janick, 1991; Anandarajah and McKersie, 1990a; Senaratna *et al.*, 1989; 1990). Application of various stress treatments, such as heat shock, nutrient stress,

and cold stress, which have been found to confer desiccation tolerance, may act by stimulating the synthesis of ABA (Senaratna *et al.*, 1989; Anandarajah and McKersie, 1990b). The mechanism by which ABA or other stresses increase desiccation tolerance is not well understood. It may act as a signal transducer for the transcription of protectants (see Vertucci, 1995), for example in inducing mRNAs for proteins associated with water stress, such as LEAs (see Section 2.3).

Desiccation tolerance in angiosperm somatic embryos has also been induced by treatment with sucrose and other sugars (Tessereau *et al.*, 1991; Lecouteux *et al.*, 1993; Anandarajah and McKersie, 1990a; 1990b; Florin *et al.*, 1994). Conversely, slow drying treatments have induced the accumulation of the raffinose series of sugars in developing soybean seeds (Blackman *et al.*, 1992). It is not known whether tolerance is induced as a result of the osmotic stress imposed by the sugars which leads to an increase in endogenous ABA, or whether the sugars act by direct action, for example, by stabilizing membranes (see Section 2.3).

In my system, all drying treatments were carried out at 5 °C so that accurate measurements of water loss could be attained without the confounding effects of weight loss due to respiration. It is possible that the low temperatures may have had an effect on desiccation tolerance, perhaps increasing tolerance through stimulation of endogenous ABA synthesis. Based on the improved germination observed between the embryos held over water at 5 °C for six days and the control embryos taken directly from the plates, it may be worth investigating replacement of the high relative humidity treatment currently used to stimulate synchronous germination in spruce somatic embryos (Roberts *et al.* 1990b; 1991) with another more conveniently imposed mild stress, such as chilling or nutrient stress.

Increased tolerance to desiccation following a cold treatment has been reported for conifer

somatic embryos as well as for angiosperms. Beardmore and Charest (1995) observed that a chilling treatment increased the tolerance of black spruce embryos to desiccation. In this cold treatment, mature somatic embryos were placed on wetted filter paper discs in petri plates at 2 °C in the dark for two days. They were then subjected to one of two drying treatments: slow desiccation, which involved step-wise transfer through a series of atmospheres of progressively lower relative humidities (79%, 58%, 31% at 25 °C), or fast desiccation, using only the saturated salt solution that generated the lowest RH. The cold treatment was found to increase the desiccation tolerance in both rapidly and slow dried embryos. Germination of slow-desiccated cold-treated embryos was 90% compared with 64% in the control slow-desiccated embryos. For the fast-desiccation treatment, germination increased from 18% in the controls to 27% in the cold-treated.

I had hoped to determine the effect of higher temperatures on desiccation tolerance. Unfortunately, this was precluded by the decline in tolerance of the embryogenic tissue, and by lack of sufficient sample material for the mature embryos. Future work using this technique should include testing the effect of drying at different temperatures on drying tolerance. If drying at higher temperatures is possible, this system might be more convenient since precise control of lower temperatures on a large scale is difficult and costly. If reduced temperature does affect the ability to dry the embryos, perhaps a chilling treatment could be imposed more inexpensively before drying, as in the studies above (Beardmore and Charest, 1995), so that low temperatures do not need to be maintained throughout the drying process. Furthermore, if embryos are not being dried to facilitate storage (i.e. drying is for improved germination), then low temperature or nutrient stress could possibly be applied as a replacement for the drying treatment.

4.4.4 Viability After Drying and Freezing

Mature embryos of white and interior spruce survived direct freezing in liquid nitrogen following a controlled-drying pretreatment. For conifer species, this is the first report of somatic embryo survival following freezing in liquid nitrogen without the use of cryoprotectants. Re-initiation of growth after freezing was best in those embryos pre-dried to water potentials of -10 MPa, -15 MPa and -20 MPa, particularly for the -15 MPa treatment. There was very minimal survival following freezing of embryos that had been pretreated at higher water potentials, and there was no survival of embryos frozen after pretreatment over silica gel. Thus, for drying alone, there is a wide range of water contents tolerated by the embryos, while for freezing following the drying treatments, the range is much narrower. This is because the freezing-tolerant range is bordered by freezing injury (due to intracellular ice formation) at high water contents, and desiccation injury at low water contents. The water content of the embryos had to be lowered to values approaching bound water in order to survive subsequent freezing.

The literature on storage of conifer somatic embryos in liquid nitrogen generally relates to the preservation of immature embryos (embryogenic tissue). The steps involved have been addressed in earlier sections (2.2, Chapter 3) and all involve the use of pre-culturing treatments, the addition of potentially cytotoxic cryoprotectants (DMSO) and slow-freezing steps. Until now, there have been no reports of true cryopreservation in the sense of regeneration after exposure to liquid nitrogen (Withers, 1985) of mature conifer somatic embryos. However, a recent paper by Attree *et al.*(1995) reported the successful storage of white spruce somatic embryos at higher sub-zero temperatures (-20 ^{o}C). A seven week pretreatment with 20 μM ABA and 7.5% PEG followed by slow drying at relative humidities of 81%, 63%, and 43% for two weeks was employed, after which petri plates containing the somatic embryos were wrapped with Parafilm and stored in a freezer.

Unfortunately, the temperature used during slow drying at these various relative humidities was not reported, nor were the water contents of the embryos prior to freezing clearly specified which might make it difficult to repeat the experiment. Additionally, in order for stable long-term preservation to be maintained, tissues must be able to survive storage in LN_2 in order to avoid growth of ice crystals during storage (Chen and Kartha, 1987). Survival rates of angiosperm somatic embryos frozen at -20 ^oC and stored at this temperature declined significantly over time (Tessereau *et al.*, 1991; Florin *et al.*, 1993).

Several papers on angiosperm species report cryopreservation of mature somatic embryos in liquid nitrogen (Lecouteux *et al.*, 1991; Dumet *et al.*, 1993; de Boucaud *et al.*, 1994; Tessereau *et al.*, 1991; 1994;). Most of these papers report use of ABA and/or sucrose preculture before freezing. It is possible that in combination with the ABA used to mature the embryos, the drying/chilling treatment imposed in my system prior to freezing induces the accumulation of soluble sugars which may mimic the effects of preculture. It has been found that slow drying of excised immature axes of soybean seeds stimulates the accumulation of large amounts of sucrose and stachyose (Blackman *et al.*, 1992). Accumulation of sugars may serve to maintain cellular integrity by osmotically decreasing cell volume or act directly to protect by stabilization of membranes (see Section 2.3). Additionally, osmotic effects could cause stresses that stimulate synthesis of ABA. It would be interesting to measure the soluble sugar contents of embryos before and after drying, and after cryopreservation, to determine whether this is a significant factor underlying tolerance.

4.5 Conclusions

Using a well-controlled closed drying system, mature somatic embryos of white and interior spruce dried to bound water by equilibration over a solution (held at 5 ^oC) with a Ψ

of -20 MPa (RH=85.8%), were still viable following rehydration. The water content of the embryos was very close to that of dry mature zygotic embryos excised from stored seed. Embryos did not tolerate direct drying over silica gel (Ψ = approximately -540 MPa, RH approximately 2%), and the observed necrosis following this treatment was likely due to severe mechanical disruption within the cells.

Once somatic embryos are dried below a critical RWC of approximately 0.20, they will tolerate direct immersion in liquid nitrogen. For conifer somatic embryos, this is the first report of embryo cryopreservation without the application of cryoprotectants and a slow-freezing step. In terms of mature conifer somatic embryos, it is the first report of survival following exposure to liquid nitrogen.

The mechanisms that enabled somatic embryos to survive desiccation and freezing are not known. The drying treatment itself, perhaps coupled with the reduced temperature at which the drying was conducted, might have stimulated the accumulation of certain sugars and/or the synthesis of ABA. The literature suggests that ABA and certain sugars may play key roles in desiccation and freezing tolerance.

The continued use of uncontrolled open systems will severely impede efforts directed at scaling up drying treatments for improved germination, dry-storage and cryopreservation protocols. The results of the present study should form a basis for new work on storage of conifer somatic embryos as well as provide a link to the existing literature that relates to improved embryo maturation and germination using drying treatments.

Figure 4.1.a. The relationship between water potential (Ψ) and relative water content (RWC) for interior spruce mature somatic embryos. Each point is the mean ($\pm$ SD) of four replicates. The slope of the linear portion of the curve, used to calculate elastic modulus (ε), is $\Psi = -4.59 + 4.76$ RWC, $R^2 = 0.94$ (n=17).

Figure 4.1.b. The relationship between the reciprocal of water potential ($1/\Psi$) and RWC for interior spruce mature somatic embryos. The linear portion of the curve (at $1/\Psi=0$) gives and estimate of bound water. The equation of the linear portion of the RWC versus $1/\Psi$ is $1/\Psi = 0.13 - 1.13$ RWC, $R^2 = 0.93$ (n=14).

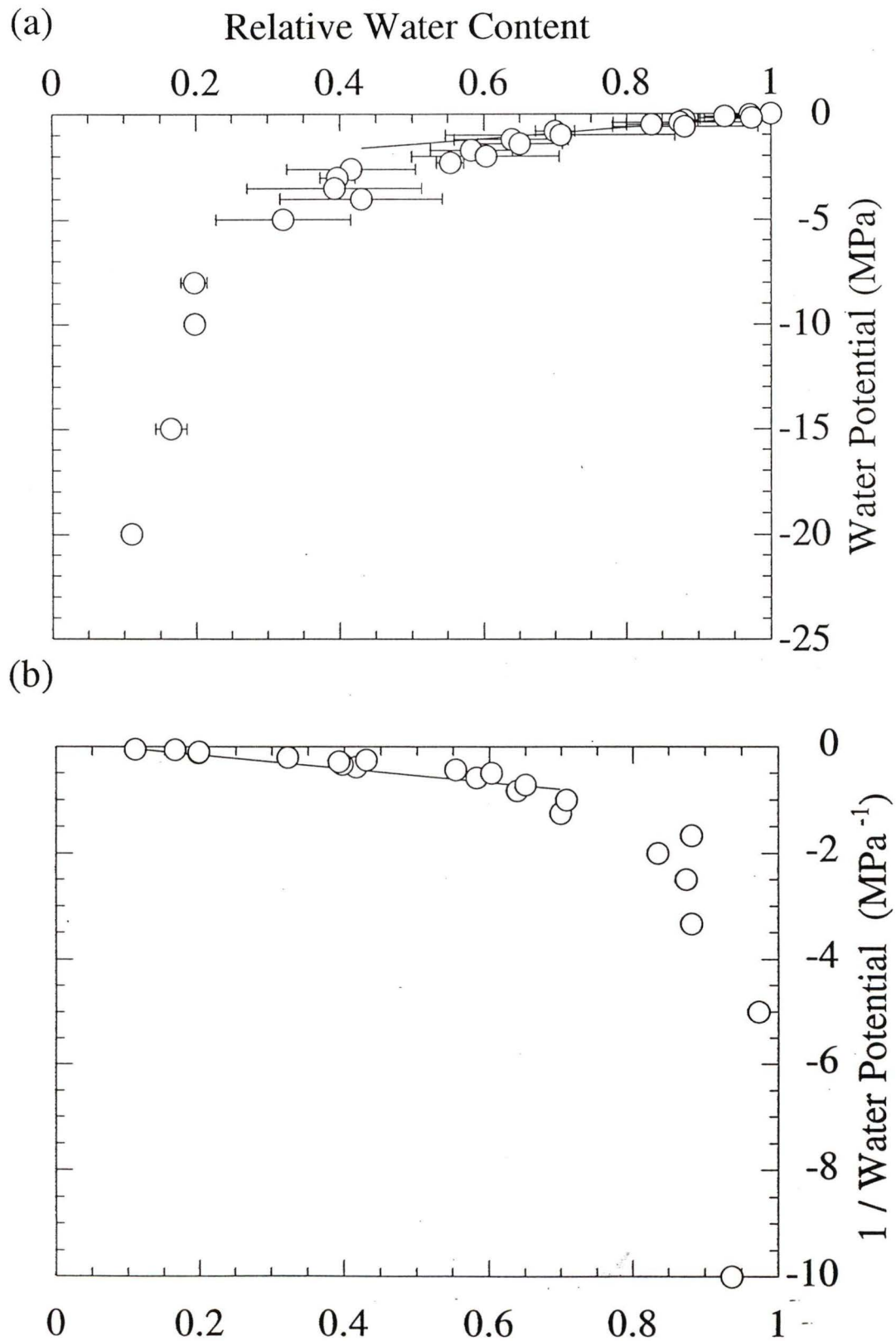

(a)
Relative Water Content
0
0.2
0.4
0.6
0.8
1
0
-5
-10
-15
-20
-25
Water Potential (MPa)
(b)
0
-2
-4
-6
-8
-10
1 / Water Potential (MPa-1)
0
0.2
0.4
0.6
0.8
1

Figure 4.2.a. Relation between relative conductivity (C_R, closed circle), water potential (Ψ) , and relative water content (RWC, open circle) for interior spruce mature somatic embryos. Each C_R data point is the mean (± SD) of ten replicates and each RWC data point is the mean (± SD) of thirty-two replicates. Open and closed squares represent RWC and C_R of the controls, respectively.

Figure 4.2.b. Survival of interior (S_x, open circle) and white spruce (S_w, open square) mature somatic embryos, as based on TTC staining, following drying to various relative water contents using a series of salt solutions of known water potential. Each point is the mean (± SD) of ten (S_w) or fifteen (S_x) replicates. For clarity, only the data points for S_x have been joined.

Figure 4.2.c. Regrowth after drying of interior spruce somatic embryos based on the presence of root, hypocotyl and cotlyedon elongation. For each treatment, n=10 samples and the experiment was repeated three times. S.D. are shown. In control samples (data not shown), 33% ± 11.5 of germinants produced roots, 77% ± 20.8 exhibited hypocotyl elongation and greening and 70% ± 17.3 possessed fully expanded cotyledons

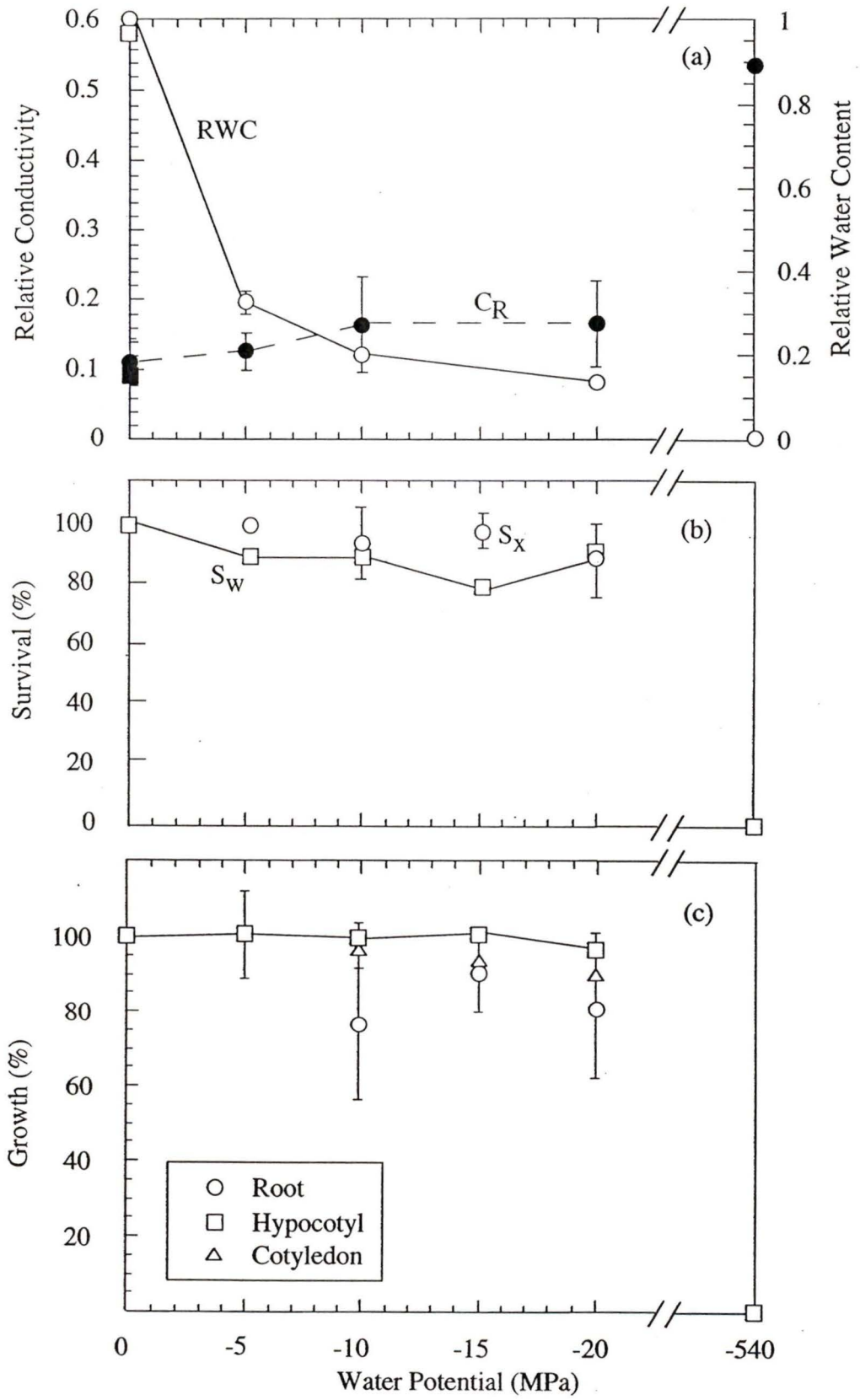

(a)
RWC
C_R
Relative Conductivity
Relative Water Content
0.6
0.5
0.4
0.3
0.2
0.1
0
1
0.8
0.6
0.4
0.2
0
(b)
S_x
S_w
Survival (%)
100
80
60
40
20
0
(c)
Growth (%)
100
80
60
40
20
Root
Hypocotyl
Cotyledon
0
-5
-10
-15
-20
-540
Water Potential (MPa)

Figure 4.3. Regrowth of interior spruce somatic embryos following drying to (A) 0 MPa, (B) -5 MPa, (C) -10 MPa, (D) -15 MPa, (E) -20 MPa and (F) -540 MPa. Silica gel was used to generate a water potential of -540 MPa. Magnification is [5x], with the exception of (F), which is [11x].

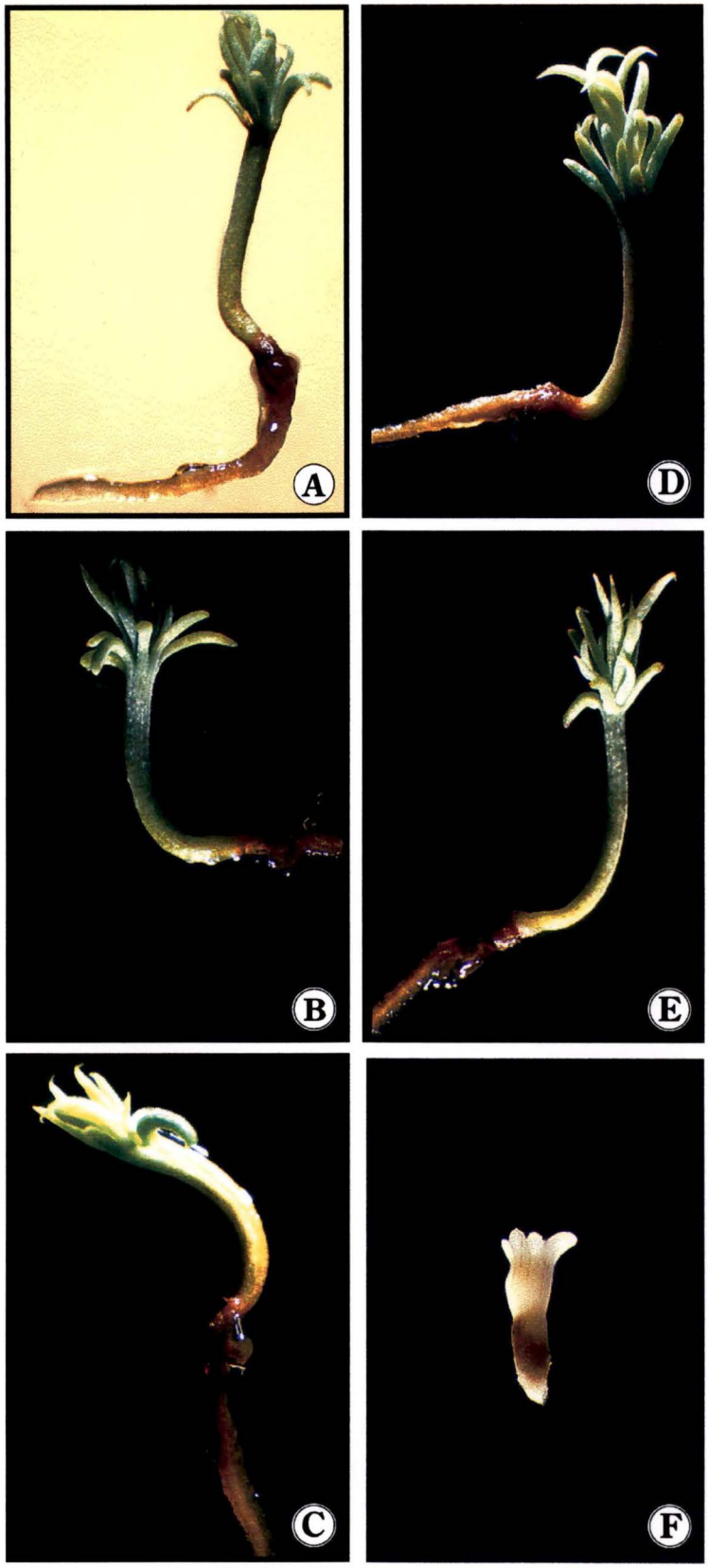

A
D
B
E
C
F

Figure 4.4.a. Survival of white (S_w, open square) and interior (S_x, open circle) spruce mature somatic embryos following drying to various relative water contents (RWC, closed circle) using salt solutions of known water potential and freezing in liquid nitrogen, as assessed using TTC staining. Each survival point is the mean (± SD) of ten (S_w) or fifteen (S_x) replicates. Each RWC data point is the mean (± SD) of thirty-two replicates. For clarity, only the data points for S_x have been joined. The water potential of -540 MPa was generated using silica gel.

Figure 4.4.b. Regrowth of interior spruce somatic embryos following drying and freezing in liquid nitrogen. Germinants were scored on the basis of root, hypocotyl and cotyledon elongation. For each treatment, n=10 samples and the experiment was repeated three times. S.D. are shown. In control samples (data not shown), 10% (SD=0) of the embryos exhibited hypocotyl elongation and greening, but none possessed roots or cotyledons.

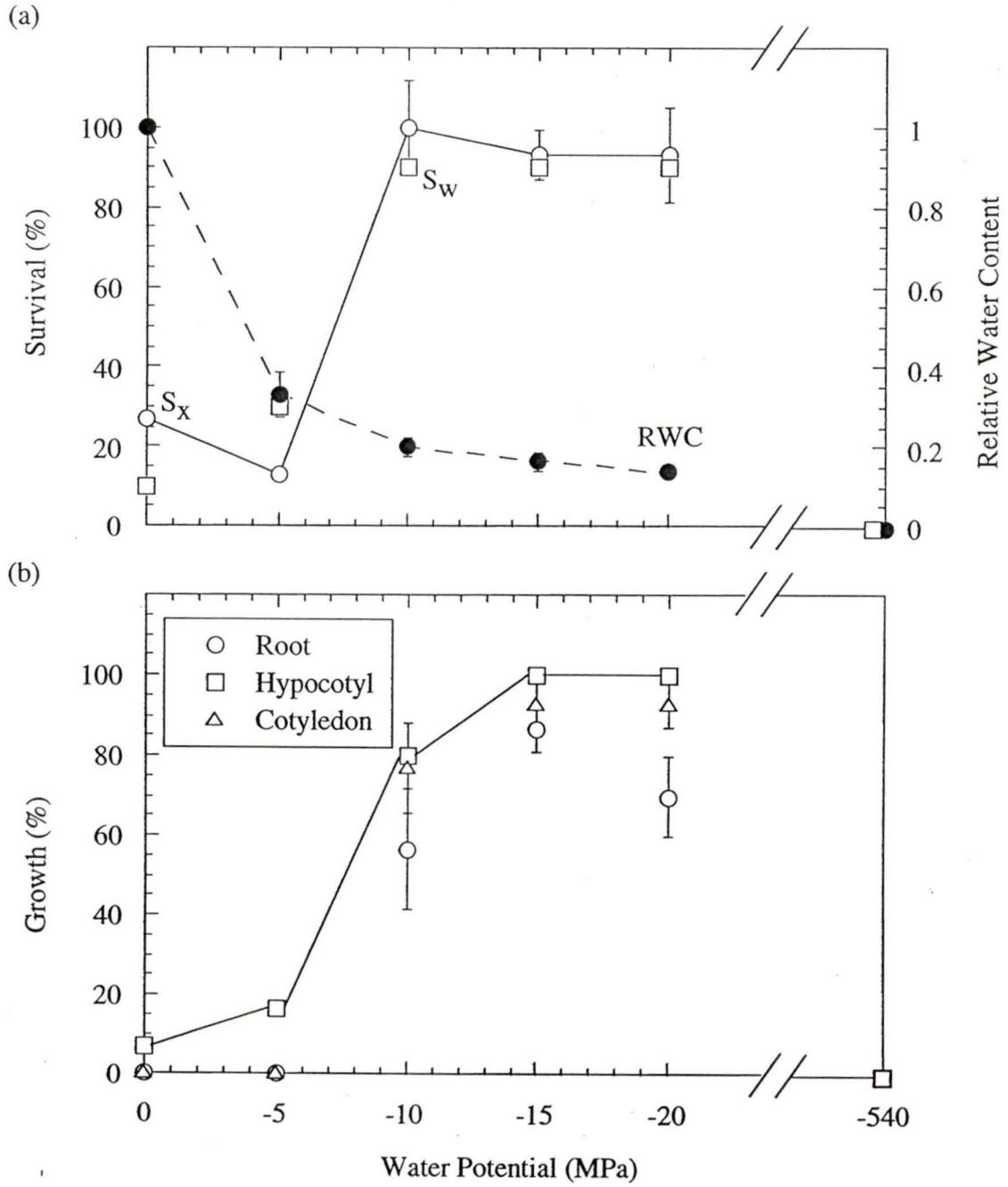
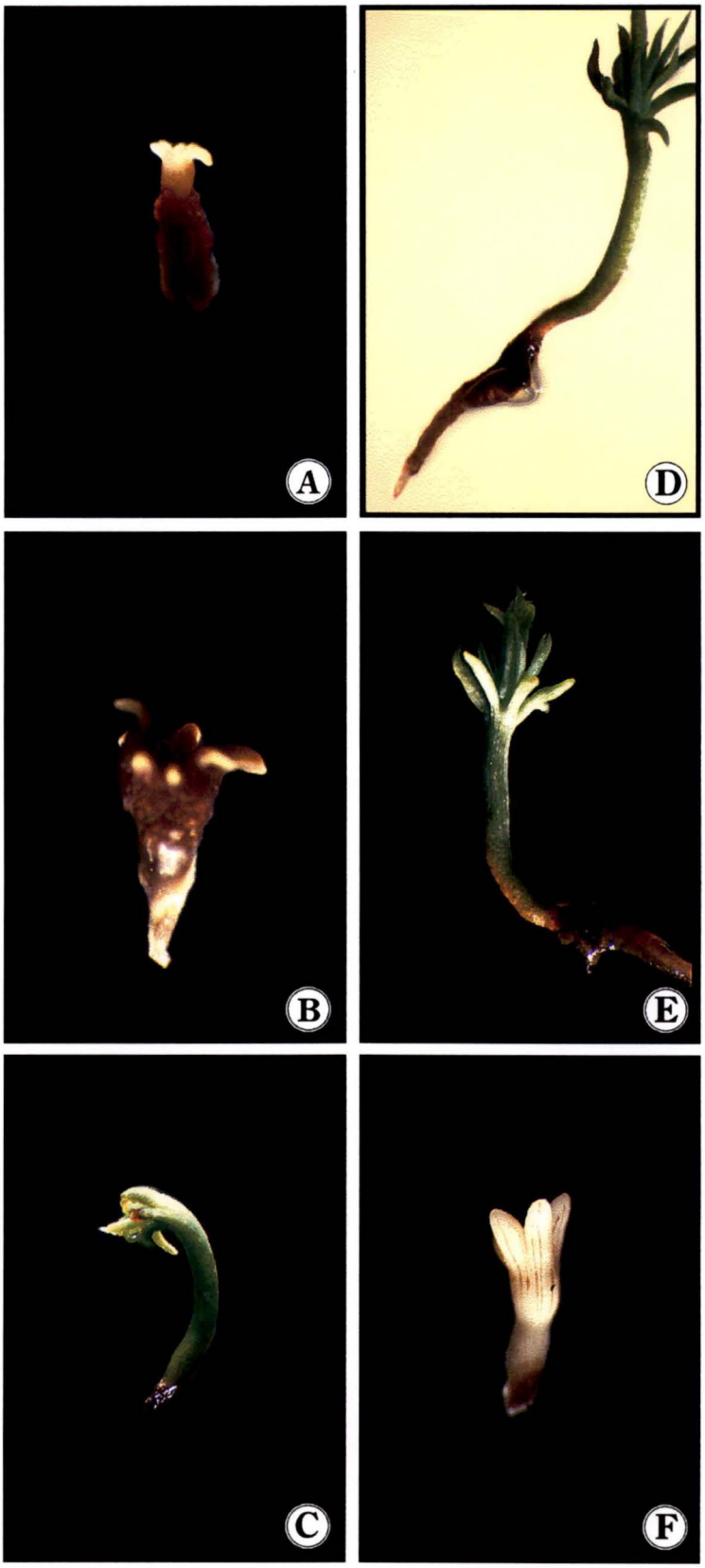

(a)
Survival (%)
Relative Water Content
S_x
S_w
RWC
100
80
60
40
20
0
1
0.8
0.6
0.4
0.2
0
(b)
Growth (%)
Root
Hypocotyl
Cotyledon
0
-5
-10
-15
-20
-540
Water Potential (MPa)

Figure 4.5. Regrowth of interior spruce somatic embryos following a drying pretreatment and then freezing in liquid nitrogen. Embryos were predried to water potentials of (A) 0 MPa [mag. 13x], (B) -5 MPa [mag. 10x], (C) -10 MPa [mag. 10x], (D) -15 MPa [mag.5x], (E) -20 MPa [mag. 5x], and (F) -540 MPa [mag. 9x]. Silica gel was used to generate a water potential of -540 MPa.


A
D
B
E
C
F

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Appendix I. Molality, Water Potential and Mass of NaCl (g) per 500g water at 5 ^{o}C.

SOLUTION	POTENTIAL (-MPa)	MOLALITY	MASS of NaCl
1	0.05	0.011	0.333
2	0.10	0.023	0.660
3	0.20	0.046	1.337
4	0.30	0.069	2.021
5	0.40	0.093	2.710
6	0.50	0.116	3.401
7	0.60	0.155	4.529
8	0.80	0.200	5.844
9	1.00	0.250	7.305
10	1.20	0.300	8.766
11	1.40	0.350	10.228
12	1.70	0.403	11.772
13	2.00	0.480	14.026
14	2.30	0.546	15.943
15	2.60	0.617	18.018
16	3.00	0.711	20.771
17	3.50	0.828	24.187
18	4.00	0.934	27.573
19	5.00	1.172	34.246
20	8.00	1.827	53.386
21	10.00	2.237	65.372
22	15.00	3.174	92.733
23	20.00	3.999	116.855

Appendix II-Statistical analysis of mature somatic embryos
-Relative Conductivity

Type III Sums of Squares

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Drying Treatment	5	.699	.140	53.542	.0001
Residual	24	.063	.003		

Dependent: Rel. Conductivity

Means Table
Effect: Drying Treatment
Dependent: Rel. Conductivity

	Count	Mean	Std. Dev.	Std. Error
-10	5	.156	.062	.028
-20	5	.165	.053	.024
-5	5	.125	.026	.012
0	5	.111	.034	.015
Control	5	.099	.042	.019
s.gel	5	.536	.074	.033

Student-Newman-Keuls
Effect: Drying Treatment
Dependent:Rel.Conductivity
Significance level: .05

	Vs.	Diff.	Crit. diff.	
Control	0	.011	.067	
	-5	.026	.081	
	-10	.057	.089	
	-20	.065	.095	
	s.gel	.437	.100	S
0	-5	.015	.067	
	-10	.045	.081	
	-20	.054	.089	
	s.gel	.426	.095	S
-5	-10	.031	.067	
	-20	.040	.081	
	s.gel	.411	.089	S
-10	-20	.009	.067	
	s.gel	.380	.081	S
-20	s.gel	.371	.067	S

S = Significantly different at this level.

Appendix III-Water Content of Plant Tissue based on Measurements of Water Mass

Relative Water Content (**RWC**)

$$\frac{\text{fresh mass(g) - dry mass*(g)}}{\text{saturated mass(g) - dry mass(g)}}$$

		Units
Dry Mass Basis (**dmb**)	$\frac{\text{fresh mass(g)-dry mass(g)}}{\text{dry mass(g)}}$	g H_2O g^{-1} dm
Fresh Mass Basis (**fmb**)	$\frac{\text{fresh mass(g)-dry mass(g)}}{\text{fresh mass(g)}}$	g H_2O g^{-1} fm

* Dry mass should be obtained using an oven temperature of 60 to 80 ^{o}C (for 24 to 48 hours) since higher temperatures might cause the loss of volatiles and nitrogenous compounds.

Converting between water contents expressed on dmb or fmb:

dmb to **fmb**

$$\frac{\text{water content } \mathbf{dmb}}{(\mathbf{1} + \text{water content } \mathbf{dmb})}$$

fmb to **dmb**

$$\frac{\text{water content } \mathbf{fmb}}{(\mathbf{1} - \text{water content } \mathbf{fmb})}$$

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Title of Thesis:

Desiccation and Cryopreservation of Spruce Somatic Embryogenic Tissue and Mature Somatic Embryos

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