CRYOPRESERVATION OF *PINUS PATULA* SCHEIDE ET DEPPE EMBRYOGENIC TISSUE

BY

CATHERINE SUSAN FORD

Submitted in fulfilment of the requirements for the degree of
MASTERS IN SCIENCE

in the Department of Botany, Faculty of Science
University of Natal, Pietermaritzburg

August 1999

PREFACE

The experimental work described in this thesis was conducted in the Botany Department, University of Natal, Pietermaritzburg, from January 1997 to March 1999 under the supervision of Professor J van Staden and the co-supervision of Doctor N B Jones.

The results have not been submitted in any other form to another University and except where the work of others is acknowledged in the text, are the result of my own investigation.

CATHERINE SUSAN FORD

AUGUST 1999

We certify that the above statement is correct.

PROF. J VAN STADEN

(SUPERVISOR)

DR NB JONES

(CO-SUPERVISOR)

PUBLICATIONS FROM THIS THESIS

FORD CS, JONES NB and VAN STADEN J (1999) Cryopreservation and plant regeneration from somatic embryos of *Pinus patula*. *Plant Cell Reports* (In Press)

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

1. **FORD CS, JONES NB AND VAN STADEN J** (1998) Cryopreservation of somatic embryos of *Pinus patula* (Paper). **Twenty-fourth Annual Congress of South African Association of Botanists, University of Cape Town, Cape Town.**

2. **FORD CS, JONES NB AND VAN STADEN J** (1999) A comparison of cryopreservation techniques used on embryogenic tissue of *Pinus patula* (Poster). **Twenty-fifth Annual Congress of South African Association of Botanists, University of Transkei, Umtata.**

ACKNOWLEDGEMENTS

I am grateful to Professor J van Staden for giving me the opportunity and the facilities in which to conduct my studies.

I wish to thank Dr Nicky Jones for all of her help and encouragement. Her active enthusiasm and interest in this project were of immense help.

My thanks to Sappi for providing me with plant material for this study, and the Technology and Human Resources for Industry Programme (THRIP) for providing funds for buying the equipment needed for this study.

I wish to thank Dr Jeff Finnie for his time and input to this research.

The financial assistance of the FRD is gratefully acknowledged.

I wish to thank Wayne Jones for helping me out with facts and figures on Pine propagation in South Africa.

To all of my friends: thanks for all of your encouragement, enthusiasm, bonding sessions and support. Special thanks to Jos and Shelagh for their help and support.

Thanks also to my folks. Thanks for putting up with me while I was writing up, and for your encouragement.

This thesis is dedicated to my sister, Robynne Hayes, and my nephew Tristan. Your strength and courage are an inspiration

ABSTRACT

Embryogenic tissue of *Pinus patula* Scheide et Deppe was initiated from immature green female cones during the months of November 1996 to February 1997 and December 1997 to January 1998. Tissue was maintained on MSG3 medium (BECWAR, NAGMANI and WANN 1990) supplemented with maltose. A comparison of various sugars as a carbohydrate source for maintaining embryogenic tissue showed that maltose was far superior to sucrose and the other sugars tested.

Embryogenic tissue was successfully cryopreserved for up to 8 weeks using 0.3 M sorbitol and 5 % DMSO. Recovered tissue initially underwent a lag phase in tissue regrowth, but by the end of 5 weeks post-thaw, tissue proliferation was as vigorous as the unfrozen, untreated control. Fluoresceine diacetate (FDA) staining revealed that the embryonal head survived cryopreservation, but the highly vacuolated suspensor tissue had ruptured and died. Embryogenic tissue from two different families and four genotypes were successfully cryopreserved using this protocol.

A comparison of commonly used cryopreservation techniques was conducted. It was found that the slow addition of the cryoprotectants over two days slowed the recovery rate of the tissue and increased the chances of contamination. Embryogenic tissue did not respond well to cryopreservation using a combination of the cryoprotectants PEG, glucose and DMSO (10-8-10%). Only a small proportion of the tissue survived, and initial tissue regrowth took up to 5 weeks. Embryogenic tissue was also set in gel and immersed directly in liquid nitrogen in an effort to cryopreserve tissue using the process of vitrification. However, none of the tissue survived, possibly due to insufficient dehydration prior to immersion in liquid nitrogen.

Tissue recovery was highest when the tissue was precooled to -70°C in a container filled with isopropyl alcohol placed in a static freezer prior to immersion in liquid nitrogen. Recovery of tissue was improved by suspending the tissue on polyester grids

and removing the liquid medium prior to placing onto MSG3 medium.

Recovered tissue was bulked up using suspension cultures, and then paced onto MSG5 (BECWAR, NAGMANI and WANN 1990) or 240 medium (PULLMAN and WEBB 1994) to mature. Mature embryos were isolated from both media and germinated. Somatic plantlets were successfully hardened-off under greenhouse conditions.

The successful cryopreservation of a number of genotypes and lines, and the maturation of recovered tissue has been achieved. This technique is now being actively incorporated into *P. patula* somatic embryo research, enabling the long-term storage of juvenile reference tissue while field trials are carried out and evaluated.

CONTENTS

Page

LIST OF COMMONLY USED ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
BA	Benzyladenine
d	Day
2,4-D	2,4 - Dichlorophenoxyacetic acid
DCR	Douglas-fir Cotyledon Revised medium
DMSO	Dimethylsulfoxide
FDA	Fluoresceine diacetate
h	Hour
min	Minute
MSG	Modified Murashige and Skoog (1962) medium
PEG	Polyethylene glycol
SE	Standard error

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. Forestry in South Africa

It is estimated that 1.3% of the total land area of South Africa is used for forestry (Forest Owners Association 1996/97). Forest products generate much needed revenue in South Africa from the export of pulp, paper and wood products (Forest Owners Association 1996/97). Approximately half of the land under afforestation is owned by large companies such as Sappi, Mondi, and Safcol, with the rest of the plantation holdings belonging to private individuals, and public and government owned companies. The majority of tree species under afforestation in South Africa are softwood species (such as *Pinus patula*, *Pinus elliottii* and *Pinus radiata)*, making up approximately 40% of the total new trees under afforestation (Forest Owners Association 1996/97).

1.2. *Pinus patula* Scheide et Deppe

Pinus patula is the most important, commercially grown softwood species in South Africa (JONES and VAN STADEN 1997). It was brought from Mexico around 1907, but was only propagated on a large scale after World War II. It is commonly known as the Mexican weeping pine, Mexican red pine or "patula" pine, due to the nature of the needles which droop in a weeping fashion. The species is evergreen, although deciduous, since needles are gradually shed in autumn (WORMALD 1975). Pines can reach 20-30 m in height. The bark on the lower part of the trunk is broken into irregularly shaped plates while higher up, it becomes thin, papery and reddish brown in appearance (DALLIMORE and JACKSON 1966). The microsporangia and megasporangia in most conifers are borne in separate cones on the same tree. The microsporangiate cones are borne on the lower branches of the tree, while the

megasporangiate or ovulate cones are borne on the upper branches. This arrangement encourages outcrossing (RAVEN, EVERT and EICHHORN 1986). *Pinus patula* grows best in summer rainfall areas (30°30' 29°40'), including the Northern Province, Mpumalanga, KwaZulu-Natal, Eastern Cape and Western Cape (GOLDSMARK pers. comm. 1999)(Figure 1).

1.3. Propagation

Propagation by seed is widely used, however, the clonal option is gaining favour. VERRYN (1997) defines a clone as "a group of genetically identical individuals asexually propagated from a single ancestor". Clonal propagation is favoured in forestry for a number of reasons: (i) a more uniform crop can be produced, enabling greater control of wood properties, and hence more efficient processing and better products (VERRYN 1997); and (ii) trees with superior genetic characteristics such as disease resistance and drought tolerance can be multiplied. Companies such as Sappi initiated their own tree improvement programme for the production of high quality seeds from selected superior trees. The superior individuals were selected and grafted in July 1988 and the first Sappi *P. patula* seed orchard was established at Tweedie, KwaZulu - Natal in December 1988 (BUTTERFIELD 1990).

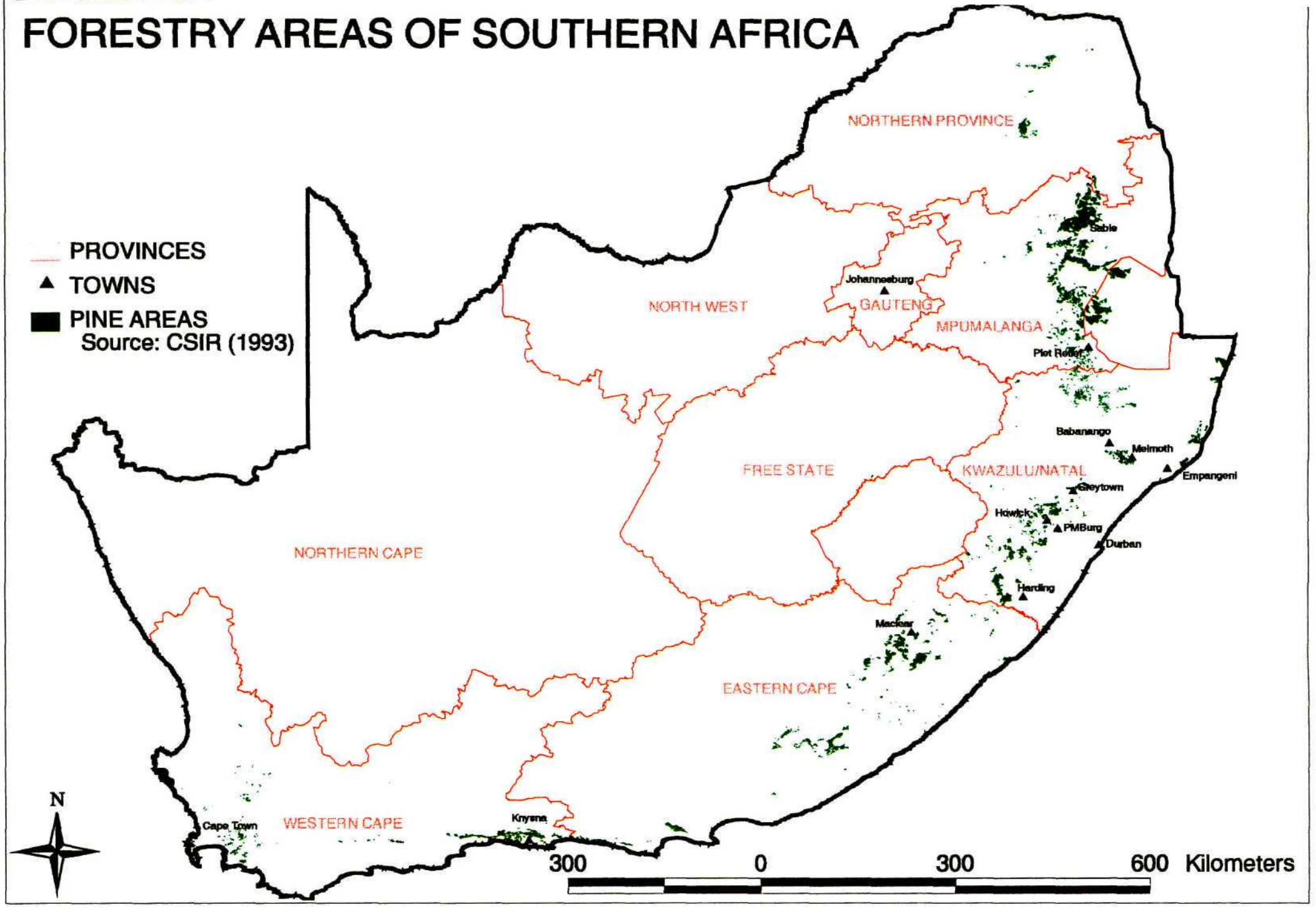
1.3.1. Macropropagation

Commercially produced *P. patula* saplings can be propagated by means of seeds from seed orchards, grafting of superior scion material onto rootstocks, and rooting of cuttings using hormones. The use of seed to produce seedlings is common, however, it is limited by the amount harvested from orchards. Grafting is used in tree breeding programmes as a means of improving genetic gain. As physiologically older material can be grafted successfully, the period required before orchards can produce seeds is significantly reduced (DONALD 1987).

Second to the use of seed, the propagating *P. patula* is through the rooting of cuttings

Figure 1. The distribution of commercially propagated *Pinus* in South Africa. Illustration produced from satellite images of land cover in 1993 (1:250 000 scale) by the Environmentek Division of the CSIR.

FORESTRY AREAS OF SOUTHERN AFRICA



taken from the ortet (parent plant). Scions taken from mature trees are preferred over cloning of embryos or seedlings because it is not always possible to determine if juvenile material has the genetic potential to develop the desired qualities later on in its life cycle (BONGA 1987). However, in ortets older than four years, maturation becomes a limiting factor for successful rooting (JONES and VAN STADEN 1997). Maturation is a developmental process relating to woody plants during which growth rate and rooting of cuttings is reduced by the onset of morphological changes (HAFFNER, ENJALRIC, LARDET and CARRON 1991). Also, only a limited number of ramets (cuttings) can be produced from each ortet (VON ARNOLD, EGERTSDOTTER, EKBERG, GUPTA, MO and NØRGAARD 1995). In order to overcome these difficulties, once a superior tree has been selected, hedging (repeated pruning back of shoots to a specific height) can be used to retard maturation and hence maintain the ortet in a juvenile state (DOOLEY 1986). Using this method buds less mature than the tissue being removed are stimulated to develop. However, ageing is only temporarily delayed. The other method employed to retard maturation is serial propagation. This involves taking cuttings from a selected tree. The cuttings are then rooted and established. These then become the secondary ortet and source of further cutting material (JONES 1994). This means that more space is required to sustain sufficient hedges.

1.3.2. Micropropagation

Micropropagation can overcome a number of the problems encountered with macropropagation. BONGA and VON ADERKAS (1992) give the following points in favour of micropropagation:

1) Tissue cultures can be cryopreserved for long periods of time, which is exploited in tree improvement schemes and in longterm germplasm storage;
2) Micropropagation is an essential part of most genetic engineering programmes; and
3) Micropropagation allows the manipulation of environmental and chemical factors under controlled circumstances.

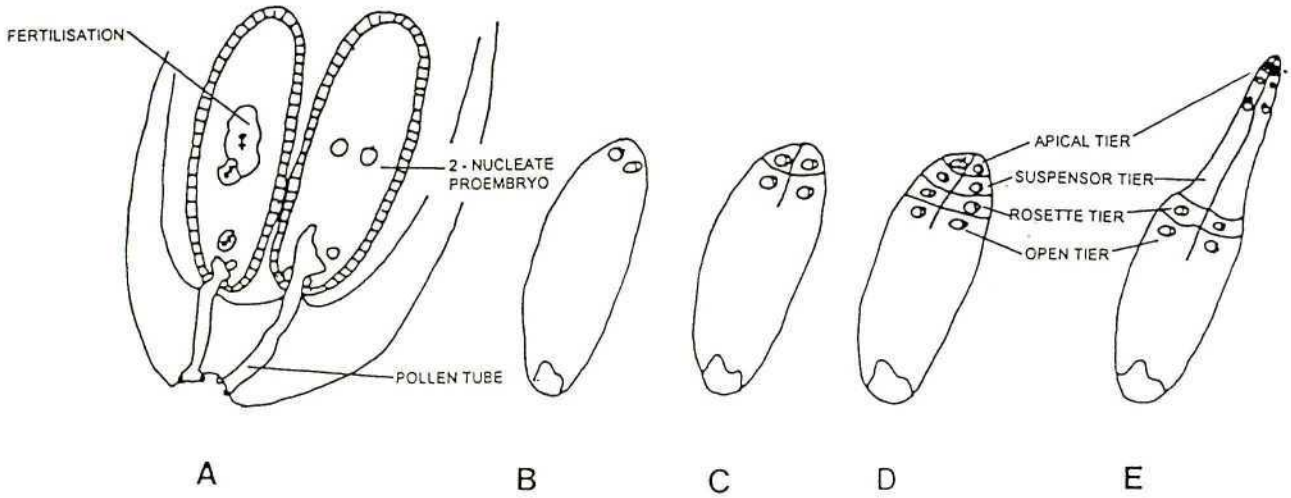
The three most commonly used methods of micropropagation are: axillary shoot elongation, organogenesis and somatic embryogenesis.

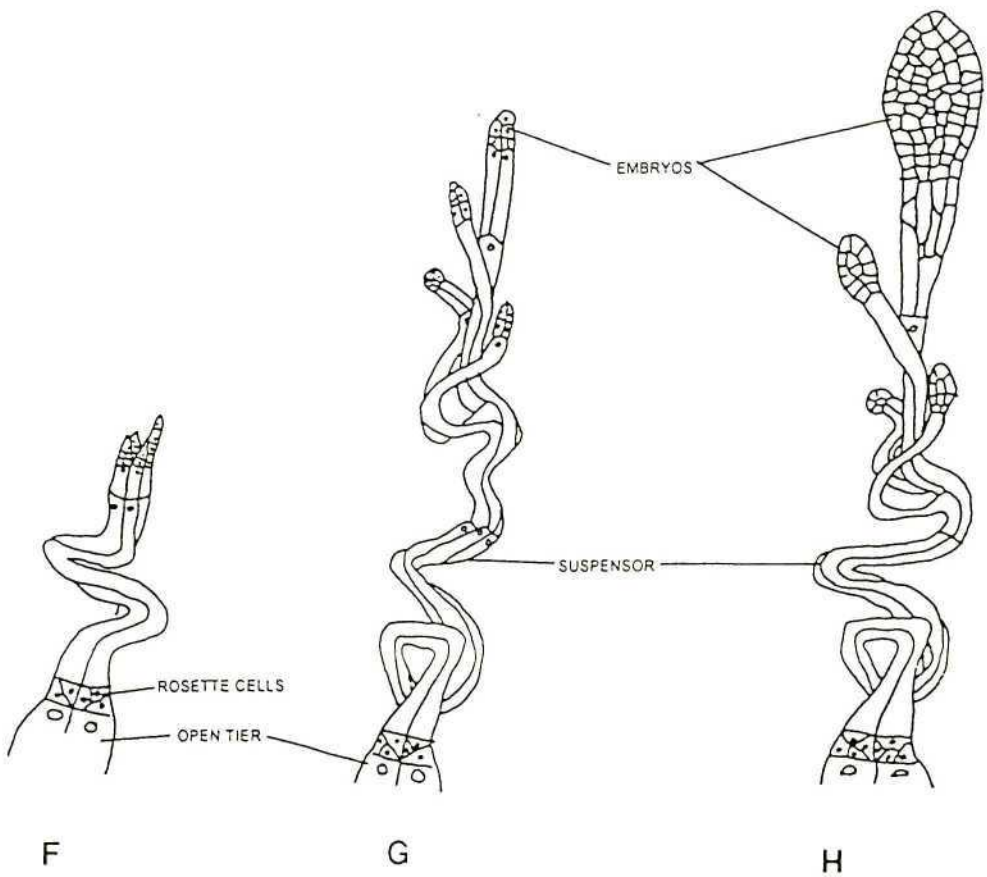
1.3.3. Somatic Embryogenesis

Every living cell is a potential embryo, based on the theory of totipotency (MEHRA-PALTA and THOMPSON 1988). DODDS and ROBERTS (1985) define totipotency as " the ability to regenerate an entire organism from a single cell or plant part". The discovery of somatic embryogenesis was first accredited to the independent research of Frederick C. Steward and Jacob Reinert. However, later investigations found that Harry Willis was the first to describe the phenomenon of plants arising from plant cells (which he termed 'neomorphs'), a realisation of Haberlandt's dream (KRIKORIAN and SIMOLA 1999). Production of somatic embryos (derived from asexual or adventitious embryogenesis) in conifers involves the culture of zygotic embryos. As in zygotic embryogenesis, the somatic precursor cells exhibit bipolarity (i.e. have root and shoot apices) (TAUTORUS, FOWKE and DUNSTAN 1991). Somatic embryogenesis and plantlet regeneration was first reported in *Picea abies*, for which zygotic embryos were used as explants to establish the cultures (CHALUPA 1985; HAKMAN, FOWKE, VON ARNOLD and ERIKSSON 1985; GUPTA and DURZAN 1986). Somatic embryogenesis has the potential to be widely used in large-scale reforestation as large numbers of identical trees can be produced, and somatic embryos can be encapsulated to produce large quantities of artificial seeds (BONGA and VON ADERKAS 1992; ATTREE and FOWKE 1993; VON ARNOLD, EGERTSDOTTER, EKBERG, GUPTA, MO and NØRGAARD, 1995). Somatic embryogenesis has been introduced on a semi-commercial basis in Canada and New Zealand. Megagametophytes, immature and mature zygotic embryos, young seedlings, and reinduced cotyledonary somatic embryos have been used to initiate somatic embryogenesis in numerous species of conifers (TAUTORUS, FOWKE and DUNSTAN 1991), as is summarised in Table 1.

It is believed that somatic embryos in *Pinus* arise from a mechanism similar to cleavage polyembryogenesis, with the initial separation occurring in the embryonic region (TAUTORUS, FOWKE and DUNSTAN 1991). In cleavage polyembryony, the apical tier separates into four files of cells, each genetically identical to the other. One of these embryos becomes dominant and the rest degenerate (Figure 2) (TAUTORUS, FOWKE and DUNSTAN 1991). Provision of the correct physical and environmental conditions

Figure 2. Fertilisation and cleavage polyembryony in *Pinus*. Proembryo development begins when the fertilised egg nucleus divides into two (A), then four, free nuclei in the centre of the egg cell. The nuclei become orientated into a single tier (B), and each nucleus divides. Cell walls form between the eight nuclei to create two tiers of four walls each (C). Each cell divides again, forming a 16-celled proembryo consisting of four tiers of four cells each (D). The third tier of cells (suspensor tier) elongates and thrusts the distal apical tier out of the archegonial jacket into the female gametophytic tissue (E), ending the proembryo stage. Cells in the apical tier of the embryo begin to separate into four files of cells (cleavage polyembryony) (F). One of the embryos becomes dominant and the other three degenerate (G,H). The surviving embryo is pushed by the suspensor to the middle of the female gametophyte tissue and enlarges (H). (Adapted from OWENS and BLAKE 1985).


FERTILISATION
2 - NUCLEATE PROEMBRYO
POLLEN TUBE
APICAL TIER
SUSPENSOR TIER
ROSETTE TIER
OPEN TIER
A
B
C
D
E


EMBRYOS
SUSPENSOR
ROSETTE CELLS
OPEN TIER
F
G
H

in culture, promotes an "embryo rescue" situation which may result in somatic embryogenesis (JONES, VAN STADEN and BAYLEY 1993).

1.4. Cryopreservation in Forest Biotechnology

One of the most valuable properties of somatic embryos is their use in genetic engineering. Through the use of transformation, plants that are more resistant to disease and adverse environmental stresses can be cloned (ATTREE and FOWKE 1993). Somatic embryos can also be used for the long-term storage of germplasm, thereby conserving valuable germplasm of rare and endangered species (TAUTORUS, FOWKE and DUNSTAN 1991; ATTREE and FOWKE 1993). Cryopreserving embryogenic cell lines at the beginning of progeny tests would also overcome the maturation constraint (VON ARNOLD, EGERTSDOTTER, EKBERG, GUPTA, MO and NØRGAARD 1995) experienced in traditional family forestry. At present, cryopreservation has become an integral part of conifer biotechnology, enabling breeders to incorporate somatic embryogenesis into breeding programmes. Some of the international companies that are actively incorporating cryopreservation int their tree breeding programmes are: New Zealand Forest Research Institute (FRI); Silverglen Inc. Canada; and Weyerhaeuser, USA. In most cases, the combination of the cryoprotectants sorbitol and DMSO, coupled with a slow cooling regime, has proved to be successful with the long term storage of a number of forest trees.

Table 1. Summary of embryogenic tissue induction in coniferous species from 1990 to date.

Species	Explant	Plantlet Production	References
Abies alba	Immature embryos	Not reported	HARTMANN *et al.* (1992)
Abies alba X Abies cephalonica	Immature and mature zygotic embryos	Germination	SALAJOVA *et al.* (1996)
Abies alba X Abies numidica	Immature and mature zygotic embryos	Germination	SALAJOVA *et al.* (1996)
Abies balsamea	Mature zygotic embryos	Not reported	GUEVIN *et al.* (1994)
Abies fraseri	Immature zygotic embryos	No	RAJBHANDARI and STOMP (1997)
Abies normanniana	Immature zygotic embryos	Yes	NØRGAARD and KROGSTRUP (1991)
Larix decidua	Immature zygotic embryos (2n) and megagametophytes (n)	Not reported	VON ADERKAS *et al.* (1990)
Larix decidua	Protoplasts	Not reported	VON ADERKAS (1992)
Larix decidua	Protoplasts	Yes	ZOGLAUER *et al.* (1992)
Larix decidua	Immature and mature zygotic embryos	Not reported	LELU *et al.* (1994)

Species	Explant	Plantlet Production	References
Larix decidua	Immature and mature zygotic embryos; young seedlings; megagametophyte tissue	Yes	BENKRIMA *et al.* (1995)
Larix X *eurolepis*	Immature zygotic embryos	Not reported	LELU *et al.* (1994)
Larix eurolepis	Immature and mature zygotic embryos; young seedlings; megagametophyte tissue	Yes	BENKRIMA *et al.* (1995)
Larix laricina	Immature zygotic embryos	Yes	KLIMASZEWSKA *et al.* (1997)
Larix X *leptoeuropaea*	Immature and mature zygotic embryos; cotyledons and needles	Not reported	LELU *et al.* (1994)
Larix leptolepis	Immature zygotic embryos (2n) and isolated megagametophytes (n)	Not reported	VON ADERKAS *et al.* (1990)
Larix leptolepis	Immature and mature zygotic embryos; young seedlings; megagametophyte tissue	Yes	BENKRIMA *et al.* (1995)
Larix occidentalis	Immature zygotic embryos	Yes	THOMPSON *et al.* (1992)

Species	Explant	Plantlet Production	References
Larix occidentalis	Immature and mature zygotic embryos; young seedlings; megagametophyte tissue	Yes	BENKRIMA *et al.* (1995)
Picea abies	Mature zygotic embryos	Not reported	SUSS *et al.* (1990)
Picea abies	Hypocotyls and cotyledons	Not reported	RUAUD *et al.* (1992)
Picea glauca	Seedlings	Yes	ATTREE *et al.* (1990)
Picea glauca	Cotyledons	Yes	LELU and BORNMAN (1990)
Picea glauca	zygotic embryos from 3-11 year old seeds	Yes	TREMBLAY (1990)
Picea glauca x *Picea engelmannii*	Immature zygotic embryos	Yes	WEBSTER *et al.* (1990)
Picea glehnii	Mature zygotic embryos	Yes	ISHII (1991)
Picea jezoensis	Mature zygotic embryos	Yes	ISHII (1991)
Picea jezoensis var. *hondoensis*	Mature zygotic embryos	Yes	ISHII (1991)
Picea mariana	Seedlings	Yes	ATTREE *et al.* (1990)
Picea mariana	Cotyledons	Yes	LELU and BORNMAN (1990)
Picea mariana	Immature zygotic embryos	Not reported	TAUTORUS *et al.* (1990)

Species	Explant	Plantlet Production	References
Picea mariana	Mature zygotic embryos	Not reported	TAUTORUS *et al.* (1990)
Picea meyeri	Mature zygotic embryos	Yes	YANG *et al.* (1997)
Picea omorika	Shoots	Yes	BUDIMIR and VUJICIC (1992)
Picea pungens	Mature zygotic embryos	Yes	AFELE *et al.* (1992)
Picea rubens	Mature zygotic embryos	Yes	HARRY and THORPE (1991)
Picea sitchensis	Immature zygotic embryos	Yes	INGRAM and MAVITUNA (1998)
Picea wilsonii	Immature zygotic embryos	Yes	LI and GUO (1990)
Pinus banksiana	Immature zygotic embryos	Not reported	KRAJCOVA (1993)
Pinus bungeana	Immature zygotic embryos	Not reported	KRAJCOVA (1993)
Pinus caribaea var. *hondurensis*	Immature embryos and protoplasts	Yes	LAINÉ and DAVID (1990)
Pinus elliottii	Immature zygotic embryos	Yes	LIAO and AMERSON (1995)
Pinus elliottii	Immature zygotic embryos	Yes	TANG-WEI *et al.* (1997)
Pinus koraiensis	Mature zygotic embryos	No	BOZHKOV *et al.* (1997)
Pinus massoniana	Mature zygotic embryos	Yes	HUANG-JAIN *et al.* (1995)
Pinus nigra	Immature zygotic embryos	No	JASIK *et al.* (1995)
Pinus patula	Immature zygotic embryos	No	JONES *et al.* (1993)

Species	Explant	Plantlet Production	References
Pinus patula	Immature zygotic embryos	Yes	JONES and VAN STADEN (1995)
Pinus palustris	Immature zygotic embryos	Not reported	NAGMANI *et al.* (1993)
Pinus strobus	Immature zygotic embryos	Not reported	KLIMASZEWSKA and SMITH (1997)
Pinus sylvestris	Immature zygotic embryos	Yes	KEINONEN-METTÄLÄ *et al.* (1996)
Pinus sylvestris	Immature zygotic embryos	Germination	HARRY and THORPE (1998)
Pinus taeda	Mature zygotic embryos	Not reported	TANG-WEI *et al.* (1998)

1.5. Aims of the Study

As indicated above, somatic embryogenesis provides a valuable means of mass producing clones, as well as providing material for genetic manipulation. The ability to store transformed tissue in a state of suspended animation for long periods of time while field trials are conducted and evaluated, is an invaluable tool to tree breeders. The major objective of this study was to devise an optimal protocol for the long-term storage of *P. patula* embryogenic tissue in liquid nitrogen. The approach involved:

i) Experimenting with a number of concentrations and combinations of cryoprotectants to determine the optimal combination and concentration for successful cryopreservation;
ii) Freezing tissue over a range of temperatures to determine an optimal freezing temperature;
iii) Determining the effect of storage time on tissue recovery;
iv) Attempting to produce plantlets from recovered tissue;
v) Successfully recovering tissue from long term storage; and
vi) Determining whether the optimal cryopreservation technique could be successfully applied to various embryogenic lines of different genetic origins.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Any biological material will eventually deteriorate and die. The rate at which material decays has been retarded as a result of the development of refrigeration. However, elaborate storage facilities, together with the assurance of a reliable electricity source are required (BLAKESLEY, PASK, HENSHAW and FAY 1996). The use of ultra-low temperatures (such as the temperature of liquid nitrogen, -196°C) has provided a successful means of storing living organisms in a state of suspended animation for extended periods of time (GROUT 1995; McLELLAN and DAY 1995). Fifty years have passed since spermatozoa were first cryopreserved (POLGE, SMITH and PARKES 1949). Since then a number of living tissues and organs have been successfully cryopreserved, including bull spermatozoa, erythrocytes, plant cell cultures, plant callus and microorganisms (McLELLAN and DAY 1995).

Preservation of both animal and plant germplasm is an integral component of sustainable agricultural systems. From a crop improvement perspective, preservation of all valuable germplasm is being accorded a high priority (IBPGR 1985). Cryopreservation offers an alternative approach to the long-term storage of genetic resources (thereby maintaining biodiversity) in various forms, including whole seeds, embryonal axes, pollen or microspores, meristems, somatic and zygotic embryos, and anthers (KARTHA and ENGELMANN 1994; BLAKESLEY, PASK, HENSHAW and FAY 1996). Cryopreservation of zygotic embryos, embryo axes, and even whole seeds is used for the preservation of recalcitrant and tropical species (KARTHA and ENGELMANN 1994).

GROUT (1995) lists the properties that are required for a successful storage system as the ability to:

- Minimise growth and development *in vitro* in the plant subjects to enable intervals between subculture and other handling to be significantly extended;
- Maintain the viability of the stored material at the highest possible level, together with the minimum risk to genetic stability;
- Maintain the full developmental and functional potential of the stored material when it is returned to normal physiological temperatures; and
- Make significant savings in labour input, materials and commitment of specialised growing equipment.

The goal for successful cryopreservation is to maintain a level of integrated structure and function compatible with high viability and normal activity upon restoration to physiological temperatures (KARTHA and ENGELMANN 1994; GROUT 1995). Given the great stability of tissues at very cold temperatures (e.g. -196°C), the challenge is in manipulating the tissues through the transient freezing and thawing operations with minimum ultrastructural and metabolic changes (FINKLE, ZAVALA and ULRICH 1985).

2.2. Cooling

Living plants and plant tissues succumb to freezing at just a few degrees below 0°C, the freezing point of pure water. "Freeze hardening" or the treatment of plant material with cryoprotective additives can help in avoiding this freezing injury (FINKLE, ZAVALA and ULRICH 1985). In general, the basic steps involved in the cryopreservation of living material by slow cooling are:

- Cold acclimatization of the donor material where possible;
- Pretreatment of explants with cryoprotectants;
- Incubation in the cryoprotectant solution in which the explants will be frozen and stored;
- Slow-cooling at a controlled rate (0.5 to 2°C per minute) to a temperature of between -30°C and -40°C, before being plunged into liquid nitrogen (-196°C);
- Thawing by immersing samples in a waterbath at 35-40°C; and

- Removal of cryoprotectants and restoration of osmolarity of culture medium to physiological levels (KARTHA and ENGELMANN 1994; GROUT 1995).

During cooling, the extracellular solution freezes first. Ice crystal latices form in the solution, trapping the as yet unfrozen plant material in channels between the ice crystals. The channels are filled with residual solutions containing solutes that composed the original external solutions. Thus, as more water freezes, the concentration of the solutes increases resulting in an increasingly negative water potential in the residual solution and significant osmotic water loss from the plant material. This process is described as cryodehydration (MAZUR 1969; GROUT 1995). Controlled, slow cooling allows cryodehydration to progress without intracellular freezing, removing water from cells to a point where their contained solutions will not form ice when taken to the final cryogen temperature (KARTHA and ENGELMANN 1994; GROUT 1995; BLAKESLEY, PASK, HENSHAW and FAY 1996).

Tissues with a naturally low water content, such as pollen and 'orthodox' seeds, can readily be cryopreserved without lethal ice crystal formation (BLAKESLEY, PASK, HENSHAW and FAY 1996). Cryopreservation of cell cultures is easier to accomplish compared to organised structures such as meristems where structural conformity has to be maintained in order to ensure faithful regeneration of plantlets (KARTHA and ENGELMANN 1994). Highly vacuolated and quiescent cells are extremely sensitive to cryoprotectant treatment and freezing, and such cells are naturally eliminated during the specimen preparation stage (KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988).

Ice crystals also have potentially lethal, disruptive effects when in an extracellular situation, such as outside tissues or between the cells of a tissue or organ. These include:

- Mechanical stresses on material entrapped in the channels between crystals, causing deformations and tissue damage (GROUT 1991a);
- Ice formed between cell walls and the shrinking protoplast that can adhere to the membrane surface generating adhesion energies sufficiently strong to result in

lysis (TANDORF, McGRATH and OLIEN 1987; GROUT 1991a);

- Electrical interactions at membrane surfaces close to ice fronts (the Workman-Reynolds effect) resulting from the different solubilities of ions in the ice and liquid phases. This leads to perturbation of biological membranes (STEPONKUS 1984); and
- Intracellular gas bubble formation that may occur under a number of circumstances and can be observed microscopically during cooling and warming of isolated protoplasts and unicellular algae (GROUT 1991b). Chemical injuries related to liquid peroxidation and localised pH changes have been proposed as causes of "bubble-related" contact injuries (MORRIS and McGRATH 1981).

Cold hardening or cold acclimation enables plants to withstand severe freezing stress. Hardy plants undergo a series of metabolic and physiological changes during cold acclimation (KARTHA and ENGELMANN 1994). It has been suggested that the preferential synthesis of metabolites such as proteins, sugars and nucleic acids is induced by low temperatures during cold hardening (BROWN 1978; KACPERSKA-PALACZ 1978; LEVITT 1980). Cold acclimation can be exploited in two possible ways in the context of cryopreservation. The first involves utilisation of explants from plants which are naturally acclimated; and the second is to artificially induce cold acclimation in plants that are capable of doing so, and to use such material for cryopreservation (KARTHA and ENGELMANN 1994). Cold acclimation is influenced by seasonal changes, thus explants isolated from their donor plants around late autumn and winter would have a better chance at cryosurvival than explants isolated during the warmer seasons. While cold acclimation is an excellent way of enhancing cryosurvival, its application is limited to those species that have the trait for low temperature acclimation. It is doubtful if tropical species would respond in a similar way (KARTHA and ENGELMANN 1994).

2.3. Vitrification

The other form of cryopreservation used is vitrification. Vitrification is the phase transition of water from a liquid, directly into a crystalline or amorphous phase, a glass,

by an extreme elevation in viscosity of cytoplasmic and extracellular solutions during cooling (FAHY, McFARLANE, ANGELL and MERYMAN 1984; MERYMAN and WILLIAMS 1985). It is not to be confused with the term that describes organs and tissues that have an abnormal morphological appearance and physiological function, which has been renamed 'hyperhydricity' (DEBERGH, AITKEN-CHRISTIE, COHEN, GROUT, VON ARNOLD, ZIMMERMAN and ZIV 1992). The conversion of cytoplasmic and extracellular solutions to a glass by vitrification following cooling avoids the damaging stresses associated with crystalline ice formation and cryodehydration , and so increases viability following recovery from the cryogen (GROUT 1995). Successful recovery of vitrified material is also dependant on rapid thawing, as this ensures that the glass returns to an aqueous solution without recrystallisation (FAHY 1987; McFARLANE and FORSYTH 1987; GROUT 1991a; FRETZ, JÄHNE and LÖRZ 1992).

Excised meristems, small shoot tips, somatic embryos, protoplasts and cells in suspension are all suited to this technique (GROUT 1995). As with slow cooling, the concentration of the cellular solutes must be manipulated. This can be achieved by:

- Incubating the tissues in a hypertonic solution of cryoprotectants at non-freezing temperatures, drawing water from the cells and raising the glass transition temperature, so that a point is reached at which transition occurs before ice nucleation; or
- The explants may be encapsulated in alginate beads prior to vitrification. Water is removed from the system by controlled air drying, thereby increasing the concentration of solutes in the intracellular solutions.

These methods should increase the concentration of the intracellular solutes , without sacrificing viability, to the point where direct immersion in liquid nitrogen will bring about vitrification of both extracellular and intracellular solutions (KARTHA and ENGELMANN 1994; GROUT 1995).

KARTHA and ENGELMANN (1994) state that there are advantages and disadvantages to vitrification. The main advantages are that it is a simple process, and it does not require regulated cooling so that the need for expensive programmable freezing equipment is eliminated. The main disadvantage is that the highly concentrated levels

of many cryoprotective agents required for vitrification may cause extreme toxicity to cells. Toxicity of the vitrification solution is relevant to its osmotic potential and therefore formulation of vitrification solutions, differing in osmotic potential, may alleviate the problem of toxicity (LANGIS and STEPONKUS 1990).

2.4. Cryoprotectants

To protect against the desiccation injuries resulting from cryodehydration, storage and thawing, a range of chemical cryoprotectants are used as additives to the extracellular medium, and many of these are low molecular weight permeating agents (KARTHA and ENGELMANN 1994; MERYMAN and WILLIAMS 1985). Some of the most commonly and successfully used cryoprotectants are: dimethylsulfoxide (DMSO), glycerol, methanol, polyvinylpyrolidone, ethylene glycol, polyethylene glycol (PEG), hydroxyethyl starch (HES), sugars and sugar alcohols (FINKLE, ZAVALA and ULRICH 1985; FRETZ, JÄHNE and LÖRZ 1992; KARTHA and ENGELMANN 1994; GROUT 1995; McLELLAN and DAY 1995; REINHOUD, URAGAMI, SUKAI and VAN IREN 1995). These compounds are used alone or in combination to afford protection to living cells during freezing and thawing (KARTHA and ENGELMANN 1994).

Dimethylsulfoxide (DMSO) has become a universal cryoprotectant, used in the long-term storage of animal and plant material (FINKLE, ZAVALA and ULRICH 1985). Although it is more toxic than glycerol, DMSO is a far superior permeating additive as it permeates the cells more rapidly than glycerol and thus requires a shorter treatment duration (KARTHA and ENGELMANN 1994). When a cryoprotective compound is able to penetrate the cells, the resulting decrease in the equilibrium concentration of salts then applies to the constituent salts or other potentially toxic compounds within the cell as well as outside the cell. If enough protective compound is present, the salt concentration does not rise to a critically damaging level until the temperature becomes so low that the damaging reactions are slow enough to be tolerated by the cells (FINKLE, ZAVALA and ULRICH 1985). Non-penetrating compounds may act by dehydrating the cell by osmotic action (TOWILL and MAZUR 1976). Dimethylsulfoxide has been implicated in generating genetic and / or epigenetic changes in higher plants

(HERVÁS and GIMÉNEZ-MARTIN 1973; IHRKE and KRONSTAD 1975; BOSE and BASU 1977). However, DMSO is often applied only at ice temperature for a short interval, then chilled to and stored at very low temperatures where both physical and chemical reaction rates are generally so slow that all interactions essentially stop (FINKLE, ZAVALA and ULRICH 1985).

WITHERS (1980) found that cryoprotectants generally work well in the 5 to 20 % range. Generally a concentration of 5 - 10 % DMSO and 10 - 20 % glycerol provides adequate protection without becoming too cytotoxic. ULRICH, FINKLE, MOORE and GINOZA (1979) successfully cryopreserved sugar cane callus using a combination of PEG, glucose and DMSO (10-8-10 %). This combination has successfully been used to cryopreserve other cell or callus lines (FINKLE, ZAVALA and ULRICH 1985), including *Ulmas americana* (ULRICH, MICKLER, FINKLE and KARNOSKY 1984), and *Picea abies* and *Pinus taeda* (GUPTA, DURZAN and FINKLE 1987). A summary of forest species explants, cryoprotectants used and freezing procedure is outlined in Table 2.

Cryoprotectants act by reducing the water content of the cell, which in turn, reduces the effective ion/solute concentrations that can occur as a result of cryodehydration (GROUT 1995). When combined with slow cooling, this has the beneficial effect of reducing the amount of cell shrinkage that will occur at any given temperature following extracellular freezing, thus reducing mechanical injuries. If, however, the hypertonicity of the residual solution is too great, potentially lethal effects result. These include:

- Irreversible surface area reduction of the limiting cell membrane following osmotic shrinkage (STEPONKUS 1984; GROUT and MORRIS 1987);
- An increase in the concentration of the cytoplasmic solutes; and
- Osmotic shrinkage of organelles.

Although opinions differ as to the method of application of cryoprotectants to the cells, it is preferable to apply them very gradually in an ice bath at 4°C in order to minimise injury. Cryoprotectants are generally added slowly to the tissues to avoid an osmotic shocking effect and a damaging excess of pressure across the cell membrane (FINKLE, ZAVALA and ULRICH 1985). Similarly, the removal of cryoprotectants from

the thawed samples should also be a gradual process to alleviate the problems associated with deplasmosis (KARTHA and ENGELMANN 1994). The temperatures of addition and removal of cryoprotective solutions can also affect survival and/or cell ultrastructure. This is dependant on species and tissue type (FINKLE, ZAVALA and ULRICH 1985).

2.5. Thawing

When thawing samples, it is very important that recrystallisation and ice crystal growth are avoided. If thawing is carried out at slow rates, structural changes may occur that may or may not affect cell viability (KARTHA and ENGELMANN 1994). Thus, samples are defrosted rapidly in a water bath at 35-40°C for 1-2 minutes.

Two different techniques are employed in the removal of the cryoprotectants post thaw. The first technique involves gradually washing the cells with chilled nutrient medium prior to their return to liquid or solid culture. However, this technique is deleterious in a number of cultures (KARTHA and ENGELMANN 1994). The other technique involves plating the thawed cells onto filter paper placed over regrowth medium for a specified period of time to facilitate slow diffusion of cryoprotectants. The filter paper can then easily be moved to two fresh changes of nutrient medium.

To determine viability of the recovered tissue, the tissue may be stained with fluoresceine diacetate (FDA) or triphenyltetrazolium chloride (TTC) reduction assays (KARTHA and ENGLEMANN 1994). The FDA stain is taken up by living cells and fluoresces under UV light (WIDHOLM 1972). In the TTC method (STEPONKUS and LANPHEAR 1967) determination of survival is based on the reduction of TTC by the mitochondria of viable cells, to form formazan, a water insoluble red compound. This reduction is considered to be quantitative and the formazan is made soluble by addition of ethanol. The supernatant can thus be examined spectrophotometrically.

The most convincing test for viability of recovered tissue would be their ability to regrow upon return to culture without exhibiting a lag phase, but rather showing identical growth

characteristics as the original culture. A long lag phase is a direct reflection of freezing injury resulting from the use of sub-optimal freezing conditions (KARTHA and ENGELMANN 1994). A low rate of survival following cryopreservation may be an indication that cryoselection is in operation.

Table 2. Summary of the protocols used to cryopreserve important forest tree species. Unless otherwise stated, tissue was plunged into liquid nitrogen after the slow cooling process.

Species	Explant	Cryoprotectants	Freezing	Plantlet regeneration	References
Abies alba	Seeds	None	Taken directly to -196°C	Yes	JÖRGENSEN (1990)
Abies nordmanniana	Embryogenic cultures	5 % DMSO and 0.4 M sorbitol	Slow cooled to -35°C	No	NØRGAARD *et al.* (1993)
Betula pendula	Shoot tips	10 % PEG, 10 % glucose and 10 % DMSO	Slow cooled to -38°C	Yes	RYYNÄNEN (1996)
Eucalyptus sp.	Meristems	Encapsulated in algenate beads; 1 M sucrose	Taken directly to -196°C	Yes	PAQUES *et al.* (1997)
Fraxinus excelsior	Zygotic embryos	0.4 M sucrose; dehydration	Taken directly to -196°C	Not reported	BREARLY *et al.* (1995)
Larix decidua	Seeds	None	Taken directly to -196°C	Yes	JÖRGENSEN (1990)
Larix x *eurolepis*	Embryogenic cultures	10 % DMSO and 0.4 M sorbitol	Slow cooled to -40°C	Yes	KLIMASZEWSKA *et al.* (1992)
Picea abies	Embryogenic cultures	10 % PEG, 8 % glucose and 10 % DMSO	Slow cooled to -30°C	No	GUPTA *et al.* (1987)

Species	Explant	Cryoprotectants	Freezing	Plantlet regeneration	References
Picea abies	Embryogenic tissue	5 or 7.5 % DMSO and 0.5 M sucrose	Slow cooled to -40°C	No	GALERNE and DEREUDDRE (1988)
Picea abies	Seeds	None	Taken directly to -196°C	Yes	JÖRGENSEN (1990)
Picea abies	Pollen	Desiccated over silica gel	Pre-cooled to -18°C	Not reported	LANTERI *et al.* (1993)
Picea glauca	Somatic embryos	5 % DMSO and 0.4 M sorbitol	Slow cooled to -35°C	Yes	KARTHA *et al.* (1988)
Picea glauca	Embryonic cotyledons	0.4 M sorbitol, 5 % DMSO and 2 % sucrose	Slow cooled to -40°C	Yes	TOIVONEN and KARTHA (1989)
Picea glauca engelmanni complex	Embryogenic cultures	5 % DMSO and 0.4 M sorbitol	Slow cooled to -35°C	No	CYR *et al.* (1994)
Picea mariana	Embryogenic cultures	10 % DMSO and 0.4 M sorbitol	Slow cooled to -40°C	Yes	KLIMASZEWSKA *et al.* (1992)
Picea sitchensis	Embryogenic suspension cultures	5 % DMSO and 0.4 M sorbitol	Slow cooled to -40°C	Yes	FIND *et al.* (1993)
Pinus canariensis	Seeds	None	Taken directly to -196°C	Yes	PITA *et al.* (1998)

Species	Explant	Cryoprotectants	Freezing	Plantlet regeneration	References
Pinus caribaea	Embryogenic cell suspensions	5 % DMSO and 0.4 M sorbitol	Slow cooled to -35°C	Yes	LAINÉ *et al.* (1992)
Pinus halepensis	Seeds	None	Taken directly to -196°C	Yes	PITA *et al.* (1998)
Pinus nigra	Pollen	Desiccated over silica gel	Pre-cooled to -18°C	Not reported	LANTERI *et al.* (1993)
Pinus nigra	Seeds	Desiccated over silica gel	Taken directly to -196°C	Yes	PITA *et al.* (1997)
Pinus nigra	Seeds	None	Taken directly to -196°C	Yes	PITA *et al.* (1998)
Pinus pinea	Pollen	Desiccated over silica gel	Pre-cooled to -18°C	Not reported	LANTERI *et al.* (1993)
Pinus pinea	Seeds	None	Taken directly to -196°C	Yes	PITA *et al.* (1998)
Pinus radiata	Embryogenic cultures	10 % DMSO and 0.4 M sorbitol	Slow cooled to -80°C	Yes	HARGREAVES *et al.* (1995)
Pinus strobus	Pollen	Desiccated over silica gel	Pre-cooled to -18°C	Not reported	LANTERI *et al.* (1993)
Pinus sylvestris	Seeds	None	Taken directly to -196°C	Yes	JÖRGENSEN (1990)

Species	Explant	Cryoprotectants	Freezing	Plantlet regeneration	References
Pinus sylvestris	Pollen	Desiccated over silica gel	Pre-cooled to -18°C	Not reported	LANTERI *et al.* (1993)
Pinus sylvestris	Buds	None	Slow cooled to -39°C, stored at -80°C	Not reported	KUOKSA and HOHTOLA (1991)
Pinus sylvestris	Seeds	Desiccated over silica gel	Taken directly to -196°C	Yes	PITA *et al.* (1997)
Pinus sylvestris	Embryogenic cultures	10 % PEG, 10 % glucose and 10 % DMSO	Alow cooled to -38°C	No	HÄGGMANN *et al.* (1998)
Pinus sylvestris	Seeds	None	Taken directly to -196°C	Yes	PITA *et al.* (1998)
Pinus taeda	Embryogenic cultures	10 % PEG, 8 % glucose and 10 % DMSO	Slow cooled to -30°C	No	GUPTA *et al.* (1987)
Pinus uncinata	Pollen	Desiccated over silica gel	Pre-cooled to -18°C	Not reported	LANTERI *et al.* (1993)
Pinus uncinata	Seeds	None	Taken directly to -196°C	Yes	PITA *et al.* (1998)
Pseudotsuga menziesii	Suspension culture	5 % DMSO, 1 % glycerol and 4 % sucrose	Slow cooled to -40°C	Not reported	BINDER and ZAERR (1980)

Species	Explant	Cryoprotectants	Freezing	Plantlet regeneration	References
Pseudotsuga menziesii	Seeds	None	Taken directly to -196°C	Yes	JÖRGENSEN (1990)
Ulmus sp.	Dormant meristems	Encapsulated in calcium alginate beads; 1 M sucrose	Slow cooled to -40°C	Yes	PAQUES *et al.* (1997)
Ulmus americana	Callus culture	10 % PEG, 8 % glucose and 10 % DMSO	Slow cooled to -30°C	Yes	ULRICH *et al.* (1984)
Quercus faginea	Embryonic axes	Desiccated, or pre-treated in 15 % DMSO	Taken directly to -196°C	Not reported	GONZALEZ-BENITO and PEREZ-RUIZ (1992)
Quercus petraea	Embryos	10 % DMSO and 5 % sucrose	Slow cooled to -40 or -50°C	Yes	JÖRGENSEN (1990)
Quercus robur	Embryonic axes	Encapsulated in calcium alginate beads, cryoprotectants *not reported*	Slow cooled to -20°C	Yes	CHMIELARZ *et al.* (1995)

CHAPTER 3

PLANT MATERIAL AND INDUCTION OF EMBRYOGENESIS

3.1. Introduction

Somatic embryogenesis and plantlet regeneration was first reported in *Picea abies*, for which zygotic embryos were used as explants to establish the cultures (CHALUPA 1985; HAKMAN, FOWKE, VON ARNOLD and ERIKSSON 1985). Immature and mature zygotic embryos were cultured on a modified MURASHIGE and SKOOG (1962) medium supplemented with the growth regulators 2,4-D and BA, with the immature embryos producing the most embryogenic tissue (TAUTORUS, FOWKE and DUNSTAN 1991). Since then a number of explants have been used to induce somatic embryogenesis in conifers (Table 1). Somatic embryos consist of long vacuolated suspensor-like cells subtending dense meristematic cells of the embryonal axis (HAKMAN, FOWKE, VON ARNOLD and ERIKSSON 1985). This has been termed a callus by many authors; however, because this tissue is composed of organised structures, the term embryogenic tissue is more appropriate (TAUTORUS, FOWKE and DUNSTAN 1991).

Somatic embryogenesis in conifers involves a reactivation of much of the developmental programme of normal embryogeny. Proliferating embryogenic tissues consist primarily of immature somatic embryos, which resemble immature zygotic embryos. These immature somatic embryos continuously initiate embryos by cleavage polyembryogenesis (ATTREE and FOWKE 1993). JONES, VAN STADEN and BAYLEY (1993) found that the combination of genotypic responses to growth regulators and the developmental stage of the explant play an important role in the induction of

embryogenic tissue in *P. patula*.

Correct media formulation is important in establishing embryogenic cultures. Some of the critical components include a suitable nitrogen source, usually in the form of L-glutamine as has been shown for numerous conifer species (SMITH 1995). Furthermore, an adequate carbon and energy source is required. Sucrose is one of the most abundant and important disaccharides. This disaccharide is composed of α-D-glucose and β-D-fructose linked via a α,β (1→2) glycosidic link (BOHINSKI 1987). When organisms require a carbon and an energy source, sucrose is hydrolysed to glucose and fructose, which enter the mainstream of metabolism. In tissue culture, sucrose and glucose are commonly used as the carbohydrate source, although nearly all cultures preform better on the disaccharide, sucrose (DODDS and ROBERTS 1985). However, current research has shown that maltose (comprising two glucose monomers linked together to form a homogenous disaccharide (BOHINSKI 1987)) is a superior carbohydrate source in the culture of conifer somatic embryos (GUPTA 1996). This brief investigation served to determine which carbohydrate source proved to be the most beneficial for *P. patula* embryogenic tissue maintenance.

3.2. Materials and Methods

3.2.1. Initiation of Embryogenic Tissue

Green female cones were collected from four selected *P. patula* families on a weekly basis. The material was supplied by Sappi Forests Research, from the grafted seed orchard situated at Tweedie in the Natal midlands (29°29' S 30°11' E). The area lies 1100 m above sea level. The collection periods were during the South African summer months from 17 November 1996 to 26 February 1997, and from 7 December 1997 to 19 January 1998. The collected cones were transported in brown paper bags and maintained at 4°C until the seeds were placed. All cones were placed into culture within 4 days of being collected. Explants from 4 genotypes, selected from two 2 different families, were placed into culture for initiation.

The cones were washed briefly in running water before being immersed into 75% ethanol (containing a few drops of Tween® 20) for 4-5 min. They were then transferred to 1.3% NaOCl (containing a few drops of Tween® 20) for 20 min before being rinsed several times in sterile distilled water. The seeds were extracted aseptically and the female gametophyte (containing the immature zygotic embryo) excised microscopically, and used as the explant source.

The explants were placed onto two different media for embryogenic induction. The first was MSG3 (BECWAR, NAGMANI and WANN 1990), a modification of MS (MURASHIGE and SKOOG 1962) in which the NH_4NO_3 was replaced by L-glutamine and the KNO_3 level reduced. The second was Douglas-fir Cotyledon Revised medium, DCR1 (GUPTA and DURZAN 1985) which also contained L-glutamine. The components of the two media are outlined in Table 3.

The pH of the medium was adjusted to 5.8 prior to autoclaving. The pH of the L-glutamine was also adjusted to 5.8 and filter sterilised prior to being added to the warm medium. Approximately 15 ml of medium was poured into 65 mm plastic petri dishes (Labotec). Dishes containing cultures were sealed with plastic cling wrap (Glad® Wrap). Cultures were kept in the dark at 25°C, and were monitored weekly for embryogenic activity (JONES, VAN STADEN and BAYLEY 1993; JONES 1994; JONES and VAN STADEN 1995).

3.2.2. Tissue Maintenance

Once embryogenic tissue had been induced, all the tissue was subcultured onto MSG3 maintenance medium every fortnight, irrespective of the medium on which the tissue was initiated. Maintenance medium consisted of the same components as the initiation medium (Table 3) but the carbohydrate source was changed to 30 g l^{-1} maltose (Associated Chemical Enterprises). The pH of the maltose was adjusted to 5.8 and autoclaved separately before being added to the medium, to prevent caramelisation of the sugar.

Table 3. Formulation of MSG and DCR basal media according to BECWAR, NAGMANI and WANN (1990).

Inorganic Compounds	MSG3 (mg l^{-1})	DCR1 (mg l^{-1})
NH_4NO_3	-	400.0
KNO_3	100.0	340.0
$Ca(NO_3)_2.4H_2O$	-	556.0
$MgSO_4.7H_2O$	370.0	370.0
KH_2PO_4	170.0	170.0
$CaCl_2.2H_2O$	440.0	85.0
KCl	745.0	-
KI	0.83	0.83
H_3BO_3	6.2	6.2
$MnSO_4.H_2O$	16.9	22.3
$ZnSO_4.7H_2O$	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25
$CoCl_2.6H_2O$	0.025	0.025
$NiCl_2.6H_2O$	-	0.025
$FeSO_4.7H_20$	27.8	27.8
Na_2EDTA	37.3	37.3
Vitamins and Amino Acids (mg l^{-1})		
Nicotinic acid	0.5	0.5
Pyridoxine-HCl	0.1	0.5
Thiamine-HCl	0.1	1.0
Glycine	-	2.0
Supplements (g l^{-1})		
Myo-inositol	0.1	0.2
Casein Hydrolysate	-	0.5
L-glutamine	1.5	0.25
Plant growth regulators (mg l^{-1})		
2,4-D	2.0	3.0
BA	1.0	0.5
Carbohydrate and Gelling Agent (g l^{-1})		
Sucrose	30.0	30.0
Gelrite®	3.0	3.0

3.2.3. Suspension Cultures

Embryogenic tissue was taken from solid MSG3 maintenance medium and introduced into MSG3 liquid medium. The principle components remained the same (Table 3), but 30 g l^{-1} sucrose was used instead of maltose and Gelrite was omitted. Embryogenic suspension cultures were initiated by inoculating 100-200 mg of tissue into 35 ml of liquid medium in 100 ml Erlenmeyer flasks. The flasks were sealed with a cotton wool bung and covered with tin foil. The flasks were placed on a rotary shaker at 120 rpm and maintained in the dark at 25°C. The suspensions were subcultured approximately every two weeks. This technique was used to bulk tissue up for experimentation.

3.2.4. The Effect of Various Carbohydrate Sources on Tissue Maintenance

Embryogenic tissue from line G1 was bulked up using suspension culture, as described above. One ml aliquots of the liquid culture were then pipetted onto Whatman No.1 filter paper and the supernatant was drawn off using a vacuum. The tissue was then placed onto solid MSG3 medium supplemented with 0.1 M of one of the following sugars: fructose, galactose, glucose, lactose, maltose, sucrose and trehalose. The sugars were autoclaved separately and added to the warm medium prior to pouring.

3.2.5. Anatomical Studies

Embryogenic tissue was stained with 0.5 % acetocarmine and viewed under a light microscope (Olympus BH-2) to record the growth and development of the embryos.

3.2.6. Data Collection

Tissue samples were weighed every 3 days for 15 days and the percent weight increase (Wi) was determined as follows (LAINÉ, BADE and DAVID 1992):

$$Wi = 100 \times \frac{\text{weight at day i - weight at day 0)}}{\text{weight at day 0}}$$

Day i was the number of days after commencement of the experiment.

3.2.7. Data Analysis

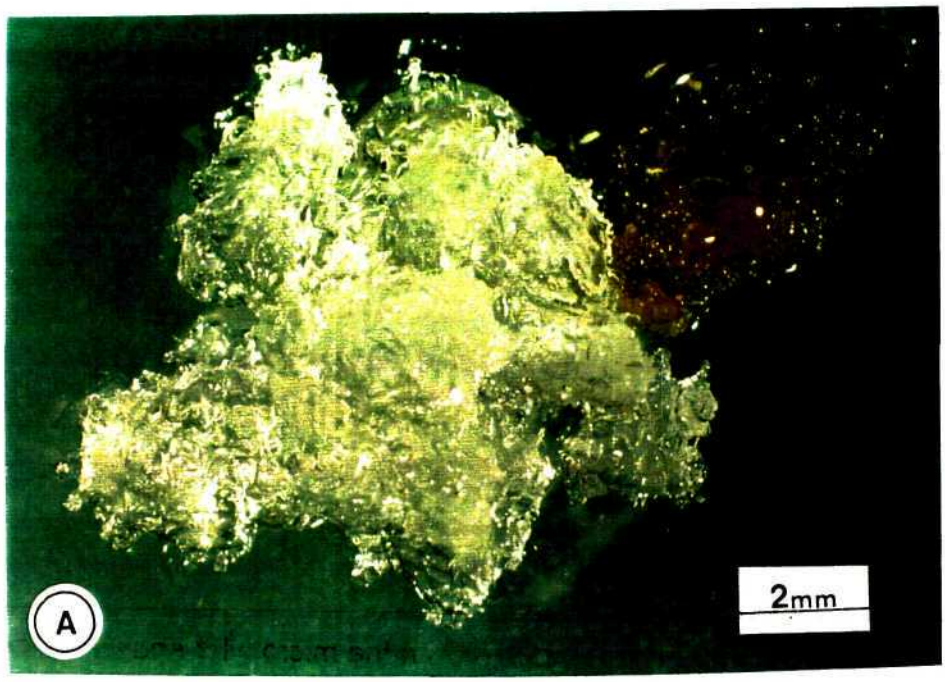
There were 10 replicates per sample for each of the sugars examined. The data collected were analysed using the Quattro Pro programme to determine the standard error (SE). A one-way Analysis of Variance (ANOVA) was conducted on the data using the Minitab statistical programme. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

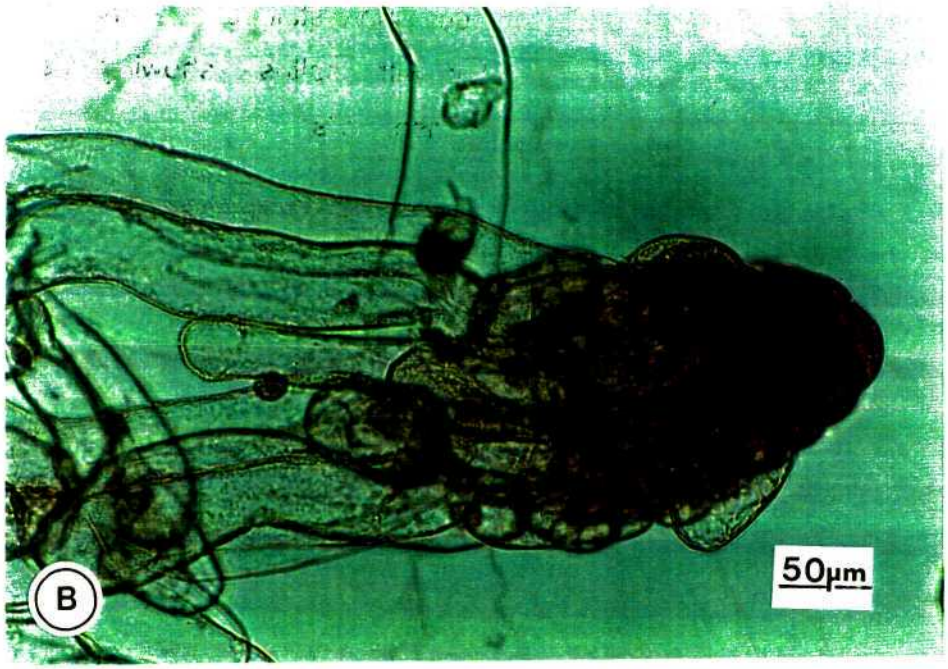
3.3. Results

After 4-6 weeks of culture, translucent embryogenic tissue was extruded from the micropylar region of the female gametophyte (Figure 3A). This tissue continued to proliferate normally when placed onto maintenance medium containing maltose. Placement of the tissue into liquid medium resulted in embryo singulation and rapid proliferation. Samples of tissue were stained with 0.5 % acetocarmine (Figure 3B) to observe the structure of the embryos more closely. The dense cytoplasm of the embryonal apex was stained red by the dye. The elongated suspensor cells subtend from the embryonal axis, giving the embryogenic tissue its translucent, mucilaginous appearance.

The growth of the embryogenic tissue on MSG3 medium supplemented with different sugars (Figure 4) showed that the best form of carbohydrate for tissue proliferation was maltose. Large, healthy masses of tissue proliferated vigorously and covered the entire surface of the filter paper. Although there was no significant difference ($p<0.05$) between the tissue grown on maltose (which is composed of two glucose monomers) and the tissue grown on glucose alone, visual observations of the tissue grown on the two media indicate that maltose was the superior carbohydrate source. When compared to the tissue grown on the other carbohydrate sources, the tissue grown on maltose showed significantly better growth ($p<0.05$). Cultures grown on sucrose (comprised of α-D-glucose and β-D-fructose monomers) and fructose initially proliferated healthy translucent tissue that covered the entire surface of the filter paper.

Figure 3. (A) Induction of embryogenic tissue from the micropylar end of an isolated female gametophyte of *P. patula*. (B) Acetocarmine stain of a somatic embryo maintained on MSG3 medium supplemented with maltose, showing the dense embryonal head subtended by elongated suspensor cells.


A
2mm


B
50µm

However, towards the end of the treatment period the tissue had begun to senesce slightly from the central core of the tissue. Tissue cultured on lactose (comprised of β-D-galactose and D-glucose monomers) turned yellow from the beginning and lost its characteristic semi-translucent appearance. The tissue cultured on the lactose containing medium proliferated poorly and exhibited white stress spots. The tissue grown on galactose also performed very poorly. Although the tissue appeared to proliferate and was semi-translucent and mucilaginous, the areas in contact with the filter paper showed signs of browning. Cleavage polyembryony seemed to have slowed considerably, as the tissue did not take over the entire surface of the filter paper as had been observed in the tissue treated with other sugars. The tissue cultured on trehalose (composed of 2 α-D-glucose monomers) showed stunted growth and white stress spots. The tissue had a dry appearance, possibly as a result of stress.

The tissue grown on maltose was still undergoing sigmoidal growth by day 15, suggesting that this carbohydrate had not become limiting and could sustain further growth. The decline in growth observed in tissue grown on sucrose and fructose may have been due to the carbohydrates becoming a limiting factor.

3.4. Discussion

Successful embryogenic induction was observed from gametophytic explants containing immature zygotic embryos of *P. patula*. ATTREE and FOWKE (1993) state that for the conifers, immature zygotic embryos yield somatic embryos more readily than mature zygotic embryos, which in turn are more responsive than explants from young seedlings. The genus *Pinus* is embryogenically responsive at a very early developmental stage of the zygotic embryo (i.e. at the stage of polyembryony) (GUPTA and DURZAN 1987; LAINÉ and DAVID 1990; JONES 1994). VON ARNOLD and HAKMAN (1988b) described the ways in which embryogenic cultures proliferate in *Picea abies* as: (1) somatic embryos can arise by a mechanism similar to cleavage polyembryogenesis, with the initial separation occurring in the embryogenic region. (2) Somatic embryos can develop from small meristematic cells within the suspensor. These initials could arise by asymmetric division of suspensor cells or from

Figure 4. Growth of *P. patula* embryogenic tissue on MSG3 maintenance medium supplemented with a variety of sugars (mean ± SE). Bar indicates Least Significant Difference (LSD) at the 95% confidence interval.

meristematic cells of the embryogenic region that have failed to elongate while being integrated into the suspensor. (3) Somatic embryos can arise from single cells or small cell aggregates by an initial asymmetric division that delimits the embryogenic region and suspensor region. JONES, VAN STADEN and BAYLEY (1993) found that initiation of embryogenic tissue of *P. patula* in culture was a direct result of cleavage polyembryony. Somatic embryo proliferation in liquid also appears to occur via cleavage polyembryony, and agitation helps to separate the developing embryos (JONES 1994).

GUPTA (1996) cultured Douglas fir embryogenic tissue in media supplemented with sucrose and maltose, and concluded that maltose was preferred over sucrose as a carbohydrate source. The advanced early stage embryos, produced using maltose in the maintenance medium, had significantly larger embryonal heads than those produced using sucrose. The associated suspensor cells were more elongated, resulting in more robust embryos and cotyledonary embryos. Staining of embryogenic tissue with acetocarmine showed that the *P. patula* tissue proliferated on maltose-containing medium also produced more robust embryos, compared with any of the other sugars tested (Figure 4). GUPTA and PULLMAN (1991) suggested that sucrose, glucose, fructose, maltose or galactose were metabolised and suitable for osmotic potential control in cotyledonary embryo development media. In *Daucus carota* several mono-, as well as di- and tri- saccharides supported embryogenesis, although fructose was found to be inhibitory if autoclaved with other media components (VERMA and DOUGALL 1977). Galactose, raffinose and stachyose only supported growth and embryogenesis after a marked delay (THOMPSON and THORPE 1987). Sucrose is the major transport and (to some extent) storage carbohydrate of most plants, so it is not really surprising that it is the most commonly used carbohydrate source in plant tissue culture (THOMPSON and THORPE 1987). Sucrose is hydrolysed by the enzyme invertase into glucose and fructose. The two monomers then enter glycolysis which degrades them to pyruvic acid in the cytosol (SALISBURY and ROSS 1992). The disaccharide is hydrolysed to its two glucose monomers by the enzyme maltase (SALISBURY and ROSS 1992). Trehalose is composed of 2 α-D-glucose molecules, which are then hydrolysed into D-glucose by the enzyme trehalase. This enzyme has been found in the pollen of several species of higher plants. In general, trehalose

mainly occurs in fungi, blue-green and red algae, the pteridophytes *Selaginella* spp. (where trehalase is used as the main translocator of fixed carbon and energy) and *Botrychium lunaria* and the spermatophytes *Echinops persicus*, *Carex brunescence* and *Fagas silvatica* (GOODWIN and MERCER 1983). A small amount of the sugar could have hydrolysed during autoclaving, allowing the embryogenic tissue to access limited amounts of glucose.

TREMBLAY and TREMBLAY (1995) found that the hydrolysis of sucrose in the medium occurred only in the presence of embryogenic tissues and continued after the tissue was removed from the medium. The enzymatic system was not inhibited by the very high concentrations of sucrose sometimes present in the maturation medium. Other studies reported that the optimum level of sucrose in the medium was in excess of the requirements for tissue growth, and relatively large amounts of sucrose and its monosaccharides accumulated in the medium (THORPE and MEIER 1973; THORPE 1974).

The use of maltose in the maintenance of *P. patula* embryogenic tissue maintenance was found to be beneficial. Although sucrose was successfully used in the initiation medium, tissue maintained on it was found to be slightly inferior to the tissue maintained on maltose. Tissue cultured on glucose (the basic monomers of maltose) did not proliferate as vigorously as the tissue cultured on the maltose. NAGMANI, DINER and WANN (1993) found that embryogenic tissue induction in *Pinus palustris* was equally as effective on media supplemented with glucose, maltose or sucrose, although slightly higher induction was observed in explants cultured on maltose or sucrose.

CHAPTER 4

SORBITOL AND DIMETHYLSULFOXIDE AS CRYOPROTECTANTS

4.1. Introduction

Pre-culture of cells in medium enriched with an osmoticum such as sorbitol, sucrose or proline has been shown to enhance freezing tolerance (WITHERS and STREET 1977; WITHERS and KING 1979; KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988). Sorbitol is a widely used prefreezing additive (WITHERS 1986). It operates a combined dehydrative and physiological effect upon individual cells, rather than by providing an environment within which cells develop a higher degree of freeze tolerance (WITHERS 1985).

It is believed that DMSO can lower the temperature at which freezing first occurs and can alter the crystal habit of ice when it separates (KLIMASZEWSKA, WARD and CHELIAK 1992). There appears to be a synergistic protective effect when DMSO and sorbitol are used in combination (KLIMASZEWSKA, WARD and CHELIAK 1992), as sorbitol tends to reduce the toxic effect of DMSO as a cryoprotectant (CHEN, KARTHA, CONSTABEL and GUSTA 1984b; CHEN and KARTHA 1987).

Dimethylsulfoxide and sorbitol are commonly used cryoprotectants in the cryopreservation of conifer species (Table 2). This chapter reports the successful determination of a protocol for the long-term storage of *Pinus patula* embryogenic tissue using sorbitol and DMSO as cryoprotectants.

4.2. Materials and Methods

4.2.1. Determination of Sorbitol and DMSO Concentrations

Embryogenic tissue was bulked up using suspension cultures as described in Chapter 3. The tissue was then plated out onto MSG3 maintenance medium and allowed to proliferate normally. For the experiment, 1 g of healthy, vigorously proliferating tissue was placed into 6 ml of MSG3 liquid medium supplemented with 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, or 0.6 M sorbitol in 25 ml Erlenmeyer flasks. The cell clusters were gently broken up using a micropipette. The tissue was agitated on a rotary shaker for 24 h at 120 rpm. One ml aliquots of the suspension were pipetted onto polyester grids. The liquid was then drawn off using a vacuum, and the cells remaining on the polyester grid were transferred onto fresh solid MSG3 medium for 24 h. The tissue was then subcultured onto fresh MSG3 medium after 7 d and then every two weeks. The weight of the tissue was taken after 24 h and then every 7 d for 5 weeks.

Four different concentrations of DMSO were also tested on the tissue. As above, tissue was bulked up using suspension cultures and plated out onto solid MSG3 medium to proliferate. One g of healthy proliferating tissue was placed into 3 ml of liquid MSG3 medium in 25 ml Erlenmeyer flasks on a rotary shaker for 24 h. The flasks were removed from the shaker and placed onto ice for 15 min. MSG3 medium with 10 %, 20 %, 30 % and 40 % DMSO (v/v) was added to produce a final volume of 6 ml and a final concentration of 5 %, 10 %, 15 % and 20 % DMSO. The cell clusters were gently broken up using a micropipette. The cultures were allowed to equilibrate for a further 20 min before 1 ml aliquots were pipetted from the flask onto polyester grids. The supernatant was drawn off using a vacuum and the cells were transferred onto fresh MSG3 solid medium. After 24 h the grids were moved onto fresh MSG3 medium. The tissue was subcultured after 7 d and then every two weeks. The tissue was weighed after 24 h and then every 7 d.

4.2.2. Concentration Grid of DMSO and Sorbitol

One g of healthy tissue was taken from solid MSG3 maintenance medium and placed into 3 ml of liquid MSG3 medium supplemented with 0.3 M or 0.4 M sorbitol in a 25 ml Erlenmeyer flask. The flasks were wrapped in aluminium foil and placed onto a rotary shaker at 120 rpm for 24 h at 25°C. The flasks were then placed onto ice and allowed to equilibrate for 20 min. Three ml of MSG3 liquid medium supplemented with 10 % or 20 % DMSO were then added to the tissue, to give a final concentration of 5 % and 10 % DMSO. This produced a final concentration grid of 0.3 M sorbitol and 5 % or 10 % DMSO; or 0.4 M sorbitol and 5 % or 10 % DMSO. The cell clusters were gently broken apart using a micropipette, and 1 ml samples were pipetted into 1.8 ml Nunc cryovials. The cryovials were then placed into a Nalgene™ Cryo 1°C freezing container filled with isopropyl alcohol (Figure 7A). The container was placed into a -70°C freezer for 2 h. The cryovials were removed from the container, clipped onto canes and plunged into liquid nitrogen (-196°C) (Figure 7B). Samples were removed from the liquid nitrogen after 24 h, 7 d and 8 weeks. Samples were thawed by placing the vials in a waterbath at 42°C for 4-6 min (Figure 7C); and the cryoprotectants were drawn from the tissue using a vacuum (Figure 7D). One set of controls was treated with the cryoprotectants but was not frozen (control 1), while the other set were not treated with cryoprotectants or frozen (control 2).

4.2.3. The Cryopreservation of Different Lines

Three new embryogenic lines were selected on their healthy growth and vigorous proliferation. The genotypes (G1, G2, G3 and G4) were derived from two *P. patula* families. The tissue was bulked up using suspension cultures, and then plated out onto solid MSG3 medium for normal proliferation. Once sufficient tissue was generated, 1 g of tissue was placed into 3 ml of liquid MSG3 medium supplemented with 0.3 M sorbitol, in a 25 ml Erlenmeyer flask. The flasks were agitated on a rotary shaker at 120 rpm for 24 h. The cultures were then placed onto ice and allowed to equilibrate for 20 min. Three ml of MSG3 medium supplemented with 0.3 M sorbitol and 10 % DMSO were then added to the tissue. This resulted in a final concentration of 5 % DMSO. The

cell clusters were gently broken apart using a micropipette, and the cultures were left to equilibrate on ice for a further 20 min. One ml aliquots were pipetted into 1.8 ml Nunc cryovials. The cryovials were transferred into a Nalgene™ Cryo 1 °C freezing container and placed into a -70 °C freezer for 2 h. The vials were removed from the container and clipped onto canes before being plunged into liquid nitrogen. Samples were removed after 24 h and 7 d.

4.2.4. Anatomical Studies

Recovered tissue was stained with 0.5 % acetocarmine and Evans blue to study freezing damage to cell types. Embryogenic tissue was mounted on glass slides and flooded with acetocarmine and then Evans blue. The slides were then viewed under a light microscope (Olympus BH-2) and photographed.

4.2.5. Viability Determination

Tissue recovered from cryopreservation was stained with 0.05 % FDA and then viewed under an inverted UV microscope (Zeiss IM 35) to determine viability (WIDHOLM 1972).

4.2.6. Data Collection

All experimental tissue was weighed on day 0 and every 7 d thereafter for 5 weeks to determine weight increase. Tissue regrowth was determined as percentage fresh weight increase (Wi) (LAINÉ, BADE and DAVID 1992)

$$Wi = 100 \times \frac{\text{(weight at day i - weight at day 0)}}{\text{weight at day 0}}$$

Day i was the number of days after the commencement of the experiment.

4.2.7. Data Analysis

There were five replicates per treatment and the experiments were repeated twice. The

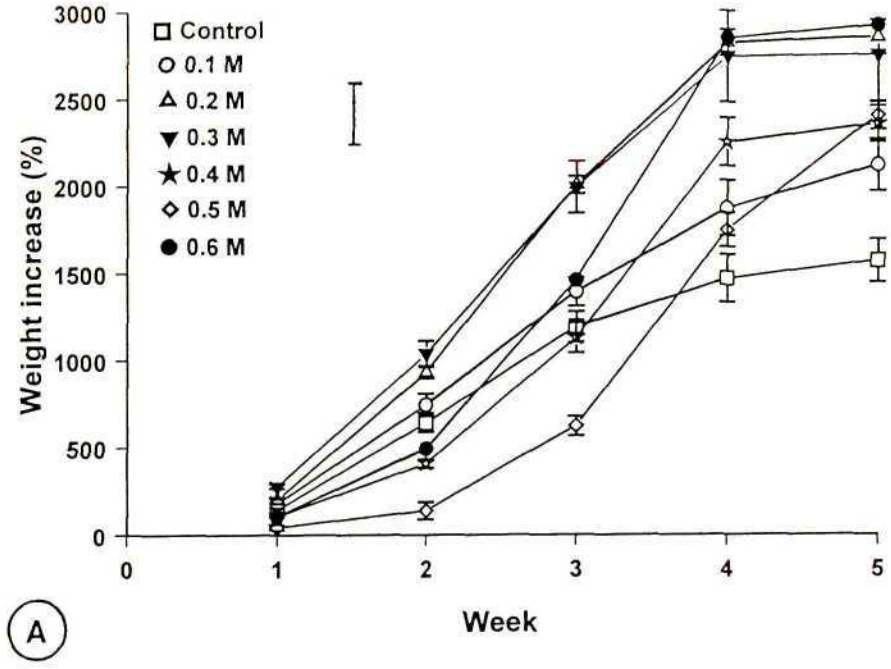
data collected were analysed using Quattro Pro to determine the standard error (SE). A one-way ANOVA was conducted on the data using the Minitab statistical programme. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

4.3. Results

Tissue regeneration was observed across all concentrations of sorbitol and DMSO tested. A lag in tissue growth was commonly observed in all sorbitol treated cultures (Figure 5A). There was no significant difference ($p<0.05$) between the tissue treated with 0.1 M sorbitol and the untreated control. The lag phase was less pronounced in tissue treated with 0.2 M and 0.3 M sorbitol compared to tissue treated with 0.4 M, 0.5 M and 0.6 M sorbitol after the second week of growth. By the end of the treatment period, there was a significant difference ($p<0.05$) between the control tissue and the tissue treated with concentrations of sorbitol greater than 0.2 M.

A lag phase was also observed in the tissue treated with DMSO (Figure 5B). Once more, there was no significant difference ($p<0.05$) between the lower concentrations of DMSO (5 - 15 %) and the untreated control tissue. The tissue treated with 20 % DMSO underwent a lag phase that lasted three weeks before a major increase in tissue weight was recorded. By the end of the treatment period, there was a significant difference ($p<0.05$) in weight increase between the tissue treated with 20 % DMSO and the untreated control. The toxicity of the highest concentrations of cryoprotectants had a significant effect on tissue growth, but based on the rate of tissue recovery, DMSO and sorbitol did not have serious deleterious effects on the proliferation of *P. patula* embryogenic tissue.

The combination of DMSO and sorbitol as cryoprotectants proved to be successful for the long-term storage of embryogenic tissue. The best results were obtained from the tissue treated with 0.3 M sorbitol and 5 % DMSO (Figure 6A). Although no tissue recovery was observed in the tissue stored in liquid nitrogen for 24 h, there was no significant difference ($p<0.05$) between the recovery of tissue stored for 7 d and



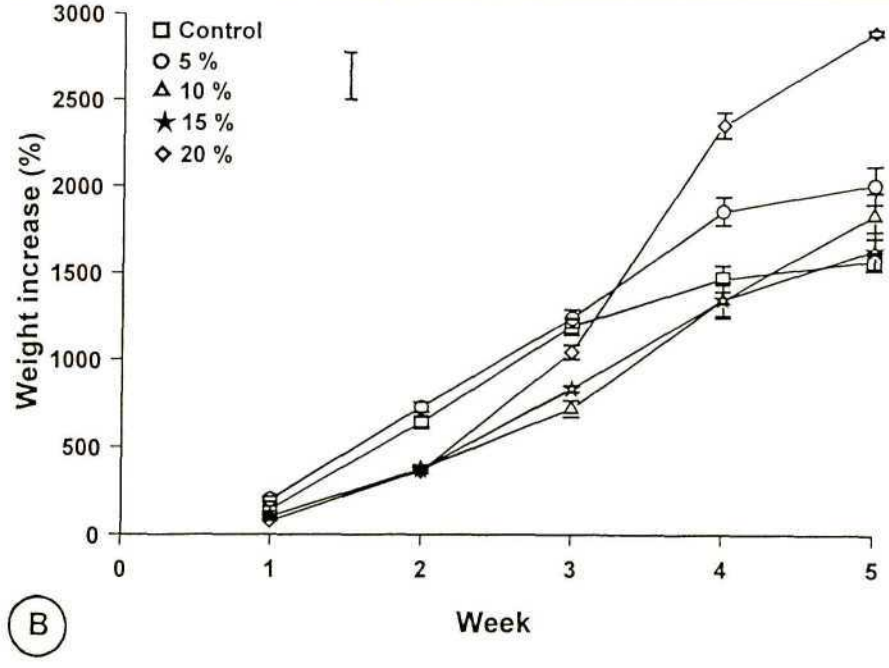
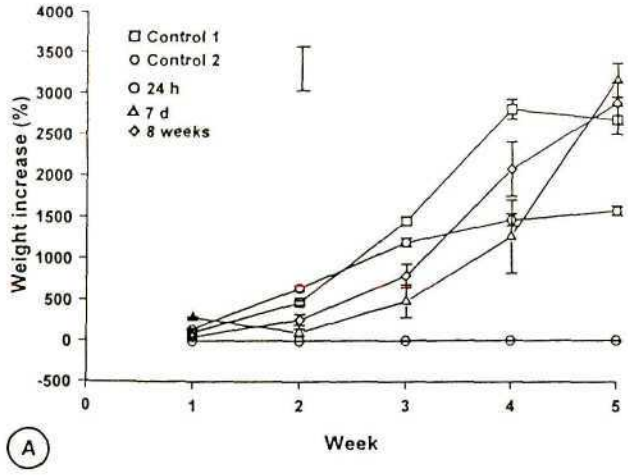


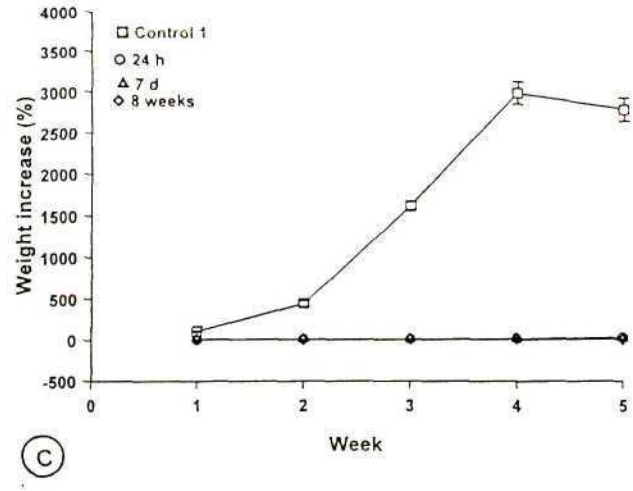
Figure 5. Growth of *P. patula* embryogenic tissue supplemented with various concentrations of (A) sorbitol and (B) DMSO (mean±SE). Bar indicates the Least Significant Difference (LSD) calculated at the 95% confidence interval.

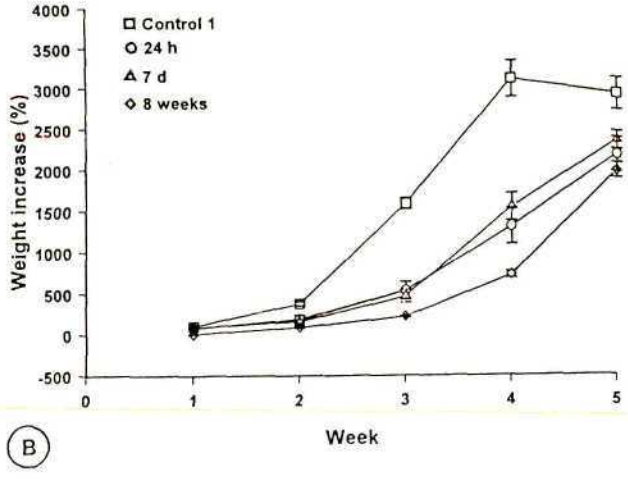
8 weeks and the growth of the treated, unfrozen control (control 1). A lag phase was observed in both the treated, unfrozen control and recovering frozen tissue, indicating that the cryoprotectants have an effect on tissue regrowth. The untreated, unfrozen control tissue (control 2) exhibited no lag phase and entered into the stationary phase towards the end of the third week. Irrespective of the cryoprotectant treatment applied, unfrozen tissue (control 1) appeared to enter a strong deceleration and negative growth rate in the final week of experimentation.

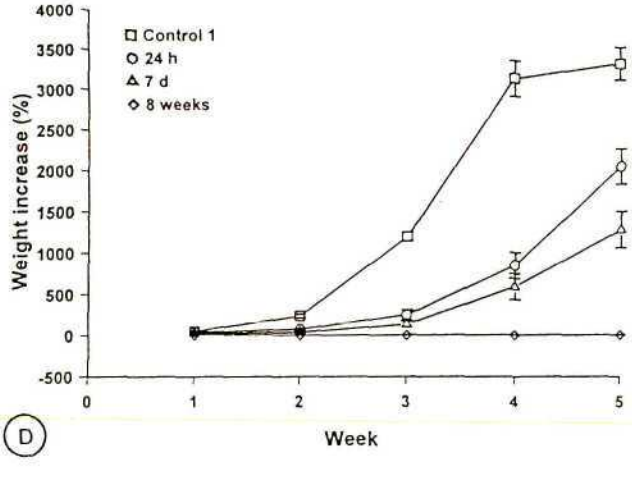
Tissue treated with 0.3 M sorbitol and 10 % DMSO (Figure 6B) also recovered after freezing, but the lag phase was much longer than when using 5 % DMSO (Figure 6A). This might imply that the freezing process itself slowed the tissue recovery more than the possible toxic effects of the addition of the cryoprotectants, as the treated, unfrozen control (control 1) tissue recovered much faster. It is possible that the higher concentration of DMSO affected tissue recovery, as it had an effect on unfrozen tissue (Figure 5B). The amount of time that the tissue was cryopreserved had no significant effect ($p<0.05$) on the recovery rate of the tissue (Figure 5B) No tissue recovery was observed in tissue treated with 0.4 M sorbitol and 5 % DMSO after thawing irrespective of storage time (Figure 6C). The control tissue recovered well with a small lag phase, thus the failure of the frozen tissue to regenerate could possibly have been due to freezing injury. The tissue treated with the highest concentration of cryoprotectants, 0.4 M sorbitol and 10 % DMSO (Figure 6D) recovered, but exhibited a very long lag phase. Storage time seemed to influence tissue recovery, as the tissue that had been frozen the longest recovered the slowest, although this effect was not observed in any of the other responsive cryoprotectant treatments (Figure 6A and 6B).

Recovered tissue was stained with acetocarmine and Evans blue to assess the damage to the various cell types (Figure 7E). The highly vacuolated suspensor cells had ruptured during the freezing and thawing process, and had taken up the Evans blue stain. The cells of the less vacuolated embryonal axis (stained red) appeared to have sustained substantially less injury. This was confirmed through the use of the FDA viability stain (Figure 7F). The embryonal heads glowed apple green when viewed under UV light, indicating that the cell membranes had remained intact and that normal









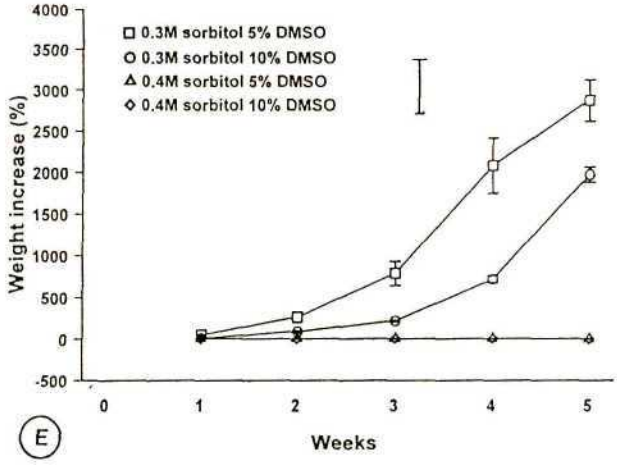


Figure 6. Growth of *P. patula* embryogenic tissue treated with (A) 0.3 M sorbitol and 5 % DMSO; (B) 0.3 M sorbitol and 10 % DMSO; (C) 0.4 M sorbitol and 5 % DMSO; and (D) 0.4 M sorbitol and 10 % DMSO. Control 1 tissue was treated with the cryoprotectants, but not frozen. Control 2 was untreated, unfrozen tissue (mean±SE). (E) A comparison of the growth of the four different treatments cryopreserved for 8 weeks (mean±SE). Bar indicates the Least Significant Difference (LSD) calculated at the 95% confidence interval.

cellular metabolism was underway. The suspensor cells did not fluoresce at all, indicating that the cell membranes had ruptured, and the cells were no longer viable.

Initially after thawing, the tissue had a mucilaginous appearance (Figure 7G). The intact embryonal heads appeared as creamy-white clumps on the polyester grid. After one week, suspensor tissue visibly proliferated from the embryonal heads (Figure 7H). By 2 weeks, healthy embryogenic tissue had proliferated from the recovered tissue, covering most of the polyester support. After 4 weeks substantial tissue proliferation was observed as a result of cleavage polyembryony (Figure 7I). The vigour and appearance of the tissue was the same as that of the unfrozen tissue.

All four lines (including the control G1) recovered from 7 d in liquid nitrogen storage (Figure 8). The lag phase observed in genotype G2 and G4 contrasted strongly with the fast recovery of the two other genotypes. However, there was no significant difference ($p<0.05$) between the final weights of all four genotypes. Genotype G4 was comprised of small, densely compacted cells that did not proliferate as vigorously as the other genotypes. The tissue often browned before the fortnightly subculturing, indicating that tissue growth may not have been as vigorous as originally anticipated. Considering that G4 may not have been an ideal candidate for cryopreservation, the fact that tissue recovery was nevertheless obtained after thawing, indicates that the protocol could be successfully used for most *P. patula* lines. If one extrapolates from the growth curve (Figure 8), one might assume that by week 6 all 4 genotypes would have achieved a 2500 % weight increase. The sigmoidal growth curve shown by the control and genotype G3 indicate that the tissue had reached the stationary phase after 5 weeks.

4.4. Discussion

In this Chapter, the successful cryopreservation of four genotypes of embryogenic tissue, from 2 different families of *P. patula*, using a combination of the cryoprotectants sorbitol and DMSO is discussed. These two cryoprotectants are widely used as a pre-treatment for conifer embryogenic tissue prior to freezing in liquid nitrogen e.g. *Picea glauca* embryogenic tissue cryopreserved with 0.4 M sorbitol and 5 % DMSO

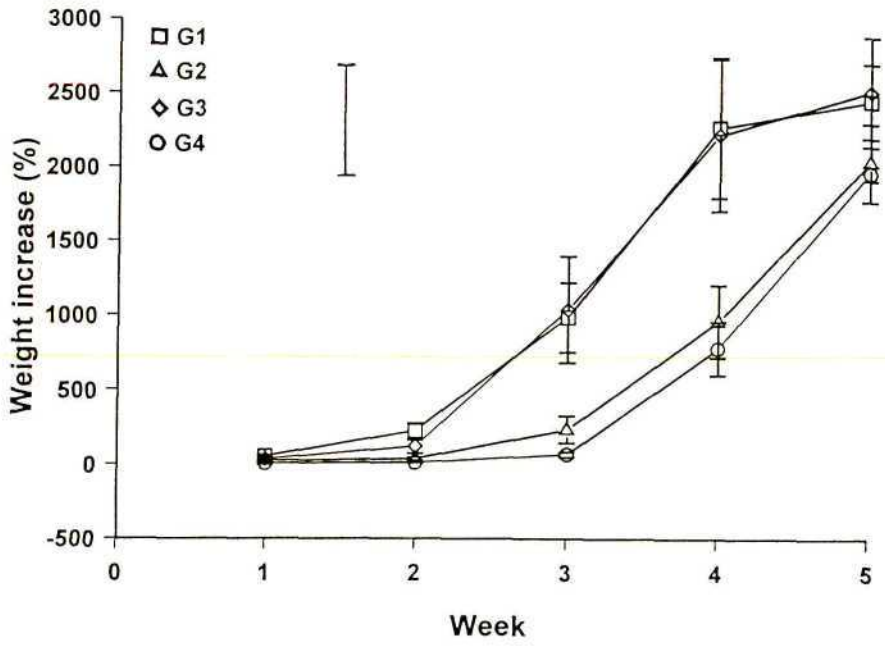


Figure 8. Growth of four different genotypes of *P. patula* embryogenic tissue cryopreserved for 7 days using 0.3 M sorbitol and 5 % DMSO (mean±SE). Bar indicates the Least Significant Difference (LSD) calculated at the 95% confidence interval.

(KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988); *Pinus caribaea* embryogenic tissue cryopreserved with 0.4 M sorbitol and 5 % DMSO (LAINÉ, BADE and DAVID 1992); and *Pinus radiata* embryogenic tissue cryopreserved with 0.4 M sorbitol and 10 % DMSO (HARGREAVES, WARR, GRACE and SMITH 1995). Although DMSO is the most widely used cryoprotectant, its cytotoxicity results in a noticeable reduction in tissue growth (KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988). This was also found to be true in the present study, where a lag phase was observed in tissue treated with concentrations higher than 5 % (Figure 5B). KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN (1988) found that the toxicity was not progressive relative to the increasing concentrations of DMSO and cells treated with all concentrations were able to recover from the toxicity and resume regrowth comparable to the untreated controls. The additive effects of sorbitol and DMSO on *Catharanthus* cells were comprehensively studied by CHEN, KARTHA, CONSTABEL and GUSTA (1984b). They concluded that a non-penetrating sugar such as sorbitol reduced cellular water and reduced the rate of initial ice crystallisation; the penetrating cryoprotectant DMSO entered the cell to reduce freeze-induced cellular dehydration; and the combination of sorbitol and DMSO minimised the effect of initial freezing stresses and subsequent dehydration. While it was observed that DMSO induced cytotoxicity, such toxic effects of DMSO were reversed by treatment of cells with sorbitol prior to (pretreatment) and during freezing (KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988). Sorbitol has previously been used as an osmotic agent in the preculture medium as well as the sole cryoprotectant during the freezing process. WEBER, ROTH and SCHWEIGER (1983) successfully cryopreserved cell cultures of *Glycine max, Datura innoxia, Brassica napus* and *daucus carota* and cell derived protoplasts of *G. max* using this procedure. Studies done on *Picea glauca* embryogenic cell cultures showed that the highly vacuolated suspensor cells were not viable subsequent to treatment with sorbitol and DMSO even prior to freezing (KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988).

Light microscope investigations have shown that vacuolated suspensor cells of the embryos were destroyed while cells with dense cytoplasm from the embryogenic region survived the cryopreservation and were responsible for regrowth of the cultures

(KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN 1988; GALERNE and DEREUDDRE 1988; FIND, FLOTO, KROGSTRUP, MØLLER, NØRGAARD and KRISTENSEN 1993). This was also observed in *P. patula* embryogenic tissue that had been cryopreserved, where the suspensor cells had ruptured (Figure 7E) and the embryonal head had remained intact (Figure 7F). KRISTENSEN, FIND, FLOTO, MØLLER, NØRGAARD and KROGSTRUP (1994) studied the regrowth of *Picea sitchensis* embryogenic cells recovering from cryopreservation. They found that the observed symmetrical divisions of surviving embryogenic cells leading to embryogenic cell clusters from which suspensor cells developed closely resembled somatic embryo development from *Picea mariana* protoplasts (TAUTORUS, ATTREE, FOWKE and DUNSTAN 1990) and *Abies alba* (HARTMANN, LANG and REUTER 1992). They also found that the densely cytoplasmic, embryonal cells underwent organised growth and differentiation with first divisions occurring after 24 hours, and embryo formation 6-8 days after thawing from liquid nitrogen. The surviving embryonal axes in recovered *P. patula* embryogenic tissue also began normal cleavage polyembryony, and tranlucent suspensor tissue was clearly visible.

LAINÉ, BADE and DAVID (1992) found that in *Pinus caribaea* for optimal recovery of tissue it was important to use highly embryogenic starting material. When using suitable material, it was possible to achieve 100 % recovery of cryopreserved samples. If non- or poorly- embryogenic lines were cryopreserved, it was difficult to obtain any regrowth after thawing. HARGREAVES and SMITH (1994) found that *Pinus radiata* lines that were brown and less vigorous did not survive the freezing process at all. Embryogenic tissue that did not grow after thawing showed loss of fresh weight. HARGREAVES and SMITH (1994) attributed this to the collapse of highly vacuolated suspensor cells that make up most of the tissue bulk on maintenance medium, which had become plasmolysed during the pre-freezing incubation step. This loss of weight was observed in *P. patula* tissue pretreated with 0.4 M sorbitol and 5 % DMSO (Figure 6C).

In order to utilise somatic embryogenesis in a clonal forestry programme the cryopreservation protocol must be applicable to a wide range of genotypes from many families (CYR, LAZAROFF, GRIMES, QUAN, BETHUNE, DUNSTAN and ROBERTS

1994). The protocol described above initially tested on four different lines has subsequently been tested on numerous embryogenic lines and found to be suitable for the cryopreservation of a wide range of *P. patula* families and genotypes (JONES pers. comm. 1999). However, some lines do not respond well to freezing and are referred to as freeze-intolerant. The recalcitrance of freeze-intolerant genotypes may reflect a high ratio of suspensor to embryogenic cells in the cultures, since the former usually do not survive pretreatments with cryoprotectants or cryopreservation (GALERNE and DEREUDDRE 1988). *Pinus patula* embryogenic was successfully cryopreserved for 2 months in this experiment. However, the protocol is now actively being incorporated into *P. patula* somatic tissue research, and tissue has been stored for 6 months and successfully recovered. HARGREAVES, WARR, GRACE and SMITH (1995) successfully recovered tissue after 32 months in cryopreservation. This opens the way for progeny testing while reference material is kept in a juvenile state.

CHAPTER 5

A COMPARISON OF COMMONLY USED CRYOPRESERVATION PROTOCOLS

5.1. Introduction

The combination of cryoprotectants for the viable freezing of plant material has been found to be beneficial. ULRICH, FINKLE, MOORE and GINOZA (1979) demonstrated the beneficial effect of using a mixture of the cryoprotectants PEG, glucose and DMSO (10-8-10 %) in the successful cryopreservation of sugarcane callus. GUPTA, DURZAN and FINKLE (1987) successfully cryopreserved embryogenic cell masses of *Picea abies* and *Pinus taeda* using this same combination. The combination of sorbitol or sucrose and DMSO is also widely used since first published by CHEN, KARTHA, LEUNG, KURZ, CHATSON and CONSTABEL (1984a). The exposure duration of cryoprotectants to cells needs to be such that the concentration applied does not cause sudden plasmolysis which in itself is a major cause of injury to the osmotic responsiveness of the cells (KARTHA and ENGELMANN 1994). The slow addition of the cryoprotectants to the cells ensures that the cells are not damaged by the dehydrating effects of the cryoprotectants.

The conventional methods of cryopreservation involved a freeze-induced dehydration step brought about by slow-cooling, prior to liquid nitrogen storage (KARTHA and ENGELMANN 1994). An alternate approach is vitrification (RALL and FAHY 1985). This method is based on the ability of highly concentrated solutions of cryoprotectants to supercool to very low temperatures upon imposition of rapid cooling rates, to become

viscous at sufficiently low temperatures, and solidify without the formation of ice (KARTHA and ENGELMANN 1994).

Three protocols are described below. The first involved the use of a combination of sorbitol and DMSO, but the sorbitol was added over two days and the concentration was doubled before the addition of the DMSO and freezing. This protocol shall be referred to as the **two-step protocol**. In the second protocol, the cryoprotectants were added slowly over three steps to the tissue, and incubated for relatively short periods of time. The concentration of the cryoprotectants PEG, glucose and DMSO was doubled with each addition to reach a final concentration of 10 % PEG- 8 % glucose- 10 % DMSO. This protocol shall be referred as the **three-step protocol.** In the third protocol, the tissue was set in solid MSG3 medium and plunged directly into liquid nitrogen. This protocol shall be referred to as the **direct immersion protocol**. All three protocols were compared with the successful protocol devised in Chapter 4, which shall be referred to as the **one-step protocol**.

5.2. Materials and Methods

5.2.1. Two-step Addition of Sorbitol and DMSO

The protocol of KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN (1988) was adapted for *P. patula* embryogenic tissue. One g of healthy, vigorously proliferating embryogenic tissue was precultured in 3 ml of MSG3 liquid medium supplemented with 0.15 M sorbitol for 24 h on a rotary shaker at 120 rpm. The liquid medium was then decanted and the cells were resuspended in 3 ml of MSG3 liquid medium supplemented with 0.3 M sorbitol. The cells were placed on the rotary shaker for a further 24 h at 120 rpm. The tissue was then placed onto ice and 3 ml of MSG3 medium supplemented with 0.3 M sorbitol and 10 % DMSO or 20 % DMSO was added. The cells were allowed to equilibrate on the ice for 20-30 min. One ml aliquots were then pipetted into 1.8 ml Nunc cryovials, and the vials were placed into Nalgene™ Cryo 1 °C freezing container filled with isopropyl alcohol. The container was then placed into a -70°C freezer for 2 h. The vials were removed from the container and clipped onto

canes before being plunged into liquid nitrogen. Samples were removed after 24 h and 7 d. They were thawed by placing the vials into a water bath at 42°C for 4-6 minutes. The liquid suspension was drawn off using a vacuum and the tissue was collected on a polyester grid support. The recovered tissue was placed onto fresh MSG3 maintenance medium, and was subcultured onto fresh MSG3 medium 24 h later. The tissue was then subcultured 7 d later and then every two weeks.

5.2.2. Three-step Addition of PEG, Glucose and DMSO

The protocol of GUPTA, DURZAN and FINKLE (1987) was applied to *P. patula* embryogenic tissue. Three g of healthy, vigorously proliferating embryogenic tissue was placed into 10 ml of MSG3 liquid medium supplemented with 2.5 % PEG, 2 % glucose and 2.5 % DMSO. The tissue was precultured in this medium for 15 min on ice. The medium was decanted off and 10 ml of MSG3 liquid medium supplemented with 5 % PEG, 4 % glucose and 5 % DMSO was added to the tissue. The tissue was incubated in this medium for a further 15 min on ice. The medium was decanted off and 10 ml of MSG3 medium supplemented with 10 % PEG, 8 % glucose and 10 % DMSO was added to the tissue. The cultures were placed on ice for a further 30 min. One ml aliquots of the suspension culture were pipetted into 1.8 ml Nunc cryovials. The vials were placed into a Nalgene™ Cryo 1°C freezing container filled with isopropyl alcohol. The container was placed into a -70°C freezer for 2 h. The vials were then removed from the container and clipped onto canes before being plunged into liquid nitrogen. Samples were removed after 24 h and 7 d. The tissue was thawed by placing the vials into a water bath at 42°C for 4-6 min. The liquid suspension was drawn from the recovered tissue using a vacuum, and the tissue was captured on a polyester grid. The grids were placed on fresh MSG3 medium, and after 24 h they were subcultured onto fresh MSG3 medium again. One week later the tissue was transferred to fresh medium, and then every two weeks.

5.2.3. Direct Immersion of Tissue held in Gelrite®

The protocol of BECWAR, NOLAND and WANN (1987) was adapted for the

embedding of tissue in gel. One g of tissue was placed into 3 ml of MSG3 liquid medium supplemented with 0.3 M sorbitol. The tissue was placed on a rotary shaker at 120 rpm for 24 h. The tissue was placed onto ice and 3 ml of liquid MSG3 medium supplemented with 0.3 M sorbitol and 10 % DMSO was added. This brought the final concentration of the DMSO to 5 %. In the mean time, MSG3 solid medium was made and allowed to cool to 40°C. The medium was poured into petri dishes and 1 ml aliquots of the cryoprotected suspension was pipetted into the medium and allowed to set. Once the medium had set, small blocks of gel containing the tissue were cut from the plate and placed into Nunc cryovials. Liquid nitrogen was then poured directly into the vials, until the liquid nitrogen stopped bubbling. The caps were then placed onto the vials and they were clipped onto canes before being stored in the dewar. Samples were removed after 7 d and thawed in a water bath at 42°C for 4-6 min. The tissue was plated out onto fresh solid MSG3 medium, supported by a polyester grid. The tissue was subcultured onto fresh medium after 24 h, 7 d and then every two weeks.

5.2.4. Anatomical Studies

Tissue was stained with 0.5 % acetocarmine and Evans blue to study freezing damage to cell types. The tissue was then mounted on glass slides and flooded with acetocarmine and then Evans blue. The slides were then viewed under a Olympus BH-2 light microscope and photographed.

5.2.5. Viability Determination

Tissue recovered from cryopreservation was stained with 0.05 % FDA and then viewed under an inverted UV microscope (Zeiss IM 35) to determine viability (WIDHOLM 1972).

5.2.6. Data Collection

All experimental tissue was weighed on day 0 and every 7 d thereafter for 5 weeks to determine weight increase. Tissue regrowth was determined as percentage fresh

weight increase (Wi) (LAINÉ, BADE and DAVID 1992).

$$Wi = 100 \times \frac{\text{(weight at day i - weight at day 0)}}{\text{weight at day 0}}$$

Day i was the number of days after commencement of the experiment.

5.2.7. Data analysis

There were five replicates per sample for the 1-step and 2-step protocol and 20 replicates for the 3-step protocol. Experiments were repeated twice. The data collected was analysed using a Quattro Pro programme to determine the standard error (SE). A one-way ANOVA was conducted on the data using the Minitab statistical programme. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

5.3. Results

A normal sigma curve was observed in the recovering control tissue. Tissue proliferation was healthy and vigorous. In the tissue that was treated over 2 d prior to cryopreservation, there was a significant difference ($p<0.05$) between the tissue treated with 0.3 M sorbitol and 5 % DMSO and tissue treated with 0.3 M sorbitol and 10 % DMSO (Figure 9). The lag phase exhibited by the tissue treated with 0.3 M sorbitol and 5 % DMSO was greater than that of the control, although there was no significant difference ($p<0.05$) between the final weights of the two treatments. This was possibly due to the greater dehydrating effect of the cryoprotectant preculture treatment applied over 2 d. The lag phase observed in tissue precultured in 0.3 M sorbitol and 10 % DMSO over a similar period was significantly greater ($p<0.05$) than that of the control and that of the 0.3 M sorbitol and 5 % DMSO 2-step treated tissue. Once more, the addition of sorbitol over two days must have dehydrated the tissues, possibly even increasing the toxic effect of the cryoprotectants. Although normal cleavage polyembryony and tissue proliferation was observed, greater damage due to cryodehydration must have occurred in the tissue treated with 0.3 M sorbitol and 10 % DMSO.

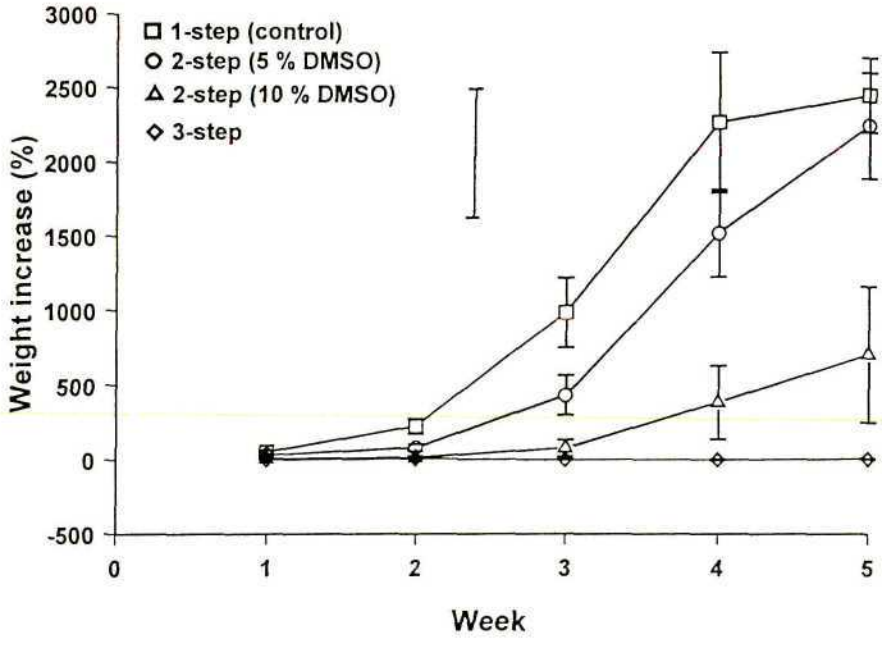


Figure 9. Growth of *P. patula* embryogenic tissue treated and cryopreserved using three different protocols and compared with tissue frozen using a 1-step method where 0.3 M sorbitol and 5 % DMSO was used as cryoprotectants (mean±SE). Bar indicates Least Significant Difference (LSD) calculated at the 95% confidence interval.

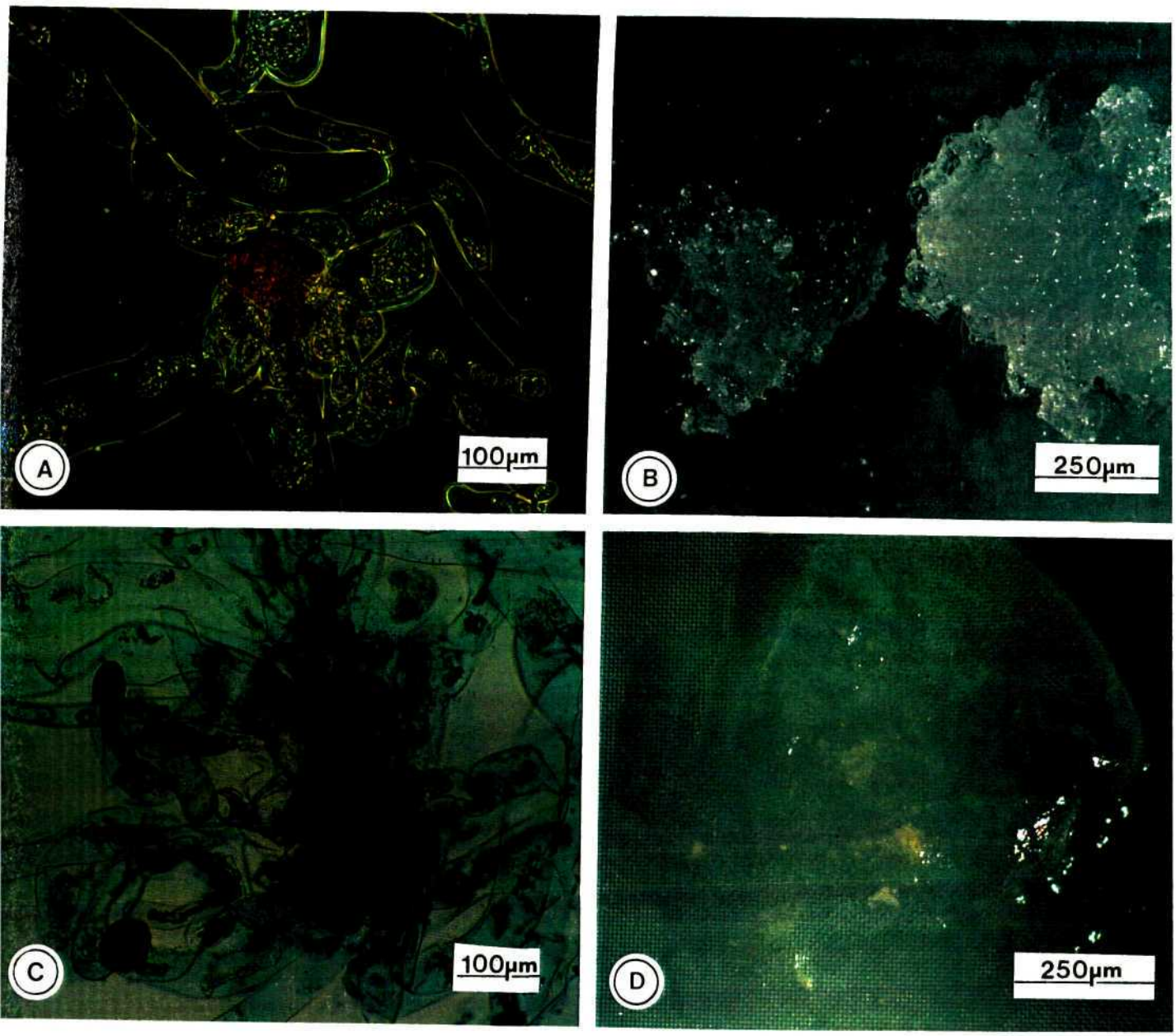
Tissue that was treated with PEG, glucose and DMSO (3-step protocol) did not respond well to freezing (Figure 9). Out of twenty samples, only three replicates produced any tissue. The recovered tissue had a slimy appearance. Anatomical studies conducted on the recovered tissue using acetocarmine and Evans blue stains, showed that the highly vacuolated suspensor tissue had been severely damaged during freezing (Figure 10A). The compact embryonal heads appeared to be intact, and when viewed under UV light, the embryonal axes fluoresced (results not shown) indicating that they had survived the cryopreservation. Tissue proliferation was only observed after 4 weeks, with small amounts of compact embryogenic tissue being proliferated (Figure 10B). The poor response observed in the tissue could have been to the toxicity of the cryoprotectants, which diffused very slowly from the tissue into the medium. The high concentrations of the cryoprotectants could also have dehydrated the tissues when they were removed from cryopreservation. A possible solution to this problem would be to rinse the tissue with liquid MSG3 medium prior to plating out the recovered tissue.

The tissue that had been set in gel and immersed directly into the liquid nitrogen did not survive cryopreservation. The suspensor tissue as well as the embryonal axes both absorbed the Evans blue stain, indicating that cell integrity had been lost in all cells present (Figure 10C). No tissue recovery was observed (Figure 10D), due to the damage to the cells. The direct exposure of the tissue to ultra low temperatures did not promote vitrification as was hoped, instead the cells possibly developed lethal ice crystals within the cytoplasm that expanded and ruptured the cells upon freezing or thawing. The possibility that the tissue was damaged by the heat of the gel was ruled out, as tissue unfrozen tissue left over from the experiment survived embedding and proliferated normally.

5.6. Discussion

The combination of sorbitol and DMSO once more proved to be the most successful treatment, even though the 2-step protocol with 0.3 M sorbitol and 5 % DMSO worked almost as well as the 1-step protocol with the same cryoprotectants. The slow addition of the cryoprotectants aids the cells in adapting to the high concentrations of

Figure 10. (A) Acetocarmine and Evans blue stain of tissue pretreated with PEG-glucose -DMSO and frozen using a 3-step protocol. (B) Compact tissue regeneration from the tissue frozen using the 3-step protocol. (C) Acetocarmine and Evans blue stain of tissue set in gel and immersed directly in liquid nitrogen. Notice how the embryonal heads have also taken the blue stain in, indicating that significant cellular damage had been incurred. (D) Embryogenic tissue embedded in gel after thawing.


A
100µm
B
250µm
C
100µm
D
250µm

cryoprotectants (GUPTA pers. comm. 1997). The 2-step protocol was used successfully by KARTHA, FOWKE, LEUNG, CASWELL and HAKMAN (1988) to cryopreserve embryogenic tissue of *Picea glauca*, where 0.4 M sorbitol and 5 % DMSO was used. Although only one genotype was tested, embryogenic tissue *P. patula* did not respond well to this treatment, and the chances of cultures becoming contaminated was increased as more preculture steps were necessary.

ULRICH, MICKLER, FINKLE and KARNOSKY (1984) cryopreserved American elm callus using the 3-step protocol. Almost 100 % recovery was found in all the lines that were slow-cooled to -23°C, however, only 50 % of one line survived slow-cooling to -30°C followed by immersion in liquid nitrogen. GUPTA, DURZAN and FINKLE (1987) cryopreserved *Picea abies* and *Pinus taeda* embryogenic cell masses using the 3-step protocol, and noted that no growth of frozen and thawed embryonal cells was observed from days 0 to 35. It was only after week 6 that tissue recovery was observed. They found that the highly vacuolated suspensor cells had been disrupted and killed, and that the embryonal cells with the dense cytoplasm and large nuclei had survived. The frozen *P. patula* embryogenic tissue also showed signs of damage to the suspensor tissue and the outer regions of the embryonal heads as seen by the uptake of Evans blue into the cells (Figure 10A). Although tissue recovery was observed, the resulting tissue was very fine and compact (Figure 10B). Tissue that had not regenerated by week 5 was scraped from the polyester grid and stained with FDA. No viable cells were found, indicating that a high proportion of cell death had occurred.

The severe damage incurred in the tissue that was directly immersed in liquid nitrogen (Figure 10C) could have been due to the low concentration of the cryoprotectants. Preculture of cells in osmotically active compounds, such as sucrose, sorbitol and mannitol, enhance the post-freezing survival of cells by reducing the cell volume and water content by inducing partial dehydration in prefreezing steps (KARTHA and ENGELMANN 1994). REINHOUD, URAGAMI, SAKAI and VAN IREN (1995) state that the vitrification solution itself should contain a very high concentration of suitable solutes in which no ice crystals are formed during cooling or rewarming. Successful vitrification solutions have a concentration of solutes in the range of 5-8 M and they

cause severe cellular dehydration. The benefits of this system, however, are that the need for expensive cooling equipment is circumvented. The direct immersion of the tissue in the liquid nitrogen was used to overcome the temperature gradient across the plastic cryotubes. ISHIKAWA, TANDON, SUZUKI and YAMAGUISHI-CIAMPI (1996) found that the plastic cryotubes had lower heat conductivity to the glass centrifuge tubes that they normally used to cryopreserve bromegrass suspension cultures. The plastic cryovials also took 3-4 times longer to thaw, slowing the processing time and exposure to high temperatures, and increasing the risk of cellular ice forming during thawing.

CHAPTER 6

OPTIMISATION OF A WORKING CRYOPRESERVATION PROTOCOL

6.1. Introduction

The establishment of a successful pretreatment of tissue with an optimal combination of cryoprotectants is very important for a successful cryopreservation protocol. However, for the protocol to be optimised, the rate of cooling and the recovery of tissue are just as important for an optimal cryopreservation technique (KARTHA 1985; BERJAK, MYCOCK, WATT, WESLEY-SMITH and HOPE 1995; GROUT 1995). The slow cooling of plant material provides a controlled amount of cryodehydration, to allow for subsequent vitrification when the sample is immersed in liquid nitrogen (GROUT 1995). If the rate of cooling is too slow, the cells would become over-dehydrated and be exposed to the damaging effects of increased concentrations of electrolytes. However, if the cooling rate is faster than optimum, the cells will not be sufficiently dehydrated and intracellular ice formation will occur (KARTHA 1985). The cells are generally cooled at optimum rates to -30°C or -40°C by which time all of the freezable water from the cell has escaped to become external ice and a subsequent drop in temperature to that of liquid nitrogen has very little adverse effect (KARTHA 1985). The optimal cooling rate for slow cooling is between 0.5 and 2.0°C/min (KARTHA and ENGELMAN 1994). SCHRIJNEMAKERS and VAN IREN (1995) studied the effectiveness of the Nalgene™ Cryo 1°C freezing container filled with ethanol (instead of isopropyl alcohol), and placed into a static freezer at -80°C. They found that the average cooling rate within the vials over the first 40 °C was close to 1°C/min when using a thermocouple, and that the final temperature in the vials after 120 to

150 minutes was close to -80°C.

Successful recovery of cryopreserved tissue often requires rapid thawing in a waterbath at 35-40°C for 1-2 min, or at least until the solid ice core has melted (KARTHA and ENGELMAN 1994). This is mainly because of the risk of crystal formation in the vitrified glass, followed by rapid and deleterious crystal growth (SAKAI 1966). The cryoprotectants were removed by washing the thawed cells with chilled nutrient medium prior to their return to culture either in liquid or semi-solid medium. However, this was later found to be deleterious to a number of cell cultures (KARTHA and ENGELMAN 1994). CHEN, KARTHA, LEUNG, KURZ, CHATSON and CONSTABEL (1984a) found that while the non-alkaloid producing *Catharanthus roseus* cell cultures recovered well after a post-thaw wash, the alkaloid producing cell cultures only recovered if they were plated out onto filter paper and placed over regrowth medium for specific amounts of time. This allowed the cryoprotectants to diffuse from the tissue into the medium, and allowed easy transfer of the tissue onto fresh medium.

The object of the experiments in this Chapter were to find the optimum pre-cooling temperature that allowed for optimal tissue recovery, as well as to determine if different supports would influence post-thaw tissue recovery.

6.2. Materials and Methods

6.2.1. Freezing of *P. patula* Embryogenic Tissue

Embryogenic tissue was precultured in 0.3 M sorbitol and 5 %DMSO before being pipetted into 1.8 ml Nunc cryovials. The samples were either plunged directly into liquid nitrogen, or placed into a Nalgene™ Cryo 1°C freezing container and frozen to -30°C or -70°C for 2 h before being plunged into liquid nitrogen. Samples were cryopreserved for 7 d, before being removed and thawed at 42°C for 4-6 min. The tissue was suspended on a polyester grid and the supernatant was drawn off using a vacuum. The recovered tissue was placed onto fresh MSG3 maintenance medium for 24 h, and then

subcultured onto fresh MSG3 medium once more. The recovered tissue was subcultured onto fresh medium after 7 d and subsequently every 2 weeks.

6.2.2. Supports for Recovering *P. patula* Embryogenic Tissue

Plant tissue was treated with 0.3 M sorbitol and 5 % DMSO, and cryopreserved for 7 d. Samples were then removed and thawed at 42°C for 4-6 min. The tissue was suspended on either a 26μM polyester grid, Whatman No.2 filter paper, or a combination of a polyester grid on a Whatman No. 2 filter paper disc. The supernatant was then drawn off using a vacuum. Half of the polyester grids with tissue were suspended on a nurse culture. A nurse culture is unfrozen embryogenic tissue of the same or a different genotype, above which the recovered tissue is suspended. The nurse culture supplies the recovered tissue with nutrients and removes toxins. The tissue was left on the nurse culture for 2 weeks, before being lifted off and placed directly onto MSG3 maintenance medium. The recovered tissue on the other supports was placed onto fresh MSG3 maintenance medium. The tissue was subcultured onto fresh MSG3 medium after 24 h, then 7 d and finally every 2 weeks after thawing.

6.2.3. Anatomical Studies

Tissue was stained with 0.5 % acetocarmine and Evans blue to study freezing damage to cell types. The tissue was then mounted on glass slides and flooded with acetocarmine and then Evans blue. The slides were then viewed under a Olympus BH-2 light microscope and photographed.

6.2.4. Viability Determination

Tissue recovered from cryopreservation was stained with 0.05 % FDA and then viewed under an inerted UV microscope (Zeiss IM 35) to determine viability (WIDHOLM 1972).

6.2.5. Data Collection

All experimental tissue was weighed on day 0 and every 7 d thereafter for 5 weeks to determine weight increase. Tissue regrowth was determined as percentage fresh weight increase (Wi) (LAINÉ, BADE and DAVID 1992).

$$Wi = 100 \times \frac{\text{(weight at day i - weight at day 0)}}{\text{weight at day 0}}$$

Day i was the number of days after commencement of the experiment.

6.2.6. Data Analysis

There were ten replicates per sample and the experiments were repeated twice. The data collected were analysed using the Quattro Pro programme to determine the standard error (SE). A one-way ANOVA was conducted on the data using the Minitab statistical programme. When the ANOVA indicated statistical significance, a Tukey's multiple comparison test was used to distinguish differences between treatments.

6.3. Results

The successful cryopreservation of *P. patula* embryogenic tissue requires that the tissue be slow-cooled to -70°C for tissue recovery to occur (Figure 11). No tissue recovery was observed in tissue slow-cooled to -30°C. The cooling processing time could have been too long, allowing the cells to dehydrate to a lethal level. The tissue plunged directly into liquid nitrogen did not have enough time to dehydrate sufficiently, allowing lethal ice crystals to form. Fluoresceine diacetate staining revealed that no tissue had survived slow-cooling to -30°C or direct freezing to -196°C, so substantial tissue damage must have been incurred during freezing.

Within one week of thawing, tissue proliferation was visible across all supports. Small amounts of translucent suspensor tissue was visibly proliferating from the embryonal heads. Tissue proliferation continued across all treatments, however the tissue

recovering on the polyester grids underwent major proliferation from week 3 to reach a weight increase of close to 3500 % (Figure 12). The thin polyester grid allowed the cryoprotectants to flow freely from the tissue and diffuse away from the tissue into the medium. The tissue recovering on filter paper alone seemed to recover the slowest, possibly due to the cryoprotectants saturating the surface and remaining behind instead of diffusing away from the tissue. The tissue on the nurse culture appeared to recover much faster than the other treatments. However, once the tissue was removed from the nurse culture and placed directly onto MSG3 maintenance medium, tissue proliferation appeared to undergo rapid deceleration. If the tissue had been left on the nurse culture longer, the tissue recovery might have been faster and more prolific. The tissue recovering on a combination of filter paper and polyester grid performed better than the tissue recovering on the filter paper alone. The polyester grid may have been an additional barrier to the cryoprotectants that were being held by the filter paper.

6.4. Discussion

Most successful protocols for the cryopreservation of conifer embryogenic tissue requires that the tissue be pre-cooled prior to immersion in liquid nitrogen. The most common pre-cooling temperature is between -35 and -40°C, e.g. *Picea glauca engelmanni* complex pre-cooled in a step-wise fashion using a programmable freezer to -35°C (CYR, LAZAROFF, GRIMES, QUAN, BETHANE, DUNSTAN and ROBERTS 1994); *Pinus carabaea* tissue was pre-cooled to -35°C using a cryostat (LAINÉ, BADE and DAVID 1992); *Larix* x *eurolepis* and *Picea mariana* tissue was pre-cooled to -40°C using a programmable freezer (KLIMASZEWSKA, WARD and CHELIAK 1992). HARGREAVES, WARR, GRACE and SMITH (1995) successfully cryopreserved *Pinus radiata* embryogenic tissue by pre-cooling the tissue in a Nalgene™ Cryo 1°C freezing container placed in a static -80°C freezer for 1 hour prior to plunging the samples into liquid nitrogen. This method was used successfully on *Pinus patula* embryogenic tissue (Figure 11). The tissue frozen to -30°C did not survive cryopreservation (Figure 11), possibly due to the slow rate of cryodehydration and the processing time extending to the point where damaging effects became significant (GROUT 1995). The cells taken

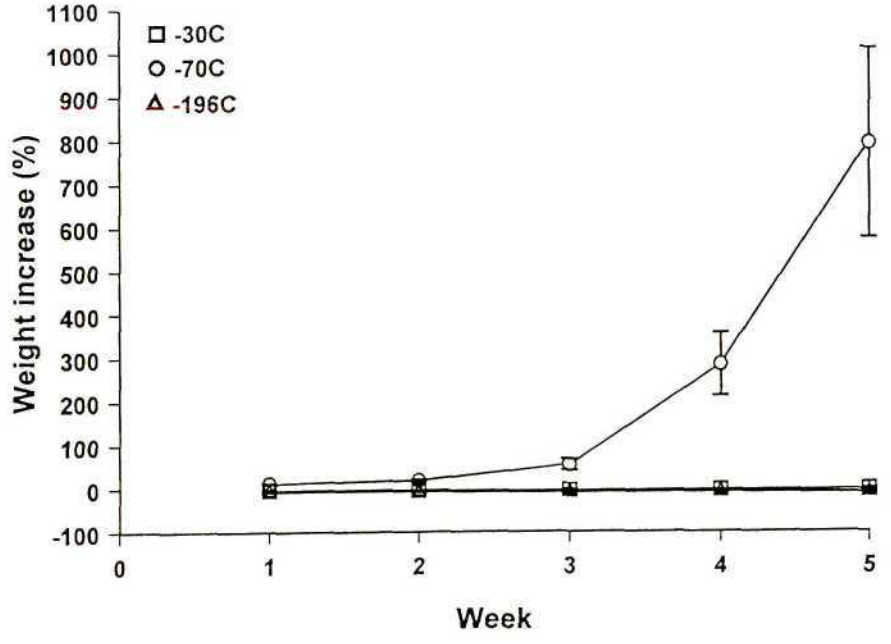


Figure 11. Growth of *P. patula* embryogenic tissue either slow-cooled prior to immersion in liquid nitrogen, or plunged directly into liquid nitrogen (mean ±SE).

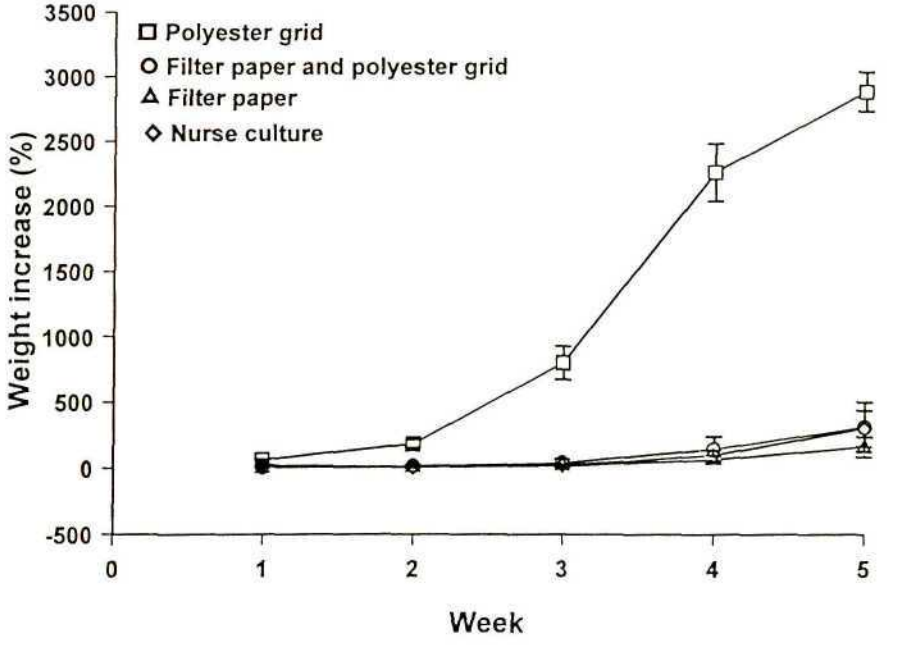


Figure 12. Growth of *P. patula* embryogenic tissue recovering on a variety of supports after 7 d in cryopreservation (mean ±SE).

directly to -196°C (Figure 11) did not survive as the intracellular solutions did not cool completely, resulting in lethal ice nucleation within the cell (GROUT 1995).

The use of supports such as filter paper (KLIMASZEWSKA, WARD and CHELIAK 1992; LAINÉ, BADE and DAVID 1992; FIND, FLOTO, KROGSTRUP, MØLLER, NØRGAARD and KRISTENSEN 1993) or nylon screens (HARGREAVES, WARR, GRACE and SMITH 1995) is widely recorded in conifer embryogenic tissue recovery, and allows the cryoprotectants to slowly diffuse away from the tissue. This allows the intercellular solutes to become redistributed to the physiological state and the normal water status of the cells to be restored (GROUT 1995). Although there is overwhelming support for the use of filter paper discs, *P. patula* tissue responded best when placed onto polyester grids (Figure 12).

HAUPTMANN and WIDHOLM (1982) used feeder plates (nurse cultures) to support growth in cryopreserved cell suspensions of *Daucus carota* and *Nicotiana tobacum*. The principle behind this procedure is that the culture medium is effectively supplemented with conditioning factors produced by unfrozen cells inoculated onto the medium from which the recovering cells are separated by a wire screen or foam pad barrier. HARGREAVES, WARR, GRACE and SMITH (1995) used nurse cultures on *Pinus radiata* embryogenic tissue and found that tissue recovery was improved between 3 to 20 times over tissue plated out directly onto the medium. The authors stressed that the integrity of the nylon mesh must be intact so as to avoid adulteration by the nurse tissue. The use of nurse cultures on *P. patula* embryogenic tissue was initially successful. However, once the cells were transferred onto MSG3 medium, the recovery rate slowed dramatically (Figure 12). Not all of the recovering cells may have been in contact with the nurse culture (given that the tissue had a 'lumpy' texture), so some of the surviving embryonal heads may have senesced as a result of poor access to sufficient nutrients. This might explain the reason thawed tissue did not continue to proliferate once transferred onto MSG3 medium.

CHAPTER 7

MATURATION AND PLANTLET REGENERATION

7.1. Introduction

Although advances in conifer embryogenesis have been marked over the last few years, maturation of embryogenic tissue has been problematic in *Pinus* species (JONES and VAN STADEN 1994). The only *Pinus* species that has successfully been matured is *Pinus radiata* (CHANDLER and YOUNG 1995). During maturation, the somatic embryos stop proliferating, increase in size, and start to accumulate storage material, including starch, proteins and lipids (FEIRER, CONKEY and VERHAGEN 1989; HAKMAN 1993). In order to improve somatic embryo maturation, numerous media modifications and supplement additions have been recommended. The addition of abscisic acid (ABA) and an osmoticum to raise the osmotic concentration of the medium have been found to be beneficial (ATTREE and FOWKE 1993).

In zygotic seeds, ABA accumulates during mid-to-late stages of seed development and prevents the developing embryo from germinating precociously (KERMODE 1990). Abscisic acid has been found to be essential for inhibiting cleavage polyembryony and promoting the synchronous maturation of somatic embryos of many conifer species (ATTREE and FOWKE 1993). Abscisic acid-treated somatic embryos of *Picea abies* became firmer and obtained a smoother surface. They resembled zygotic embryos in that they had a similar appearance, similar growth habit and similar culturing requirements under *in vitro* conditions (VON ARNOLD and HAKMAN 1988a).

High molecular weight osmotica, such as PEG and dextrans, are commonly used in conjunction with ABA to promote maturation (ATTREE and FOWKE 1993). KONG and YEUNG (1995) found that PEG can stimulate endogenous ABA production in white spruce. Osmotica subject the cells to a stress that is similar to drought conditions, which in turn stimulates the deposition of storage reserves (ATTREE and FOWKE 1993). White spruce somatic embryo maturation was improved, storage lipid levels were increased to levels of zygotic embryos, and embryo desiccation tolerance was achieved using PEG in embryo development medium (ATTREE, POMEROY and FOWKE 1992). The importance of increasing the osmolarity of the culture medium is that it aids in promoting the formation of large size embryonal heads which in turn improve the level and quality of embryo maturation (GUPTA, TIMMIS, TIMMIS, CARLSON and WELTY 1995). Maltose, either alone or in combination with glucose, was reported as a superior carbon source compared to sucrose for development of cotyledonary-stage somatic embryos of loblolly pine (UDDIN, DINUS and WEBB 1990; UDDIN 1993).

7.2. Materials and Methods

7.2.1. Maturation Medium

Two different media were used in an attempt to mature tissue recovered from cryopreservation. The first medium used was MSG5 medium (JONES 1994). This medium was comprised of the MSG basal medium (BECWAR, NAGMANI and WANN 1990) with modifications listed in Table 4. The second medium used was the improved development and maturation medium (240) of PULLMAN and WEBB (1994).

Tissue that had recovered from cryopreservation was bulked up using suspension cultures as described in Chapter 3.

Prior to tissue being placed onto MSG5 maturation medium, the tissue was cultured on hormone free pre-development medium. Pre-development medium consisted of MSG basal medium supplemented with 0.1 g l^{-1} myo-inositol; 1.45 g l^{-1} L-glutamine; 30 g l^{-1} sucrose; 5 g l^{-1} activated charcoal; and 8 g l^{-1} Uni-lab agar. The pH of the medium was

Table 4. Formulation for MSG5 and 240 maturation media.

Inorganic compounds	MSG	240
NH_4NO_3	-	200
KNO_3	100	909.9
$Ca(NO_3)_2.4H_2O$	-	236.2
$MgSO_4.7H_2O$	370.0	256.5
KH_2PO_4	170	136.1
$CaCl_2.2H_2O$	440	-
KCl	745	-
KI	0.83	4.15
H_3BO_3	6.2	15.5
$MnSO_4.H_2O$	16.9	10.5
$ZnSO_4.7H_2O$	8.6	14.4
$Na_2MoO_4.2H_2O$	0.25	0.125
$CuSO_4.5H_2O$	0.025	0.125
$CoCl_2.6H_2O$	0.025	0.125
$MgCl_2.6H_2O$	-	101.7
$FeSO_4.7H_20$	27.8	13.9
Na_2EDTA	37.3	18.65
Vitamins and amino acids (mg l^{-1})		
Nicotinic acid	0.5	0.5
Pyroxidine-HCl	0.1	0.5
Thiamine-HCl	0.1	1.0
Glycine	-	2.0
Supplements (g l^{-1})		
Myo-inositol	0.1	0.1
Casein hydrolysate	-	0.5
L-glutamine	1.5	0.45
Plant growth regulators (mg l^{-1})		
ABA	10	10
Carbohydrates and gelling agent (g l^{-1})		
Maltose	60	60
Gelrite®	3	3
pH	5.8	5.7

adjusted to 5.8 prior to autoclaving, and the L-glutamine was filter sterilised and added to the medium once it had cooled to 60°C.

Various concentrations of the osmoticum PEG 4000 (5 %, 7.5 % and 10 %) were added to the MSG5 maturation medium. In an effort to ensure proper setting of the media, the PEG component was autoclaved separately. The maltose was also autoclaved separately, and then added to the medium. The L-glutamine was dissolved in the ABA, the pH was adjusted to 5.8 and the solution was filter sterilised, prior to addition to the cooled medium.

The 240 medium was supplemented with PEG 8000 also in various concentrations (0 %, 5 %, 7.5 % and 10 %). Once more the basal medium was autoclaved separately, as was the maltose and the PEG. These components were combined after autoclaving. The pH of the L-glutamine and the ABA was adjusted to 5.7 and the solution was filter sterilised prior to being added to the cooled medium.

7.2.2. Maturation of Recovered Embryogenic Tissue

The embryogenic tissue was bulked using suspension cultures as described in Chapter 3 prior to placing onto maturation medium. One ml aliquots of the suspensions were pipetted onto Whatman No. 1 filter paper and the supernatant was drawn off using a vacuum. One genotype (G1) was placed onto MSG5 medium and four genotypes (G1, G2, G3 and G4) were placed onto 240 medium. Tissue being matured on MSG5 was first placed onto pre-development medium for 7 days. The tissue was then placed onto MSG5 maturation medium for 8-10 weeks. Tissue was subcultured onto fresh medium every 2 weeks. Tissue that was placed onto 240 medium was not subjected to a pre-development step, and was subcultured every 3 weeks. Cultures were sealed with cling wrap and kept in the dark at 25°C. There were 5 replicates per treatment. Embryogenic tissue that had not been frozen or treated with cryoprotectants was used as a control.

7.2.3. Desiccation

Once the maturing embryos showed hypocotyl extension and the cotyledons were clearly visible, they were harvested. The isolated embryos were placed onto small discs of Whatman No. 1 filter paper. The filter paper discs were then placed into the wells of Nunclon® 4 well multidishes and the space around the wells filled with 4 ml sterile distilled water. The dishes were then sealed with Parafilm and the embryos allowed to desiccate under high relative humidity in the dark for 2 weeks.

7.2.4. Germination

The embryos were removed from the multidishes and placed onto hormone-free MSG5 medium containing 2 g l^{-1} Gelrite. Embryos matured on 240 medium were placed onto hormone free 240 medium solidified with 3 g l^{-1} Gelrite. After transfer to the germination medium, the somatic embryos were left in the dark for 7 d. Thereafter, the embryos were transplanted into culture tubes containing 8 ml germination medium or placed in culture vessels with 40 ml germination medium. They were placed into a growth room with a 16 h light:8 h dark photoperiod and a PAR of 67.7 μmol m^{-2} s^{-1}.

7.2.5. Acclimatization

When the radical had emerged and elongated (6-8 weeks), the somatic embryos were transferred to the greenhouse. The plantlets were placed in a mixture of pine bark, perlite and vermiculite (1:1:1). The plantlets were then sprayed with Prevacur N®, a systemic fungicide (2 ml l^{-1}) to prevent damping off. A foliar feed was applied on a fortnightly basis (1 % Kelpak solution) and watering was conducted manually. Plantlets were covered with translucent plastic covers to reduce rapid water loss and shaded with 40 % shade cloth.

7.3. Results

Within 3 weeks, stage 1 embryos were visible on all media and across all treatments (Figure 13A). These embryos were characterised by a dense embryonal apex, suspended by more elongated, translucent suspensor cells (VON ARNOLD and HAKMAN 1988a). The embryos soon developed into smooth, opaque structures subtended by suspensor tissue (Figure 13B) typical of stage 2 embryos (VON ARNOLD and HAKMAN 1988a). Stage 3 embryos characterised by small cotyledons clustered around a central meristem became visible after 5-6 weeks (VON ARNOLD and HAKMAN 1988a). The embryos now lost their translucency and were creamy in colour although still attached to the suspensor (Figure 13C). The stage 4 embryos, consisting of well formed cotyledons and an elongated hypocotyl region (Figure 13D), were removed from the tissue mass and desiccated under high humidity before being plated out onto germination medium (Figure 13 E). Abnormal embryo development was observed across all treatments. These were characterised by malformed cotyledons and thickened hypocotyls (Figure 13F), and were therefore not counted or isolated. The selected embryos were allowed to germinate in the dark for 1 week before being placed in the light. After 4 weeks on germination medium, the radical had emerged and elongated, and the cotyledons had elongated and greened (Figure 13G). These germinated somatic seedlings were transferred to the greenhouse to acclimatise and grow (Figure 13H).

A comparison of the number of healthy embryos isolated from cryopreserved tissue matured on MSG5 medium was compared to untreated, unfrozen control tissue (Table 5). The highest number of healthy embryos was isolated from the tissue treated with 0.3 M sorbitol and 10 % DMSO, the treatment that had the best tissue recovery following 24 h cryopreservation (Chapter 4, Figure 6B). All of the other cryoprotectant treatments (frozen for 24 h) did not recover well after cryopreservation, resulting in very little tissue being isolated for maturation purposes. Although abnormal embryo development was observed across all treatments, this was especially evident in recovered tissue treated with 0.4 M sorbitol and 10 % DMSO. The highest number of

Figure 13. Maturation of recovered *P. patula* embryogenic tissue. (A) Stage 1 embryo characterised by translucent embryonal head. (B) Stage 2 embryo is smoother in appearance and opaque. (C) Stage 3 embryo showing cotyledonary initials starting to develop. (D) Stage 4 embryos showing well defined cotyledons and the hypocotyls have elongated. (E) A late stage 4 embryo on germination medium showing further hypocotyl extension. (F) An abnormal embryo characterised by a short hypocotyl and cotyledons. (G) Germinated emblings showing good root growth and greening of the needles. (H) A hardened-off somatic seedling.

somatic embryos was isolated from tissue matured on the lowest concentration of PEG (5 %).

Table 5. Number of mature embryos isolated from tissue that had been frozen for 24 h. Control tissue was not treated with cryoprotectants or frozen. Tissue was matured on the MSG5 maturation medium. Dashes indicate that no embryos were isolated.

Freezing Treatment	PEG concentration		
	5 %	7.5 %	10 %
Unfrozen control	6	3	1
0.3 M sorbitol and 5 % DMSO	2	-	-
0.3 M sorbitol and 10 % DMSO	36	20	22
0.4 M sorbitol and 5 % DMSO	-	-	-
0.4 M sorbitol and 10 % DMSO	-	-	-

The number of embryos isolated from four genotypes that had been cryopreserved for 7 d was compared with unfrozen, untreated control tissue (Table 6). The embryos matured on 240 medium were more robust and developed fewer abnormalities when compared to the embryos isolated from tissue matured on MSG5 medium (Table 5). In three of the four lines, the cryopreserved tissue produced healthier embryos than the control tissue (Table 6). The only exception was observed in genotype G3. The recovered tissue of this genotype had more deformed and precociously germinating embryos than the control tissue. The control tissue had well formed embryos, and did not produce many abnormal embryos. In general, higher numbers of embryos were isolated from tissue cultured on media supplemented with 7.5 and 10 % PEG. Tissue matured on 240 medium without PEG produced healthy embryos, but at a slower rate. Some of the embryos germinated precociously on the medium. Very few of these isolated embryos germinated and produced roots. Hypocotyl extension and greening of the embryos, but no root emergence was common. Contamination reduced the

number of surviving somatic seedling, so statistical analysis was not possible. Only two somatic seedlings were successfully acclimitised. Optimisation of the germination and acclimatisation technique would probably result in higher recovery rates of somatic seedlings.

Table 6. A comparison of the average number of embryos isolated from four genotypes cryopreserved for 7 d. Control tissue was not treated or frozen. The tissue was matured on the 240 maturation medium.

Tissue	PEG concentration			
Genotype	0 %	7.5 %	10 %	13 %
G1	3.2	4.5	11.5	contam
G1 (control)	1	1.4	2	3.8
G2	3.2	28	58.6	59
G2 (control)	1	3	5.4	1.2
G3	29.6	20.2	15	contam
G3 (control)	13.4	29	24	14.3
G4	9.8	13.2	10.8	8.8
G4 (control)	3.5	0	1	4

7.4. Discussion

The results of the above experiments indicate that cryopreserved tissue produces slightly higher numbers of embryos than untreated, unfrozen tissue (Table 5 & 6). Contamination of plates prevented the data from being statistically analysed, as there were insufficient replicates. BERCETCHE, GALERNE and DEREUDDRE (1990) reported that cryopreservation improves the numbers and rate of development of embryos. However, NØRGAARD, BALDURSSON and KROGSTRUP (1993) and CYR, LAZAROFF, GRIMES, QUAN, BETHANE, DUNSTAN and ROBERTS (1994) found these results to be transient, as they did not encounter such effects. CYR, LAZAROFF,

GRIMES, QUAN, BETHANE, DUNSTAN and ROBERTS (1994) noted that cryoprotectant treatment, cryopreservation, and the duration of storage, did not reduce the potential for embryo production. DAVID, LAINÉ and DAVID (1995) found that the emblings (somatic seedlings) performed as well as untreated, unfrozen emblings once they were transplanted outside. Although germination and acclimatisation results were poor for this experiment, later experiments have yielded high numbers of somatic seedlings from tissue recovered from cryostorage. These seedlings grew at a rate similar to somatic seedlings derived from untreated, unfrozen embryogenic tissue (JONES pers. comm. 1999). GALERNE and co-workers (as reviewed by GUPTA, TIMMIS, TIMMIS, CARLSON and WELTY 1995) have suggested that the beneficial effect of cryogenic storage on embryogenic capacity is related to the elimination of non-embryogenic cells from the cultures. It might also result from increased synchrony of development from embryo heads, which are the only tissue surviving immersion in liquid nitrogen when cryopreserved using DMSO as a cryoprotective substance.

Tissue exposed to concentrations of DMSO between 2-10 %, may incur genetic and/or epigenetic changes (FINKLE, ZAVALA and ULRICH 1985). This may be an explanation as to why the tissue treated with 0.4 M sorbitol and 10 % DMSO did not perform well at all (Table 5). The combined effect of the two cryoprotectants could have damaged the DNA sufficiently to prevent normal tissue regeneration.

The choice of the maturation medium also has an effect on the number of mature somatic embryos produced. The number of embryos isolated from MSG5 medium (Table 5) was lower in comparison with the amount harvested from 240 medium (Table 6). This finding was confirmed by JONES (pers. comm. 1999), who found that the number of embryos isolated from the latter treatment was 2-3 times greater than that isolated from the MSG5 treatment.

CHAPTER 8

CONCLUSIONS

The traditional method of propagating *Pinus patula* is through seed collected from superior trees. However, the family forestry approach is slow to realise genetic gains. Clonal forestry offers a means of producing a more uniform crop and capturing genetic gains more efficiently. Somatic embryogenesis is one of the tools used to make clonal forestry possible by eliminating the need to maintain hedge plants for long periods of time and hence reduce the need for large amounts of space for hedge propagation. Somatic embryos can also be used in genetic engineering to further enhance genetic gains in tree breeding. Cryopreservation of embryogenic tissue has been able to take clonal forestry one step further by allowing the storage of superior germplasm without the intensive labour costs, storage facilities and hedge management required for traditional breeding.

Using techniques developed in this study, embryogenic tissue from a number of different genotypes was successfully cryopreserved for up to two months. Practical application of these cryopreservation techniques has so far ensured cryostorage of tissue for six months (JONES pers. comm. 1999). The embryogenic tissue was recovered from cryopreservation and matured, producing somatic seedlings. The major limitation of this procedure was that genotypes that were freeze-intolerant did not perform well once recovered from cryopreservation.

Researchers have found that cryopreservation does not induce somaclonal variation in embryogenic tissue (CYR, LAZAROFF, GRIMES, QUAN, BETHANE, DUNSTAN and ROBERTS 1994) and that in some cases improved embryo maturation can result from cryostorage (BERCETCHE, GALERNE and DEREUDDRE 1990). This has opened new

avenues for the transformation of embryogenic tissue with genes for disease resistance, better wood pulping qualities and drought resistance. Stocks of transformed tissue could be cryogenically stored in the juvenile state eliminating the need for maintaining hedge plants, or important genotypes *in vitro* while field trials are conducted and evaluated.

Another avenue of research that could be investigated is the cryopreservation of mature embryos or the encapsulation of embryos to produce artificial seeds. The major limitation to this form of storage is that the embryos and artificial seed would be bulky and take up more room, whereas cryopreservation of embryogenic tissue enables the storage of unlimited numbers of embryos as the tissue can be bulked up for mass production.

The use of embryogenic suspension cultures in bioreactors and artificial seed technology is progressing (HARRY and THORPE 1994). Artificial seeds, obtained through the encapsulation of somatic embryos after desiccation, provide a means of lowering the cost of producing clonal material, increasing the long-term storage of somatic embryos and a mechanism through which the field planting process can be mechanised.

References

AFELE JC, SENARATNA T, McKERSIE and SAXENA PK (1992) Somatic embryogenesis and plant regeneration from zygotic embryo culture in blue spruce (*Picea pungens* Engelman). *Plant Cell Reports* **11**: 299-303.

ATTREE SM, BUDIMIR S and FOWKE LC (1990) Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings from stored seeds of black and white spruce (*Picea mariana* and *Picea glauca*). *Canadian Journal of Botany* **68**: 30-34.

ATTREE SM, POMEROY MK and FOWKE LC (1992) Manipulation of conditions for the culture of somatic embryos of white spruce for improved triacylglycerol biosynthesis and desiccation tolerance. *Planta* **187**: 395-404.

ATTREE SM and FOWKE LC (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. *Plant Cell, Tissue and Organ Culture* **35**: 1-35.

BECWAR MR, NOLAND TL and WANN SR (1987) Somatic embryo development and plant regeneration from embryogenic Norway spruce callus. *Tappi Journal*: 155-160.

BECWAR MR, NAGMANI R and WANN SR (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). *Canadian Journal of Forest Research* **20**: 810-817.

BENKRIMA L, VON ADERKAS P, SCHMIDT WC and McDONALD KJ (1995) *In vitro* embryogenesis in larch. *General Technical Report Intermountain Research Station* USDA Forest Service No. INT-GTR 319: 412-416.

BERCETCHE J, GALERNE M and DEREUDDRE J (1990) Augmentation des capacités de régénération de cals embryogénes de *Picea abies* (L.) Karst après congélation dans l'azote liquid. *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences, Paris* **310** (Ser III): 357-363.

BERJAK P, MYCOCK JD, WATT P, WESLEY-SMITH J and HOPE B (1995) Cryostorage of pea (*Pisum sativum* L.). *In*: Biotechnology in agriculture and forestry 32: Cryopreservation of plant germplasm I. (Ed) Bajaj YPS. Springer, Heidelberg. Pp 292-307.

BINDER WD and ZAERR B (1980) Freeze preservation of suspension cultured cells of a gymnosperm, Douglas-fir. *Cryobiology* **17**: 624.

BLAKESLEY D, PASK N, HENSHAW GG and FAY MF (1996) Biotechnology and the conservation of forest genetic resources: *in vitro* strategies and cryopreservation. *Plant Growth Regulation* **20**: 11-16.

BOHINSKI RC (1987) Modern concepts in biochemistry (fifth edition). Allyn and Bacon, Inc. Massachusetts.

BONGA JM (1987) Tree tissue culture applications. *In*: Advances in cell culture, Vol 5. Ed: Maramorosch K. Academic Press, Inc. Orlando, pp 209-239.

BONGA JM and VAN ADERKAS P (1992) *In vitro* culture of trees. Kluwer Academic Press Dortrecht, The Netherlands.

BOSE S and BASU SK (1977) Preliminary studies on the mitotic irregularities in onion *(Allium cepa* L.) after single and combined treatments with dimethylsulfoxide and x-rays. *Indian Biol.* **9**: 44.

BOZHKOV PV, AHN IS and PARK YG (1997) Two alternative pathways of somatic embryo origin from polyembryogenic mature stored seeds of *Pinus koraiensis* Sieb et Zucc. *Canadian Journal of Botany* **75**: 509-512.

BREARLY J, HENSHAW GG, DAVEY C, TAYLOR NJ and BLAKESLEY D (1995) Cryopreservation of *Fraxinus excelsior* L. zygotic embryos. *Cryo Letters* **16**: 215-218.

BROWN GN (1978) Protein synthesis mechanisms relative to cold hardiness. *In*: Plant cold hardiness and freezing stress. Mechanisms and crop implications. Eds: Li PH and Sakai A. Academic Press, New York. Pp 153-164.

BUDIMIR S and VUJICIC R (1992) Benzyladenine induction of buds and somatic embryogenesis in *Picea omorika* (Panicic) Purk. *Plant Cell, Tissue and Organ Culture* **31**: 89-94.

BUTTERFIELD MK (1990) Isozyme variation in a South African and a Mexican population of *Pinus patula* Scheide et Deppe. M.Sc thesis.

CHALUPA V (1985) somatic embryogenesis and plant regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. *Communicationes Instituti Forestalis Čechosloveniae* **14**: 57-63.

CHANDLER SF and YOUNG R (1995) Somatic embryogenesis in *Pinus radiata* Don. *In*: Somatic embryogenesis in woody plants, Vol. 3, Gymnosperms. Eds: Jain

SM, Gupta PK and Newton RJ). Kluwer Academic Press, Dordrecht. Pp 243-255.

CHEN THH, KARTHA KK, LEUNG NL, KURZ GWG, CHATSON KB and CONSTABEL FC (1984a) Cryopreservation of alkaloid-producing cell cultures of periwinkle (*Catharanthus roseus*). *Plant Physiology* **75**: 726-731.

CHEN THH, KARTHA KK, CONSTABEL FC and GUSTA LV (1984b) Freezing characteristics of cultured *Catharanthus roseus* L.G. don cells treated with dimethylsulfoxide and sorbitol in relation to cryopreservation. *Plant Physiology* **75**: 720-725.

CHEN THH and KARTHA KK (1987) Cryopreservation of woody species. *In*: Cell and tissue culture in forestry. Vol 2. Eds: Bonga JM and Durzan DJ. Martinim Nijhoff Publishers, Dordrecht.

CHMIELARZ P, ELLIS RH, BLACK M and MURDOCH AJ (1995) Preservation of *Quercus robar* L. embryogenic axes in liquid nitrogen. Proceedings of the Fifth International Workshop of Seeds. Reading, UK.

CYR DR, LAZAROFF WR, GRIMES SMA, QUAN G, BETHANE TD, DUNSTAN DI and ROBERTS DR (1994) Cryopreservation of interior spruce (*Picea glauca engelmanni* complex) embryogenic cultures. *Plant Cell Reports* **13**: 574-577.

DALLIMORE A and JACKSON B (1966) A handbook of Coniferae and Ginkoaecae. Forth edition. Edward Arnold Publishers Limited, London. P 464.

DAVID A, LAINÉ E and DAVID H (1995) Somatic embryogenesis in *Pinus caribaea*. *In*: Somatic embryogenesis in woody plants, Vol. 3, Gymnosperms. Eds: Jain SM, Gupta PK and Newton RJ. Kluwer Academic Press, Dordrecht. Pp 303-313.

DEBERGH P, AITKEN-CHRISTIE J, COHEN D, GROUT B, VON ARNOLD S, ZIMMERMAN R and ZIV M (1992) Reconsideration for the term 'vitrification' as used in micropropagation. *Plant Cell, Tissue and Organ Culture* **30**: 135-140.

DODDS JH and ROBERTS LW (1985) Experiments in plant tissue culture (second edition). Cambridge University Press, USA.

DONALD DGM (1987) Vegetative propagation of pines using cuttings. *South African Journal of Forestry* **140**: 1-35.

DOOLEY BJ (1986) Factors affecting rooting of conifer cuttings. Proceedings of Seminar No. 1. Department of Horticulture, University of Natal. Pp 1-27.

FAHY GM, McFARLANE DR, ANGELL CA and MERYMAN HT (1984) Vitrification as an approach to cryopreservation. *Cryobiology* **21**: 407-426.

FAHY GM (1987) Biological effects of vitrification and devitrification. *In*: The biophysics of organ cryopreservation. Eds: Pegg DE and Karow AM. Plenum Press. New York. Pp 265-300.

FEIRER R, CONKEY J and VERHAGEN S (1989) Triglycerides in embryogenic conifer calli: a comparison with zygotic embryos. *Plant Cell Reports* **8**: 207-209.

FINKLE BJ, ZAVALA ME and ULRICH JM (1985) Cryoprotective compounds in the viable freezing of plant tissues. *In*: Cryopreservation of plant cells and organs. Ed: Kartha KK. CRC Press. Boca Raton, Florida. Pp 75-113.

FIND JI, FLOTO F, KROGSTRUP P, MØLLER JD, NØRGAARD JV and KRISTENSEN MMH (1993) Cryopreservation of an embryogenic suspension culture of *Picea sitchensis* and subsequent plant regeneration. *Scandinavian Journal of Forest Research* **8**: 156-162.

FOREST OWNERS ASSOCIATION (1996) South African forestry facts. Pamphlet.

FRETZ A, JÄHNE A and LÖRZ H (1992) Cryopreservation of embryogenic suspension cultures of barley (*Hordeum vulgare* L.). *Bot acta* **105**: 140-145.

GALERNE M and DEREUDDRE J (1988) Survie de cals embryogenes d'epicea apres congelation a -196°C. *Annales de Recheres Sylvicoles* AFOCEL 1987: 7-32.

GONZALEZ-BENITO ME and PEREZ-RUIZ C (1992) Cryopreservation of *Quercus faginea* embryonal axes. *Cryobiology* **29**: 685-690.

GOODWIN TW and MERCER EI (1983) Introduction to plant biochemistry. Second edition. Pergamon Press, Oxford, England. Pp 227-272.

GROUT BWW and MORRIS GJ (1987) Freeing and cellular organisation. *In*: The effects of low temperatures on biological systems. Eds: Grout BWW and Morris GJ. Edward Arnold. London, pp 147-174.

GROUT BWW (1991a) The effect of ice during the cryopreservation of clinical systems. In: Clinical applications of cryobiology. Eds: Fuller BJ and Grout BWW. CRC Press. Boca Raton, pp 81-94.

GROUT BWW (1991b) Cryopreservation of plant cells and organs. In: Conservation of plant genetic resources through in vitro methods. Eds: Zakri AH, Normah MT, Senawi MT and Abdul Karim AG. FRIM/MNCPGR .Kuala Lumpur, pp 43-55.

GROUT B (1995) Genetic preservation of plant cells in vitro. Springer-Verlag Berlin Heidelberg, Germany.

GUEVIN TG, MICAH V and KIRBY EG (1994) Somatic embryogenesis in cultured mature zygotic embryos of *Abies balsamea*. *Plant Cell, Tissue and Organ Culture* **37**: 205-208.

GUPTA PK and DURZAN DJ(1985) Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii)* and sugar pine (*Pinus lamberiana*). *Plant Cell Reports* **4**: 177-179.

GUPTA PK and DURZAN DJ (1986) Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). *In vitro Cellular and Developmental Biology* **22**: 685-688.

GUPTA PK and DURZAN DJ (1987) Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. *Bio/Technology* **5**: 147-151.

GUPTA PK, DURZAN DJ and FINKLE BJ (1987) Somatic polyembryogenesis in embryogenic cell masses of *Picea abies* (Norway spruce) and *Pinus taeda* (loblolly pine) after thawing from liquid nitrogen. *Canadian Journal of Forestry* **17**: 1130-1134.

GUPTA PK and PULLMAN GS (1991) Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. U. S. Patent No. 5,036,007.

GUPTA PK, TIMMIS R, TIMMIS KA, CARLSON WC and WELTY EDE (1995) Somatic embryogenesis in Douglas-fir (*Pseudotsuga menziesii*). *In*: Somatic embryogenesis in woody plants, Vol. 3, Gymnosperms. Eds: Jain SM, Gupta PK and Newton RJ. Kluwer Academic Press, Dordrecht. Pp 303-313.

GUPTA PK (1996) Method for reproducing conifers by somatic embryogenesis using a maltose enriched maintenance medium. U. S. Patent No. 5,563,061.

HAFFNER V, ENJALRIC F, LARDET L and CARRON MP (1991) Maturation of woody plants: a review of metabolic and genomic aspects. *Annales des Sciences Forestieres* **48**: 615-630.

HÄGGMANN HM, RYYNÄNEN LA, ARONEN TS and KRAJNAKOVA J (1998) Cryopreservation of embryogenic cultures of Scots pine. *Plant Cell, Tissue and Organ Culture* **54**: 45-53.

HAKMAN I, FOWKE LC, VON ARNOLD S and ERIKSSON T (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). *Plant Science* **38**: 53-59.

HAKMAN I (1993) Embryogeny in Norway spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition reserves during seed development and somatic embryogenesis. *Plant Physiology* **87**: 148-159.

HARGREAVES CL and SMITH DR (1994) Cryopreservation of *Pinus radiata* embryogenic tissue. International Plant Propagators Society Combined Proceedings **42**: 327-333.

HARGREAVES CL, WARR AA, GRACE LJ and SMITH DR (1995) Cryopreservation and plant regeneration of selected genotypes and transformed embryogenic tissue of *Pinus radiata*. Conifer Biotechnology Working Group: 7th International Conference. Surfers Paradise, Queensland, Australia.

HARRY IS and THORPE TA (1991) Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. *Botanical Gazette Chicago* **152**: 446-452.

HARRY IS and THORPE TA (1994) *In vitro* culture of forest trees. *In*: Plant Cell and Tissue Culture. Eds: Vasil IK and Thorpe TA. Kluwer Academic Publishers, Dordrecht. Pp 539-560.

HARRY IS and THORPE TA (1998) Micropropagation of *Pinus sylvestris* via somatic embryogenesis. IX International Congress of Plant Tissue and Cell Culture. Jerusalem, Israel.

HARTMANN S, LANG H and REUTHER G (1992) Differentiation of somatic embryos from protoplasts isolated from embryogenic suspension cultures of *Abies alba* L. *Plant Cell Reports* **11**: 554-557.

HAUPTMANN RM and WIDHOLM JM (1982) Cryostorage of cloned amino acid analog-resistant carrot and tobacco suspension cultures. *Plant Physiology* **70**: 30-34.

HERVÁS JP and GIMÉNEZ-MARTIN G (1973) Dimethylsulfoxide effect on division of cells. *Experientia* **29**: 1540.

HUANG JQ, WEI ZM and XU ZH (1995) Somatic embryogenesis and plantlet regeneration from callus of mature zygotic embryos of masson pine. *Acta Botanica Sinica* **37**: 289-294.

IHRKE CA and KRONSTAD WE (1975) Genetic recombination in maize as affected by ethylenediaminetetraacetic acid and dimethylsulfoxide. *Crop Science* **15**: 429.

INGRAM B and MAVITUNA F (1998) *Picea sitchensis* somatic embryogenesis: proliferation and maturation bioreactors. IX International Congress of Plant Tissue and Cell Culture. Jerusalem, Israel.

International Board for Plant Genetic Resources (IBPGR) (1985). Annual Report 1984, IBPGR, Rome.

ISHII K (1991) Somatic embryo formation and plantlet regeneration through embryogenic callus from mature zygotic embryos of *Picea jezoensis* var. *hondoensis, Picea jenzoensis* and *Picea glehnii. Journal of the Japanese Forestry Society* **73**: 24-28.

ISHIKAWA M, TANDON P, SUZUKI M and YAMAGUISHI-CIAMPI A (1996) Cryopreservation of bromegrass (*Bromus inermis* Leyss) suspension cultured cells using slow prefreezing and vitrification procedures. *Plant Science* **120**: 81-88.

JASIK J, SALAJOVA T and SALAJ J (1995) Developmental anatomy and ultrastructure of early somatic embryos in European black pine (*Pinus nigra* Arn.). *Protoplasma* **185**: 205-211.

JONES NB, VAN STADEN J and BAYLEY AD (1993) Somatic embryogenesis of *Pinus patula. Journal of Plant Physiology* **142**: 366-372.

JONES NB (1994) Somatic embryogenesis of *Pinus patula* Scheide et Deppe. Ph.D thesis. University of Natal, Pietermaritzburg.

JONES NB and VAN STADEN J (1995) Plantlet production from somatic embryos of *Pinus patula. Journal of Plant Physiology* **145**: 519-525.

JONES NB and VAN STADEN J (1997) The effect of a seaweed application on the rooting of pine cuttings. *South African Journal of Botany* 63: 141-145.

JÖRGENSEN J (1990) Conservation of valuable gene resources by cryopreservation in some forest tree species. *Journal of Plant Physiology* **136**: 373-376.

KACPERSKA-PALACZ A (1978) Mechanisms of cold acclimatisation in herbaceous plants. *In*: Plant cold hardiness and freezing stress. Mechanisms and crop implications. Eds: Li PH and Sakai A. Academic Press, New York. pp 139-153.

KARTHA KK (1985) Meristem culture and germplasm preservation. *In*: Cryopreservation of plant cells and organs. (Ed) Kartha KK. CRC Press Inc.,

Boca Raton, Florida. Pp 115-134.

KARTHA KK, FOWKE LC, LEUNG NL, CASWELL KL and HAKMAN I (1988) Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). *Journal of Plant Physiology* **132**: 529-539.

KARTHA KK and ENGELMANN F (1994) Cryopreservation and germplasm storage. *In*: Plant cell and tissue culture. Eds: Vasil IK and Thorpe TA. Kluwer Academic Press, Netherlands. Pp 195 - 230.

KEINONEN-METTÄLÄ K, JALONEN P, EUROLA P, VON ARNOLD S and VON WEISSENBERG K (1996) Somatic embryogenesis in *Pinus sylvestris*. *Scandinavian Journal of Forest Research* **11**: 242-250.

KERMODE AR (1990) Regulatory mechanisms involved in the transition from seed development to germination. CRC critical review. *Plant Science* **9**: 155-195.

KLIMASZEWSKA K, WARD C and CHELIAK WM (1992) Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix* x *eurolepis*) and black spruce (*Picea mariana*). *Journal of Experimental Botany* **43**: 73-79.

KLIMASZEWSKA K, DEVANTIER Y, LACHANCE D, LELU MA and CHAREST PJ (1997) *Larix laricina* (tamarack): somatic embryogenesis and genetic transformation. *Canadian Journal of Forest Research* **27**: 538-550.

KLIMASZEWSKA K and SMITH DR (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiologia Plantarum* **100**: 949-957.

KONG L and YEUNG EC (1995) Effects of silver nitrate and polyethylene glycol on white spruce (*Picea glauca*) somatic embryo development: enhancing cotyledonary embryo formation and endogenous ABA content. *Physiologia Plantarum* **93**: 298-304.

KRAJCOVA D (1993) Tvorba embryogenneho kalusu na nezrelych embryach *Pinus bungeana* Zucc. ex Endl. a *P. banksiana* Lamb. in vitro. *Folia Dendrologica* **20**: 203-208.

KRAKORIAN AD and SIMOLA LK (1999) Totipotency, somatic embryogenesis, and Harry Waris (1893-1973). *Physiologia Plantarum* **105**: 348-355.

KRISTENSEN MMH, FIND JI, FLOTO F, MØLLER JD, NØRGAARD JV and KROGSTRUP P (1994) The origin and development of somatic embryos following cryopreservation of an embryogenic suspension culture of *Picea*

sitchensis. *Protoplasma* **182**: 65-70.

KUOKSA T and HOHTOLA A (1991) Freeze-preservation of buds from Scots pine trees. *Plant Cell, Tissue and Organ Culture* **27**: 89-93.

LAINÉ E and DAVID A (1990) Somatic embryogenesis in immature embryos and protoplasts of *Pinus caribaea*. *Plant Science Limerick* **69**: 215-224.

LAINÉ E, BADE P and DAVID A (1992) Recovery of plants from cryopreserved embryogenic cell suspensions of *Pinus caribaea*. *Plant Cell Reports* **11**: 295-298.

LANGIS R and STEPONKUS PL (1990) The toxicity of vitrification solutions by their osmotic potential. *Cryobiology* **27**: 654.

LANTERI S, BELLETTI P and LOTITO S (1993) Storage of pollen of Norway spruce and different pine species. *Silvae Genetica* **42**: 104-109.

LELU MAP and BORNMAN CH (1990) Induction of somatic embryogenesis in excised cotyledons of *Picea glauca* and *Picea mariana*. *Plant Physiology and Biochemistry Paris* **28**: 785-791.

LELU MA, KLIMASZEWSKA K and CHAREST PJ (1994) Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of *Larix*. *Canadian Journal of Forest Research* **24**: 100-106.

LEVITT J (1980) Responses of plants to environmental stress, Vol.1. Chilling, freezing and high temperature stress. Academic Press, New York.

LI YH and GUO ZS (1990) Somatic embryogenesis and plantlet formation of *Picea wilsonii* under different conditions. *Acta Botanica Sinica* **32**: 568-570.

LIAO YK and AMERSON HV (1995) Slash pine (*Pinus elliottii* Engelm.) somatic embryogenesis I. Initiation of embryogenic cultures from immature zygotic embryos. *New Forests* **10**: 145-163.

LIAO YK and AMERSON HV (1995) Slash pine (*Pinus elliottii* Engelm.) somatic embryogenesis II. Maturation of somatic embryos and plant regeneration. *New Forests* **10**: 165-182.

MAZUR P (1969) Freezing injury in plants. *Annual Review of Plant Physiology* **20**: 419-448.

McFARLANE DR and FORSYTH M (1987) Devitrification and recrystallisation of glass-forming aqueous solutions. *In*: The biophysics of organ cryopreservation. Eds: Pegg DE and Karow AM. Plenum Press. New York, pp 237-264.

McLELLAN MR and DAY JG (1995) Methods in molecular biology, Vol. 38: Cryopreservation and freeze-drying protocols. Humana Press Inc., Totowa, NJ.

MEHRA-PALTA A and THOMPSON DG (1988) *In vitro* propagation of conifers. *In*: Forest and crop biotechnology: progress and prospects. Ed: Valentine FA. Springer-Verlag, New York, pp 12-34.

MERYMAN HT and WILLIAMS RJ (1985) Basic principles of freezing injury to plant cells: natural tolerance and approaches to cryopreservation. *In*: Cryopreservation of plant cells and organs. Ed: Kartha KK. CRC Press. Boca Raton. pp 13-48.

MORRIS GJ and McGRATH JJ (1981) Intracellular ice nucleation and gas bubble formation in *Spirogyra*. *Cryo Letters* **2**: 341-352.

MURASHIGE T and SKOOG F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

NAGMANI R, DINER AM and SHARMA GC (1993) Somatic embryogenesis of longleaf pine (*Pinus palustris*). *Canadian Journal of Forest Research* **23**: 873-876.

NØRGAARD JV and KROGSTRUP P (1991) Cytokinin induced somatic embryogenesis from immature embryos of *Abies nordmanniana* Lk. *Plant Cell Reports* **9**: 509-513.

NØRGAARD JV, BALDURSSON Sand KROGSTRUP P (1993) Genotypic differences in the ability of embryogenic *abies nordmanniana* cultures to survive cryopreservation. *Silvae Genetica* **42**: 93-97.

OWENS JN and BLAKE MD (1985) Forest tree seed production. Patawawa National Forestry Information Report. P1-X-53

PAQUES M, POISSONNIER M, DUMAS E, MONAD V and ALTMANA (1997) Cryopreservation of dormant and non-dormant broad-leafed trees. *Acta Horticulturae* **447**: 491-497.

PITA JM, BARRERO S and ESCUDERO A(1997) Cryopreservation: an alternate method for the conservation of endangered populations of two Iberian pines (*Pinus nigra* Arnold and *Pinus sylvestris* L.). *Silvae Genetica* **46**: 250-252.

PITA JM, SANZ V and ESCUDERO A (1998) Seed cryopreservation of seven Spanish native pine species. *Silvae Genetica* **47**: 220-223.

POLGE C, SMITH AU and PARKES AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164: 666.

PULLMAN GS and WEBB DT (1994) An embryo staging system for comparison of zygotic and somatic embryo development. *Biological Science Symposium* pp 31-34.

RAJBHANDARI N and STOMP AM (1997) Embryogenic callus induction in Fraser fir. *HortScience* **32**: 737-738.

RALL WF and FAHY GM (1985) Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* **313**: 573-575.

RAVEN PH, EVERT RF and EICHHORN SE (1986) Biology of Plants. Forth edition. Worth Publishers, Inc. New York. P 340.

REINHOUD PJ, URAGAMI A, SUKAI A and VAN IREN F (1995) Vitrification of plant cell suspensions. *In:* Methods in molecular biology, Vol 38: Cryopreservation and freeze-drying protocols. Eds: McLellan MR and Day JG. Humana Press Inc., Totowa, NJ.

RUAUD JN, BERCETCHE J and PAQUES M (1992) First evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. *Plant Cell Reports* **11**: 563-566.

RYYNÄNEN L (1996) Cold hardening and slow cooling: tools for successful cryopreservation and recovery of in vitro shoot tips of silver birch. *Canadian Journal of Forest Research* **26**: 2015-2022.

SAKAI A (1966) Survival of plant tissues at super-low temperatures. IV . Cell survival with rapid cooling and rewarming. *Plant Physiology* **41**: 1050-1054.

SALAJOVA T, JASIK J, KORMUTAK A, SALAJ J and HAKMAN I (1996) Embryogenic culture initiation and somatic embryo development in hybrid firs (*Abies alba X Abies cephalonica*, and *Abies alba X Abies numidica*). *Plant Cell Reports* **15**: 527-530.

SALISBURY FB and ROSS CW (1992) Plant physiology. Fourth edition. Wadsworth Publishing Company, Belmont, California. Pp 172-173.

SCJRIJNEMAKERS EWM and VAN IREN F (1995) A two-step or equilibrium freezing procedure for the cryopreservation of plant cell suspensions. *In*: Methods in molecular biology, Vol. 38: Cryopreservation and freeze-drying protocols. (Eds) Day JG and McLellan MR. Humana Press, Totowa, New Jersey. Pp 103-111.

SMITH DR (1995) Growth medium for conifer embryogenic tissue. Patent No. WO 95/33822.

STEPONKUS PL and LANPHEAR FO (1967) Refinement of the triphenyltetrazolium chloride method of determining cold injury. *Plant Physiology* **4**: 1432-1436.

STEPONKUS PL (1984) Role of the plasma membrane in freezing injury and cold acclimatization. *Annual Review of Plant Physiology* **35**: 543-584.

SUSS R, EWALD D and MATSCHKE J (1990) Production of somatic embryos from Norway spruce (*Picea abies*) seed. *Beitrage für die Fortwirdschaft* **24**

TANDORF S, McGRATH JJ and OLIEN CR (1987) On the adhesive interaction between ice and cell-size liposomes. *Cryo Letters* **8**: 322-328.

TANG W, OUYANG F and GUO ZG (1997) Plantlet regeneration via somatic embryogenesis in slash pine. *Journal of Plant Resources and Environment* **6**: 8-11.

TANG W, OUYANG F and GUO ZC (1998) Changes in growth parameters of embryogenic cell suspension cultures of loblolly pine (*Pinus taeda*). *Journal of Tropical and Subtropical Botany* **6**: 30-34.

TAUTORUS TE, ATTREE SM, FOWKE LC and DUNSTAN DI (1990) Somatic embryogenesis from immature and mature zygotic embryos, and embryo regeneration from protoplasts in black spruce (*Picea mariana* Mill.).*Plant Science Limerick* **67**: 115-124.

TAUTORUS TE, FOWKE LC and DUNSTAN DI (1991) Somatic embryogenesis in gymnosperms. *Canadian Journal of Botany* **69**: 1873-1899.

THOMPSON RM and THORPE TA (1987) Metabolic and non-metabolic roles of carbohydrates. *In:* Cell and tissue culture in forestry Vol.1 General principles and biotechnology. Eds: Bonga JM and Durzan DJ. Martinus Nijhoff Publishers, Dordrecht, The Netherlands. Pp 89-112.

THOMPSON RG and VON ADERKAS P (1992) Somatic embryogenesis and plant regeneration from immature embryos of western larch. *Plant Cell Reports* **11**: 379-385.

THORPE TA and MEIER DD (1973) Sucrose metabolism during tobacco callus growth. *Phytochemistry* **12**: 493-497.

THORPE TA (1974) Carbohydrate availability and shoot formation in tobacco callus cultures. *Physiologia Plantarum* **30**: 77-81.

TOIVONEN PMA and KARTHA KK (1989) Cryopreservation of cotyledons of nongerminated white spruce (*Picea glauca* (Moench) Voss) embryos and

subsequent plant regeneration. *Journal of Plant Physiology* **134**: 766-768.

TOWILL LE and MAZUR P (1976) Osmotic shrinkage as a factor in freezing injury in plant tissue cultures. *Plant Physiology* **57**: 290.

TREMBLAY FM(1990) Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. *Canadian Journal of Botany* **68**: 236-242.

TREMBLAY L and TREMBLAY FM (1995) Maturation of black spruce somatic embryos: Sucrose hydrolysis and resulting pressure of the medium. *Plant Cell, Tissue and Organ Culture* **42**: 39-46.

UDDIN MR, DINUS RJ and WEBB DT (1990) Effects of different carbohydrates on maturation of *Pinus taeda* somatic embryos. Abstracts VII International Congress of Plant Tissue and Cell Culture, June 24-29, 1990. Amsterdam, Netherlands. P 272.

UDDIN MR (1993) Somatic embryogenesis in gymnosperms. U.S. Patent No. 5,187,092.

ULRICH JM, FINKLE BJ, MOORE PH and GINOSA (1979) Effect of a mixture of cryoprotectants in attaining liquid nitrogen survival of callus cultures of a tropical plant. *Cryobiology* **16**: 550-556.

ULRICH JM, MICKLER RA, FINKLE BJ and KARNOSKY DF (1984) Survival and regeneration of American elm callus after being frozen in liquid nitrogen. *Canadian Journal of Forest Research* **14**: 750-753.

VERMA DC and DOUGALL DK (1977) Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. *Plant Physiology* **59**: 81-85.

VERRYN (1997) Clonal forestry. Southern African institute of forestry. *Continuous Improvement in Forestry Symposium Proceedings.*

VON ARNOLD S and HAKMAN I (1988a) Regulation of somatic embryo development in *Picea abies* by abscisic acid (ABA). *Journal of Plant Physiology* **132**: 164-169.

VON ARNOLD S and HAKMAN I (1988b) Plantlet regeneration *in vitro* via adventitious buds and somatic embryos in Norway spruce (*Picea abies*). In: Genetic manipulation of woody plants. Basic life sciences 44. Eds: Hanover JW and Keathley DE. Plenum Press, New York and London. Pp 199-215.

VON ADERKAS P, KLIMASZEWSKA K and BONGA JM (1990) Diploid and haploid embryogenesis in *Larix leptolepis, L. decidua* and their reciprical hybrids. *Canadian Journal of Forest Research* **20**: 9-14.

VON ADERKAS P (1992) Embryogenesis from protoplasts of haploid European larch. *Canadian Journal of Forest Research* **22**: 397-402.

VON ARNOLD S, EGERTSDOTTER U, EKBERG I, GUPTA P, MO H and NØRGAARD J (1995) Somatic embryogenesis in Norway spruce (*Picea abies*). In: Somatic embryogenesis in woody plants Vol 3- Gymnosperms. Eds: Jain SM, Gupta PK and Newton RJ. Kluwer Academic Press. Dortrecht, The Netherlands. Pp 17-36.

WEBER G, ROTH EJ and SCHWEIGER HG (1983) Storage of cell suspensions and protoplasts of *Glycine max* (L.) Merr, *Brassica napus* (L.), *Datura innoxia* (Mill.) and *Daucus carota* (L.) by freezing. *Zeitschrift für Pflanzenphysioloy* **109**: 29-39.

WEBSTER FB, ROBERTS DR, McINNIS SM and SUTTON BCS (1990) Propagation of interior spruce by somatic embryogenesis. *Canadian Journal of Forest Research* **20**: 1759-1765.

WIDHOLM JM (1972) The use of fluoresceine diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology* **47**: 189-194.

WITHERS LA and STREET HE (1977) The freeze-preservation of plant cell cultures. *In*: Plant tissue culture and its biotechnological application. (Eds) Reinhard WE and Zenk MH. Springer, Berlin, Heidelberg, New York. Pp 226-246.

WITHERS LA and KING PJ (1979) Proline: A novel cryoprotectant for the freeze-preservation of cultured cells of *Zea mays* L. *Plant Physiology* **64**: 675-678.

WITHERS LA (1980) *Tissue Culture Storage for Genetic Conservation*. Technical report for the International Board for Plant Genetic Resources, Rome.

WITHERS LA (1985) Cryopreservation of cultured cells and meristems. *In*: Cell culture and somatic cell genetics of plants. Vol 2. Ed: Vasil JK. Academic Press, Orlando. Pp 253-316.

WITHERS LA (1986) Cryopreservation and gene banks. *In*: Plant cell technology. Ed: Yeoman MM. Blackwell Scientific Publications, Oxford. Pp 96-140.

WORMALD (1975) *Pinus patula*: Tropical Forestry Papers no.7. Department of Forestry, Commonwealth Forestry Institute, University of Oxford.

YANG JL, GUI YL, YANG YG, DING QX and GUO ZC (1997) Somatic embryogenesis and plantlet regeneration in mature zygotic embryos of *Picea meyeri*. *Acta*

Botanica Sinica **39**: 315-321.

ZOGLAUER K, DEMBNY H and BEHRENDT U (1992) Pflanzenregeneration aus Protoplasten von *Larix decidua* L. durch somatische Embryogenese. *Wissenschaftliche Zeitschrift der Humboldt Universitat zu Berlin. Reihe Matematische Naturwissenschaftliche* **41**: 51-62.