

**SOME INVESTIGATIONS TOWARDS THE
CRYOPRESERVATION OF SUGARCANE
GERMPLASM**

by

ASHIKA JAIMANGAL

Submitted in fulfillment of the
academic requirements for the degree of
Master of Science
in the
School of Biological and Conservation Sciences
Westville Campus
University of KwaZulu-Natal
Durban
February 2009

Prof. P. Berjak
Supervisor

Prof. N.W. Pammenter
Co-supervisor

Abstract

Sugarcane has become an increasingly important crop in recent years, with South Africa featuring as one of the prominent producers. This has led to a significant growth in the South African sugarcane industry, translating into an increased demand for planting material. Although this demand is now satisfied by recent biotechnological advancements such as protocols for somatic embryogenesis to increase the production of planting material, such techniques are limited as a result of the progressive loss of the embryogenic potential of calli over time. In order to facilitate management of this material, it is desirable to develop a protocol for the long-term storage of the germplasm.

This study reports on investigations of the different parameters that influenced the cryo-process in attempts to develop a protocol for the successful cryopreservation of sugarcane somatic embryos of the 88H0019 variety. Experiments were carried out to determine *in vitro* culture conditions for successful induction of somatic embryos via both the direct and indirect routes of micropropagation. A suitable regeneration medium for plantlet establishment pre- and post-cooling was established (Chapter 2). Investigations were also carried out to ascertain the responses of somatic embryos to both rapid and slow dehydration techniques (Chapter 3). Finally, several cooling techniques (both slow and rapid), were applied, on partially dehydrated somatic embryos, either without, or after cryoprotection, in an attempt to achieve survival after cryopreservation of the somatic embryos (Chapter 4).

Both directly- and indirectly-derived somatic embryos were converted, most successfully, on full strength Murashige and Skoog medium without addition of plant growth regulators. The initial mean water contents of directly- and indirectly-derived somatic embryos were not significantly different from each other ($8.38 \pm 0.19 \text{ g g}^{-1}$ and $8.45 \pm 0.33 \text{ g g}^{-1}$ [dry mass basis], respectively). The percentage conversion at these water contents was also not significantly different; 97% for directly- and 98% for indirectly-induced embryos.

Slow dehydration by culture on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h each was the most effective technique, with water content being reduced to 0.94 ± 0.03 g g⁻¹ and 0.95 ± 0.02 g g⁻¹ after dehydration on media containing 1.0 M sucrose, while maintaining between 98% and 100% conversion, respectively.

Of the various cryoprotectants tested, proline and casamino acid had the least adverse effects on the somatic embryos. The encapsulation-vitrification cooling technique was the most efficient of all techniques employed. The best conditions involved encapsulation of embryo clumps in a solution of MS medium with 3% (w/v) Na-alginate and loading solution containing 2 M glycerol plus 0.4 M sucrose, followed by infiltration and dehydration at 0°C for various time intervals (0, 5, 10, 15, 20, 25, 30 min) with 1 ml PVS2 solution and thereafter, rapid immersion in liquid nitrogen. Under such conditions, 30% of the cryopreserved somatic embryos retained viability, going on to form callus from which shoots and roots were produced.

Although somatic embryos of sugarcane of the local variety 88H0019 have proved to be recalcitrant to cryopreservation, the results obtained with explants that had been processed by encapsulation-vitrification suggest that this approach may be worth pursuing and refining.

Preface

The experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from August 2005 to December 2008, under the supervision of Professor P. Berjak and Professor N.W. Pammenter.

This study represents original work carried out by the author and no part of this work has been submitted in any form to another university. Where use has been made of the work of other authors, it has been duly acknowledged in the text.

A. Jaimangal

Declaration 1 – Plagiarism

I, Ashika Jaimangal declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. The thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
 - a. Their words have been re-written but the general information attributed to them has been referenced.
 - b. Where their exact words have been used, then their writing has been placed within quotation marks, and referenced.
5. This thesis does not contain text, graphs or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the Literature Cited section.

Signed.....

(February, 2009)

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ABSTRACT	ii
PREFACE	iv
DECLARATION 1 – PLAGIARISM	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	xi
LITS OF TABLES	xiii
ACKNOWLEDGEMENTS	xiv
LIST OF ABBREVIATIONS	xv

CHAPTER 1: General Introduction

1.1 Plant genetic resources conservation	1
1.2 <i>Ex situ</i> plant germplasm conservation	2
1.2.1 Field gene-banks	3
1.2.2 Seed storage	3
1.3 Tissue culture technology	6
1.3.1 Micropropagation	6
1.3.2 Aspects of cell and tissue culture	7
1.3.2.1 Organogenesis	8
1.3.2.2 Somatic Embryogenesis	9

1.3.3 Somaclonal variation	13
1.4 <i>In vitro</i> storage methods	14
1.4.1 Storage of actively growing cultures	14
1.4.2 Minimal-growth storage	14
1.4.2.1 Manipulation of culture media	15
1.4.2.2 Modification of the gaseous environment	16
1.4.2.3 Reduced incubation temperature	16
1.4.3 Synthetic seeds ('synseeds')	17
1.5 Cryopreservation	19
1.5.1 Type of explant	20
1.5.2 Dehydration of plant material	21
1.5.3 Developmental stage	23
1.5.4 Cryoprotection	24
1.6 Sugarcane and the rationale of this study	28

CHAPTER 2: Induction of somatic embryos

2.1 Introduction	32
2.2 Materials and Methods	33
2.2.1 Plant material	33
2.2.2 Induction of somatic embryos	34
2.2.3 Regeneration medium	35
2.2.4 Initial viability assays	35
2.2.5 Determination of yield	35
2.2.6 Water content determination	36

2.2.7 Statistical analysis	37
----------------------------	----

2.3 Results and Discussion	37
-----------------------------------	-----------

2.3.1 Somatic embryo induction	37
--------------------------------	----

2.3.2 Regeneration media	38
--------------------------	----

2.3.3 Determination of yield	44
------------------------------	----

2.3.4 Initial water content	45
-----------------------------	----

2.3.5 Concluding remarks	47
--------------------------	----

CHAPTER 3: Responses of Directly-Induced Somatic Embryos to Dehydration

3.1 Introduction	48
-------------------------	-----------

3.1.1 Pregrowth, preculture and encapsulation-dehydration	50
---	----

3.1.2 Desiccation damage	51
--------------------------	----

3.2 Materials and Methods	52
----------------------------------	-----------

3.2.1 Flash-drying	52
--------------------	----

3.2.2 Osmotic dehydration	53
---------------------------	----

3.3 Results and discussion	53
-----------------------------------	-----------

3.3.1 Response of somatic embryos to flash-drying	53
---	----

3.3.2 Osmotic dehydration	55
---------------------------	----

CHAPTER 4: Cryopreservation Studies

4.1 Introduction	60
4.1.1 Cryopreservation-induced injury	61
4.1.2 Cooling rates	62
4.1.2.1 Slow cooling/Cooling-induced dehydration	63
4.1.2.1a Vitrification	64
4.1.2.1b Encapsulation-vitrification	67
4.1.2.1c Encapsulation-dehydration	67
4.1.2.2 Rapid cooling	68
4.1.3 Thawing	69
4.1.4 Rehydration	71
4.1.5 Regeneration	71
4.2 Materials and Methods	72
4.2.1 Chemical cryoprotection	72
4.2.2 Cooling, rehydration and regeneration	73
4.2.2.1 Slow cooling	73
4.2.2.1a Cooling in cryovials and foil jackets	73
4.2.2.1b Cooling in Mr Frosty	74
4.2.2.1c Vitrification	75
4.2.2.1d Encapsulation-vitrification	75
4.2.2.1e Slow controlled cooling	76
4.2.2.2 Rapid cooling	77
4.2.3 Embryo conversion (regeneration)	77
4.3 Results and Discussion	78
4.3.1 Chemical cryoprotection	78
4.3.1.1 'Traditional' cryoprotectants	78
4.3.1.2 Amino acids as cryoprotectants	79

4.3.2 Cooling, rehydration and regeneration	90
4.3.2.1 Slow cooling	90
4.3.2.1a Cooling in cryovials and foil jackets	90
4.3.2.1b Cooling in Mr Frosty	91
4.3.2.1c Vitrification	92
4.3.2.1d Encapsulation-vitrification	93
4.3.2.1e Slow controlled cooling	96
4.3.2.2 Rapid cooling	97
4.3.2.2a Cryoprotection and rapid cooling	98

5: Concluding Comments	100
-------------------------------	-----

6: Literature Cited	102
----------------------------	-----

List of Figures

Figure No.	Title	Page No.
2.1	Somatic embryos (SE) at the late globular developmental stage (A), and callus (B), following 6 weeks culture on direct and indirect embryo induction media containing 0.6 and 3.0 mg l ⁻¹ 2,4-D, respectively	38
2.2	The percentage somatic embryo conversion of directly-induced somatic embryos on different regeneration media after 8 weeks <i>in vitro</i> cultured under either light and dark conditions	42
2.3	Mean water contents of freshly excised clumps of somatic embryos generated via the direct and indirect routes of micropropagation, and the corresponding average percentage conversion of the somatic embryos on PGR-free medium	46
3.1	Change in percentage conversion of somatic embryos with water content over drying time	55
3.2	Change in water content and percentage conversion of direct somatic embryos subjected to osmotic dehydration on basal MS medium containing various concentrations of sucrose	58
3.3	Change in water content and percentage conversion of indirect somatic embryos subjected to osmotic dehydration on basal MS medium containing various concentrations of sucrose	59

4.1	Change in water content and percentage conversion of directly-induced somatic embryos after treatment with various cryoprotectants	79
4.2	Change in water content and percentage conversion of directly-induced somatic embryos following treatment with 0.5 and then 1% proline	81
4.3	Change in water content and percentage conversion of directly-induced somatic embryos following treatment with 5 and then 10% proline	82
4.4	Direct shoot formation from embryo clumps pre-treated with proline at 5 and then 10% for 30 min at each concentration	82
4.5	Change in water content and percentage conversion of directly-induced somatic embryos following treatment with 0.5 and then 1% casamino acid	85
4.6	Change in water content and percentage conversion of directly-induced somatic embryos following treatment with 5 and then 10% casamino acid	86
4.7	Direct shoot formation from embryo clumps pre-treated with casamino acid at 5 and then 10% for 30 min at each concentration	87
4.8	Individually encapsulated somatic embryo clumps	93
4.9	Shoot (A) and root (B) formation from callus formed by embryo clumps cooled by encapsulation-vitrification protocol and converted on medium containing 3 mg l ⁻¹ 2,4-D in the dark for 8 weeks and subsequently transferred to medium without PGRs	94

List of Tables

Table No.	Title	Page No.
2.1	Effects of 2,4-D and BAP on the conversion capacity (%) of sugarcane somatic embryos	43
2.2	The interactive effect of the plant growth regulators (PGRs), 2,4-D or BAP, and light/dark regime on the conversion capacity (%) of sugarcane somatic embryos	44
2.3	Plantlet yield at the two culture venues, SASRI and UKZN laboratories	45
4.1	Effects of lower (0.5 and then 1%) and higher (5 and then 10%) proline immersion for different times on the conversion capacity (%) of sugarcane variety 88H0019 somatic embryos	83
4.2	Effects of casamino acid treatment at two different concentrations and for different times on the conversion capacity (%) of sugarcane variety 88H0019 somatic embryos	88
4.3	Interactive effects of treatment with loading solution (L), vitrification solution (V) or unloading solution (U) within the encapsulation-vitrification protocol, and light and dark culture conditions on the conversion capacity (%) of sugarcane variety 88H0019 somatic embryos	95

Acknowledgements

First and foremost, I would like to express my heartfelt gratitude and appreciation towards my supervisor, Prof. Patricia Berjak for her consistent assistance, guidance and support throughout this study.

I am also extremely grateful to Dr D.J. Mycock (University of Witwatersrand) for allowing me the opportunity to work in his laboratory and for his valuable input and suggestions.

Special thanks to Dr Sandy Snyman at the South African Sugar Research Institute (SASRI) for initiating me into sugarcane research.

I would also like to thank Prof. Norman Pammenter and Dr Joseph Kioko for their contributions towards this project.

Many thanks to Mrs Marzena Banasiak, Prof. Paula Watt, and husband Dr Derek Watt for kindly transporting my samples between SASRI and the University of KwaZulu-Natal.

Thanks are also due to Sershen Naidoo, Dr Boby Varghese and Dr Dalia Varghese, for their input and guidance during the writing-up of my thesis, as well as to Mr Atif Naim, for his invaluable assistance with the statistical analyses of experimental data.

A special thank you is due to Provain Kistnasamy who has assisted me in countless ways, from collection and processing of samples, to helping with tissue culture and data collection. I am extremely grateful for all he has done.

Finally, I would like to acknowledge, and express my gratitude to the National Research Foundation for the financial support received.

Abbreviations

ABA	abscisic acid
ANOVA	analysis of variance
BAP	benzylaminopurine
CAc	casamino acid
CaCl ₂	calcium chloride
CaCl ₂ .2H ₂ O	calcium chloride dihydrate
Ca/Mg	calcium magnesium (thawing/rehydration solution)
Ca(OCl) ₂	calcium hypochlorite
d	days
D	DMSO
2,4-D	2,4-dichloro-phenoxyacetic acid
dmb	dry mass basis
DMSO ; Me ₂ SO ₄	dimethyl sulphoxide
fmb	fresh mass basis
G	glycerol
G+D	glycerol plus DMSO
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ISTA	International Seed Testing Association
L	loading solution
LEAs	Late Embryogenic Accumulating Proteins
LN	liquid nitrogen
M	molar (moles per litre)
MgCl ₂ .6H ₂ O	magnesium chloride
min	minutes
mm	millimetre
mM	millimolar
ml	millilitre

MS	Murashige and Skoog
n	sample size
Na-alginate	sodium alginate
NH ₄ ⁺	ammonium ion
PGRs	plant growth regulators
PPFD	photosynthetic photon flux density
Pro	proline
PVP	polyvinylpyrrolidone
PVS	plant vitrification solution
S	sucrose
S+D	sucrose plus DMSO
SE	somatic embryos
S+G	sucrose plus glycerol
S+G+D	sucrose plus glycerol plus DMSO
TTZ	2,3,5-triphenyl-tetrazolium chloride
U	unloading solution
V	vitrification solution
WPM	woody plant medium
%	percent
°C	degrees Celcius
g g ⁻¹	gram of water per gram of dry matter
g l ⁻¹	gram per litre
h	hours
mg l ⁻¹	milligram per litre
min ⁻¹	per minute
μM	micromolar
μmol m ⁻² s ⁻¹	micromole per square metre per second
s	seconds
s ⁻¹	per second
v/v	volume per volume
w/v	weight per volume

Chapter 1: General Introduction

1.1 Plant genetic resources conservation

Biodiversity is described by Bibby *et al.* (1992) as “the total variety of life on earth including all genes, species and ecosystems and the ecological processes of which they are part.” The total naturally occurring genetic diversity, which encompasses both primitive cultivars and wild varieties (Ford-Lloyd and Jackson, 1991), has suffered immense depletion, particularly over recent decades. In his book, Bajaj (1995b) highlighted the need for conservation of plant genetic resources in the context of an estimated 2 000 species of higher plants believed to be endangered, rare and/or threatened with extinction. Factors contributing to this decline include replacement of these resources by newly developed varieties in efforts to sustain ever-increasing human populations, clearing of forests for agricultural land, industrialisation and other consequences of economic growth (Razdan and Cocking, 1997). In addition to traditional crops, the genetic resources of a large number of medicinal plants and forest trees also face the danger of extinction (Hussain, 1983; Sakai, 1986; Berjak, 2000). It has been suggested that approximately 25% of the estimated 250 000 (or more) vascular plants could become extinct by the mid 21st century (Schemske *et al.*, 1994). This clearly highlights the urgent need for the development of conservation strategies for the preservation of germplasm of highly utilised, as well as endangered or threatened, plant species.

There are two approaches for conservation of plant genetic resources, namely *in situ* and *ex situ* conservation. *In situ* conservation involves the maintenance of genetic resources in the natural habitat, whether as wild and uncultivated plant communities or crop cultivars in farmers’ fields as components of the traditional agricultural systems (so-called on-farm conservation) (Rao, 2004). The principle underlying this approach is the conservation of sufficient genetic diversity such that the full evolutionary potential of the species is realised (Benson, 2008). This approach is advantageous in that it allows for the continuation of natural selection such that species may evolve as components of the natural physical and

biological environment; and the ecosystem as a whole, is conserved (Krøgstrop *et al.*, 1992, Shands, 1993). These conservation areas, however, are readily exposed to natural disasters and predictably, global climate change, and therefore do not guarantee the preservation of biodiversity. A safer alternative or backup system is the removal of plants or their propagules from their natural habitat and conserving them under artificial storage conditions (traditionally in botanic gardens, arboreta, seedbanks or, more recently, as *in vitro* cultures), which is referred to as *ex situ* conservation (Frankel and Soule, 1981). This method of conservation functions well to protect plant species while allowing for their re-introduction into degraded natural areas.

1.2 *Ex situ* plant germplasm conservation

Germplasm accumulated through *ex situ* conservation methods is generally maintained as either active or base collections (Hawkes, 1987). Active collections are short-to-medium term collections that require relatively frequent regeneration and multiplication to retain vigour and viability. These are normally maintained as a field or greenhouse collection, making them both expensive and labour intensive. In contrast, base collections achieve long-term germplasm conservation most often via the storage of seeds (Harrington, 1972; Krøgstrop *et al.*, 1992). Biotechnology provides a complementary tool for broadening the scope of *ex situ* conservation. This is particularly relevant to recalcitrant-seeded species (i.e. species that produce seeds which cannot be stored at low moisture contents and/or are chilling sensitive [Ellis, 1984; Roberts *et al.*, 1984; Ellis *et al.*, 1991]), or to those species that require large areas if they are to be grown as conservation stands. Here, *in vitro* storage ensures cost-effective *ex situ* conservation in the form of minimal-growth cultures or cryopreserved explants, while facilitating a steady supply of germplasm that can be retrieved from storage as and when required.

1.2.1 Field gene-banks

The traditional use of field gene-banks for *ex situ* storage offers a useful but not ideal approach to conservation. An advantage is that genetic resources under conservation are readily accessible thereby allowing for detailed monitoring. However, the plants and hence the genetic resources they represent, are exposed to the risks of destruction by pests, diseases and other natural calamities such as drought, other weather-related damage, human error, theft and vandalism, etc. Also, the maintenance of field gene-banks incurs significant costs and is consequently prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance, and even their very survival in times of economic stringency (Withers and Engelmann, 1998). Even under the best circumstances, field gene-banks demand large areas of land (often requiring multiple sites for rotation), labour, management and materials. The problems presented by this form of *ex situ* conservation of plant genetic resources has promoted the development and application of suitable tissue culture systems of propagation and conservation, particularly in the case of economically important species or where a threat of extinction of a particular species exists.

1.2.2 Seed storage

Seeds represent the natural means of propagation of the great majority of higher plant species, and in many (or perhaps, most) cases, offer a suitable vehicle for germplasm conservation. However, simple seed storage is not universally applicable across species. Differences in the post-harvest physiological responses to desiccation and low temperature have resulted in the division of seeds into two broad categories, i.e. 'orthodox' and 'recalcitrant' (Roberts, 1973), while another category, that of seeds showing 'intermediate' post-harvest behaviour, was later defined (Ellis *et al.*, 1990, 1991). Seeds that are tolerant to desiccation and can be stored at low moisture (water) content under low relative humidity and low temperature conditions for predictably long periods while still maintaining viability, are termed orthodox (Roberts, 1973; Ellis, 1984; Roberts *et al.*, 1984;

Ellis *et al.*, 1991). Those that are sensitive to desiccation and therefore cannot be stored in the dehydrated condition are classified as non-orthodox or recalcitrant (Roberts, 1973; Roberts *et al.*, 1984). While viability loss is the immediate response of recalcitrant seeds to dehydration, those categorised by Ellis *et al.* (1991) and Hong and Ellis (1996) as intermediate are short-lived in the desiccated condition, when they also may be chilling-sensitive.

Storage of orthodox seeds is presently the most ideal plant germplasm conservation method since seeds occupy a relatively small space, are easily transported to various introduction centres and genebanks, and most importantly, allow for rigorous biodiversity conservation in that each seed represents a genetically unique individual. Under less than ideal storage conditions (which are the norm) the disadvantages of this mode of conservation are a loss of viability of seeds with time and susceptibility of stored seeds to insect and/or pathogen attack.

However, three categories of crops are not amenable to seed storage, and for these, *in vitro* storage appears to be most suited (Razdan, 1993). The first category includes those crop species that do not produce seeds at all, and are vegetatively propagated, for example, banana and plantain (*Musa* spp.) (Dodds, 1991). Secondly, there are crop species that have some sterile genotypes; while others may produce seeds that are highly heterozygous and therefore of limited use for cultivation. Such species are propagated vegetatively, generally in the form of tubers, roots, cuttings etc. (Dodds, 1991). Crops that belong to this category include yams (*Dioscorea* spp.) (Asiedu *et al.*, 1992), potatoes (*Solanum* spp.), cassava (*Manihot* spp.) (Asiedu *et al.*, 1992; Gonzales *et al.*, 1998), taro (*Colocasia esculentum*), sweet potato (*Ipomoea batatas*) (Kartha, 1985) and sugarcane (*Saccharum* spp.) (Dodds, 1991). The third category includes a large number of economically important tropical fruit and timber species, for which conventional seed storage strategies are not possible since they produce recalcitrant seeds (Roberts, 1973). Some of these species include coconut (*Cocos nucifera*) (King and Roberts, 1980), avocado (*Persea americana*) (King and

Roberts, 1980), mango (*Magnifera indica*) (Jana, 1994), cocoa (*Theobroma cacao*) and members of the Dipterocarpaceae (Tompsett, 1987).

In the case of sugarcane, which is the focus of this study, the lack of multiplication procedures has presented a serious problem in conventional breeding programmes. Primarily, the time spent to complete a selection cycle, which may take between 12 to 15 years, is considered a serious economic drawback (Liu *et al.*, 1984; Lee, 1987). Additionally, the maintenance of large numbers of sugarcane clones as growing plants not only demands a large facility and adequate staff and resources, but also plants grown under field conditions are at risk from environmental hazards (Taylor and Dukic, 1993).

The development of *in vitro* techniques for sugarcane has facilitated more efficient and effective means for exchange of germplasm internationally. The resources are in the form of pest- and disease-free plants produced after thermotherapy and chemotherapy of meristem tissue, the reduced mass of plant material being transferred, and the control over material being transferred (Hendre *et al.*, 1975; Waterworth and Kahn, 1978; Wagih, 1989). The propagation of sugarcane by sexual means is undesirable for the purposes of retaining the elite genetic characteristics of populations. Thus, vegetative, clonal propagation by means of somatic embryos has become the method of choice over seed production and it provides a useful source of germplasm that can be encapsulated as synthetic seeds (Nieves *et al.*, 2001). However, to obviate continuous *in vitro* maintenance of cultured material, some alternative form of long-term storage is imperative. Presently, only cryostorage in liquid nitrogen, or in its vapour phase, is considered to offer this possibility (Engelmann, 2004). It is, however, imperative that micropropagation be optimised for any species prior to the cryo-process and sugarcane is no exception to this rule.

1.3 Tissue culture technology

The genetic diversity of the majority of crop species is conventionally maintained as field plantings. However, there are drawbacks, as described above. *In vitro* germplasm conservation offers a reliable approach for only short and medium-term storage of genetic stocks since the morphogenetic potential of embryogenic cultures is lost with time, although the period is species-dependent (Witjaksono and Litz, 1999).

Current plant cell and tissue culture work aims at achieving efficient exploitation of specific properties of plant genotypes. This includes generation of variation, selective transfer of genes, identification of desirable traits and the generation of plants from *in vitro* cultures (Engelmann, 1994). The products of these *in vitro* procedures are, however, prone to variation and perhaps even accidental loss (Bajaj, 1995b). Clones obtained from elite genotype cell lines with special attributes, and genetically transformed material, are as vulnerable, demanding the development of efficient techniques to ensure the perpetuation of their genetic fidelity and conservation.

1.3.1 Micropropagation

Traditional methods of propagating plants include vegetative reproduction, by which plants bearing elite genotypes are produced by the use of cuttings, divisions and grafting, and propagation by seed. Plant tissue culture (micropropagation) presents a more recent and increasingly technically-efficient method for the propagation of plants. It involves the aseptic culturing of small parts of plants (explants) on a nutrient medium *in vitro* to produce shoots and then rooted plantlets that can ultimately be transferred to the external environment (*extra vitrum*) (George, 1993). The basis of this form of propagation is the stimulation of multiple shoot growth from cultured shoot-tip and nodal explants (e.g. Murashige, 1974; Brown and Thorpe, 1995). Currently, the diverse applications and numerous benefits of this technique have contributed to its being preferred over conventional breeding methods.

These benefits include:

- (i) Mass propagation of genotypes of interest with a minimal space requirement (Vasil and Vasil, 1994);
- (ii) production of pathogen-free plants by aseptic propagation, resulting in minimal loss of plants through disease and contamination (Vasil and Vasil, 1994; Bajaj and Jian, 1995);
- (iii) ability to manipulate growth conditions that contribute to the rate of propagation such that numerous plants may be produced within a given time (Ammirato, 1987);
- (iv) production of plants at any time, since production is independent of the external environment and seasonal changes (George *et al.*, 2008a);
- (v) minimisation of cost of labour and material required for watering, spraying and weeding, etc, since plants require very little maintenance between subcultures (George *et al.*, 2008a);
- (vi) variability in the term of storage for propagated material, depending on the conditions to which they are exposed (Engelmann, 1997); and
- (vii) conservation of germplasm.

1.3.2 Aspects of cell and tissue culture

Individual cells possess the ability to regenerate into whole plants because of the property of totipotency whereby division and differentiation of the products of a cell, under specific conditions, are able to regenerate an entire organism (Mantell *et al.*, 1985; Duncan and Widholm, 1986; Lindsey and Jones, 1990; Allan, 1991). In this manner, a small piece of parent material (somatic cells or tissue) may produce a large number of clonal individuals, given the appropriate culture conditions.

At any given time, the state of an individual cell or tissue can be described as being either determined or undetermined. Determined cells or tissues are highly differentiated, e.g. vascular elements, whereas undetermined cells are usually at a very early stage of development, e.g. cells of the meristem. Although the latter are preferred as explant material, dedifferentiation is possible in various cell/tissue types, followed by active cellular division and then redifferentiation into specialised cells, which form organs and eventually whole plants (Davies *et al.*, 1997).

Plant regeneration may occur via two routes *viz.*, organogenesis which is the production of shoots, followed by root formation (Evans *et al.*, 1981a; Thorpe, 1983; Ammirato, 1986; Christianson, 1987), or via somatic embryogenesis whereby fully formed embryos are generated and subsequently induced to germinate (Ammirato, 1985, 1987), a process more recently described as somatic embryo conversion (Mycock pers. comm.¹).

1.3.2.1 Organogenesis

Organogenesis refers to the formation of a variety of organs *de novo*, e.g. roots, shoots, leaves and flowers from cell and tissue cultures (Reinert, 1973). It involves two main types of morphological differentiation, *viz.* shoot and root formation, occurring sequentially and in response to appropriate culture conditions (mainly to type, combination and concentration of plant growth regulators in the culture medium) (Jiménez, 2001). Following the production of shoot and root tissues, cells that lie between them initiate vascular connections between the mother tissue and the regenerating structure (Terzi and Lo Schiavo, 1990), producing an autonomous plant once these connections are established as a result of vascularisation. Organogenesis can occur either directly where organs (usually shoots) are generated directly from the meristematic primordia of the explant, or indirectly, via a callus stage (George, 1993; Davies *et al.*, 1997).

¹ Mycock, D.J. School of Animal, Plant and Environmental Sciences, University of Witwatersrand, Gauteng, South Africa.

The presence, concentration and interaction of plant growth regulators in the medium directly influence both the direct and indirect routes of organogenesis. For example, adventitious root formation can be induced in stem and leaf cuttings by auxins such as indole-3-acetic acid (IAA), whereas cell division can be initiated by cytokinins (Davies *et al.*, 1997). Organogenesis, however, generally involves the interaction of auxins and cytokinins where high auxin:cytokinin ratios promote rooting or rhizogenesis (Gresshoff, 1978; George, 1993), while a high cytokinin:auxin ratio promotes shoot development (Vardjan and Nitsch, 1961; George, 1993). This response however, is not typical for all plant species (Davies *et al.*, 1997).

Callus is defined as the growth of unorganised cell masses arising from small plant organs or parts of plant tissue, and may occur naturally around wounds (George, 1993). Callus can be induced in culture by placing the explant on a medium with a high auxin concentration (Skoog and Miller, 1957; George, 1993; Chawla, 2002; Feher, 2008). It was Dodds (1983) who first reported that genetic aberrations may occur during mitotic divisions necessary for callus growth, thereby resulting in some regenerated plantlets being genetically dissimilar from the parent plant, as well as from each other.

1.3.2.2 Somatic Embryogenesis

Somatic embryogenesis is described by Zimmerman (1993) as the ability of haploid or diploid somatic cells to produce embryos that are morphologically, and appear developmentally, similar to zygotic embryos. Embryoids may develop from vegetative cells of mature plants; reproductive tissues other than the zygote, and hypocotyls and cotyledons of embryos and young plantlets without any intervening callus development (Kohlenbach, 1978). Although morphological similarities between somatic and zygotic embryos exist, in the former, there is no involvement or fusion of gametes (Williams and Maheshwaran, 1986; Emons, 1994; Raemakers *et al.*, 1995) or the production of a zygote. “Embryos are derived through the process of somatic embryogenesis, which is the initiation of embryos from plant somatic tissues [thus producing clones of a single parent], that

closely resemble their zygotic (i.e. sexually produced) counterparts” (Ammirato, 1983). As opposed to zygotes that are intrinsically embryogenic, somatic embryogenesis requires the induction of that competence in cells which are not naturally embryogenic (Dodeman *et al.*, 1997). In the context of somatogenesis, the term ‘embryogenic’ describes cells which have achieved the transition from the somatic state to a stage where no further external stimuli are required to produce a somatic embryo (Komamine *et al.*, 1990). These cells can be identified microscopically by a dense cytoplasm containing a large nucleus with a darkly stained nucleolus and by characteristically large starch grains (Dodds and Roberts, 1985).

Somatic embryogenesis may occur directly where somatic embryos are formed without an intervening stage from the explant tissue, or indirectly, where the embryos are formed from callus tissue or suspension cultures. George (1993) has identified explants derived from the female gametophyte tissue, as well as ovules and nucellus tissue as those tissue types that readily give rise to somatic embryos. It is indirect somatic embryogenesis that is more commonly observed, however, and the following requirements need to be met for successful embryo induction from callus and suspension cultures:

- (i) The plant genotype must be capable of inducing embryo formation on the medium of choice;
- (ii) auxin levels need to be high to induce embryogenesis (Reinert, 1973);
- (iii) sugar concentrations should not be too high since this may retard embryogenesis (Reinert, 1973);
- (iv) prolonged periods between subcultures should be avoided (Reinert *et al.*, 1977);
and
- (v) nitrogen must be supplied in the form of the ammonium ion (NH_4^+) or the amino acid, glutamine (Wetherell and Dougall, 1976).

Somatic embryogenesis occurs through a series of developmental stages, and was demonstrated for the first time in *Daucus carota* (Steward *et al.*, 1958). There are essentially two main stages of differentiation during somatic embryogenesis: stage 1, which

involves the differentiation of pro-embryoid cells in the presence of high auxin concentrations; and stage 2, which includes the developmental transformations of the developing embryo from globular to heart shaped and torpedo at low auxin concentrations (Ammirato, 1987). Zygotic embryos are categorised as being either orthodox or recalcitrant on the basis of their ability to withstand desiccation and, when desiccated, the former enter a state of developmental arrest (Bewley and Black, 1994). Somatic embryos, whether produced from tissues of species producing recalcitrant or orthodox seeds, behave as recalcitrant embryos, and germinate when they have apparently reached their final stage of development (Litz and Gray, 1992).

The development of somatic embryos from pro-embryonal complexes tends to occur asynchronously such that embryos at several stages are present in cultures at any given time (Gray, 1995). Several studies have shown that cell polarity and asymmetric cell division are involved in the initiation of somatic embryogenesis. For example, in alfalfa, stimulation by auxins promotes asymmetric division in embryogenic cultivar-derived protoplasts, while protoplasts of non-embryogenic lines divide symmetrically (Bögre *et al.*, 1990; Dudits *et al.*, 1991). In carrot, it has been shown that the asymmetric division of auxin-induced embryogenic cells gives rise to small daughter-cells from which somatic embryos develop (Komamine *et al.*, 1990).

It is the transition from somatic cells to the embryogenic state that is mediated by auxins and these are therefore the principal agents used to induce embryogenesis (Feher, 2008). For example, indole-3-butyric acid (IBA) has been found to promote the normal development of somatic (and zygotic) embryos *in vitro* (Crouch and Sussex, 1981; Ammirato, 1983). There have been reports, however, of other stimuli with the ability to affect cell polarity or the division plane position (Dodeman *et al.*, 1997). Maheswaran and Williams (1985) reported that cytokinin induces a change of the normal anticlinal division plane in favour of oblique periclinal divisions, thereby promoting the formation of embryogenic cells from the epidermis of immature zygotic embryos of white clover (*Trifolium repens*). A shift in pH (Smith and Krikorian, 1990), or an application of an

electric field is also thought to affect cell polarity (Dijak *et al.*, 1986). It has been suggested that exogenous growth regulators modify the cell polarity by interfering with pH gradients or electrical fields around the cells (Dijak *et al.*, 1986; Smith and Krikorian, 1990). Several studies have also demonstrated stimulation of somatic embryogenesis from immature zygotic embryos by amino acids such as L-proline (Armstrong and Green, 1985; Duncan *et al.*, 1985; Lowe *et al.*, 1985). Similar stimulation was reported for carrot, using serine (Nutti *et al.*, 1984).

The applications of somatic embryogenesis are extensive, the widest being in large-scale propagation, facilitating the production of large numbers of units representing the germplasm (Ammirato, 1987). Somatic embryogenesis requires relatively few manipulation steps (Snyman *et al.*, 2000a), affords the option of dormancy induction and long-term storage (Bajaj and Jian, 1995), and facilitates encapsulation as packaging and delivery system as applied in synthetic seed technology (Gray and Purohit, 1991; Gray *et al.*, 1995; Litz and Gray, 1995; Engelmann, 2000). Somatic embryogenesis can also facilitate production of plants with different levels of ploidy; for example, haploid embryos may be obtained by cultivating anthers, and triploid/polyploid embryos raised from endosperm tissue (Terzi and Lo Schiavo, 1990). In addition, the use of embryogenic cultures has proven to be increasingly valuable in providing a source of regenerable protoplasts in graminaceous species (Finch *et al.*, 1991; Chang and Wong, 1994; Lyznik and Hodges, 1994; Funatsuki *et al.*, 1996), citrus species (Jiménez, 1996), and forest trees (David, 1987; McCown and Russell, 1987).

Work done by several researchers has shown that graminaceous plants can form somatic embryos in cultures derived from young/immature leaves (Ho and Vasil, 1983a; Snyman *et al.*, 2000a), inflorescences (Vasil *et al.*, 1985) and immature zygotic embryos (Vasil *et al.*, 1985; Fransz and Schel, 1991). It was discovered that these embryos were derived from epidermal and sub-epidermal regions of young explants (Vasil *et al.*, 1985). In graminaceous crops, somatic embryogenesis is promising for breeding purposes, particularly with regards to cell fusion, the stable introduction of foreign genetic

information, and tolerance to salt and herbicides (Brisibe *et al.*, 1994). This, in addition to the many other applications of somatic embryogenesis, makes this technique an extremely useful means of propagation and storage of germplasm of vegetatively propagated plants in particular.

1.3.3 Somaclonal variation

Variations may arise within clones, either spontaneously or they may be induced in *in vitro* cultures (Partenen, 1963; Widholm, 1974; Carlson and Polacco, 1975). Somaclonal variation is a phenomenon that occurs in most *in vitro* cultures that involve a callus stage, regardless of whether regeneration occurs through somatic embryogenesis or by adventitious shoot formation (Larkin and Scowcroft, 1981; Scowcroft, 1984). Theoretically, micropropagated plants derived from the same explant should constitute a clone, i.e. they should be genotypically and phenotypically identical. However, spontaneous genetic variations may be manifested in regenerated plantlets as a consequence of culturing procedures, as follows: the actual procedure employed (callus, cell suspension or protoplast culture); the chemical composition of the plant growth medium; the ratio of plant growth regulators in the growth medium; the culture conditions; and the duration of the culture period (George, 1993; Karp, 1994). These randomly-occurring genetic variations pose a potentially major problem, particularly in genetic manipulation systems such as transformation, and may be minimised by careful selection of explant material from young, actively growing regions of the parent plant, which possess fewer inherent genetic mutations (Sweby *et al.*, 1994).

Some of the earliest reports of somaclonal variation in *in vitro*-derived plantlets of sugarcane, included changes in morphology such as presence or absence of hairs, differences in isozyme profiles, as well as variation in crop parameters such as cane diameter, stalk length and weight, and cane and sugar yield (Bailey and Bechet, 1989; Lyndsey and Jones, 1989). Although often undesired, somaclonal variation can be potentially advantageous for the production of agronomically useful mutants resulting in

increased crop yields and resistance to pests and diseases (Masirevec *et al.*, 1988). These are especially beneficial to plant propagators in the horticultural and agricultural industries.

1.4 *In vitro* storage methods

The three main approaches to *in vitro* storage of germplasm include maintenance of actively growing cultures, slow or minimal growth storage, and cryopreservation (Krøgstруп *et al.*, 1992). Although the objective of the present study was ultimately to develop a cryopreservation protocol for a test variety of sugarcane, 88H0019, an introduction to alternative means of *in vitro* storage is presented in some detail here. This is because somatic embryos of variety 88H0019 proved quite intractable to cryopreservation, and this may prove true for other locally important varieties as well.

1.4.1 Storage of actively growing cultures

This type of *in vitro* technology requires that plant material be maintained as actively growing tissues in culture systems. As this method requires constant maintenance and often involves monthly transfer to fresh culture medium, it poses a significant risk of loss of individuals, particularly by means of microbial contamination. The advantage of this method, however, is the potential for rapid multiplication of plantlets by micropropagative techniques.

1.4.2 Minimal-growth storage

As mentioned earlier, maintenance of germplasm collections in the field is expensive and potentially risky. The *in vitro* minimal-growth method offers an immediate solution for short- to medium-term storage of genetic resources of vegetatively propagated species. It involves the imposition of growth restrictions on *in vitro* cultures by exposure to growth limiting chemicals and/or physical factors, involving the use of growth regulators such as abscisic acid (ABA), and/or the provision of non-metabolisable sugar alcohols such as

mannitol and sorbitol, and/or by maintenance at reduced temperatures (2-3°C for temperate species and 14-18°C for tropical species) (Wilkins and Dodds, 1983; Ford-Lloyd and Jackson, 1991; Krøgstrop *et al.*, 1992; Mycock *et al.*, 2003). Minimal growth conditions may also be imposed by reducing the quantity of oxygen available to cultures for growth and development (George, 1993). Depending on the species, slow-growth procedures allow for the storage of clonal plant material for 1-8 years under tissue culture conditions with periodic sub-culturing (Henshaw and Blakesley, 1996; Taylor *et al.*, 1996).

Plant material amenable to this method of storage includes meristems, shoot apices, buds, cell cultures, callus, protoplast cultures, somatic and zygotic embryos, anthers, pollen and even whole seeds (Mandal, 1997); with unrooted shoot clusters, somatic embryos and rooted plantlets being the most suitable (George, 1993).

According to Dodds (1991), the advantages associated with maintenance of germplasm under slow-growth conditions are:

- (i) visual inspection of cultures, allowing for timely action to avoid losses;
- (ii) availability of material for international exchange, since material can be pathogen-tested;
- (iii) significantly reduced space for the storage of *in vitro* materials compared with that required for field gene-banks,
- (iv) potentially high multiplication rate; and
- (v) avoidance of loss of germplasm due to natural disasters.

1.4.2.1 Manipulation of culture media

Under normal conditions, the choice of medium is dependent on the size of the explant, with smaller explants requiring more complex media (Kane, 2004). The most commonly used formulation is Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) or a modification of it. The initiation medium for small explants, e.g. meristems and shoot

tips, serves to support optimal growth and usually contains a basal salt mixture, sucrose, vitamins, inositol and plant growth regulators (auxin, cytokinin or gibberellic acid) (George, 1993; George *et al.*, 2008b, c). Reduced growth rates, however, are achieved by using a medium with lower salt concentrations, such as White's medium (White, 1954) or Heller's medium (Heller, 1953) or by reducing the strength of normal MS basal medium (Ng and Ng, 1991; George *et al.*, 2008b, c). Increasing or decreasing the concentration of sucrose in the medium, or adding osmotica (such as mannitol and sorbitol) or growth retardants are other means of achieving slow growth (Ng and Ng, 1991; Gopal *et al.*, 2002). ABA may be incorporated into the culture medium to retard growth of *in vitro* cultures by inducing organ dormancy, reducing cellular metabolism or preventing nuclear division, but success depends on the species (Jarret and Gawel, 1991; Taylor *et al.*, 1996). Successful reduction in growth by manipulation of the culture media has been reported for several plant species. For example, coffee (*Coffea arabica*) plantlets were reported to be only 30-40 mm in height after two years of storage on a medium containing half-strength MS without sucrose but with 1 μ M IBA (Kantha *et al.*, 1981).

1.4.2.2 Modification of the gaseous environment

This alternate slow-growth technique involves a reduction in the quantity of oxygen available to cultures, which influences their growth and development (George, 1993). This approach is noted here for completeness but, as it has no bearing on the present investigation, is not discussed further.

1.4.2.3 Reduced incubation temperature

In most cases growth limitation is achieved by exposing cultures to a low storage temperature, often in combination with low light intensity or in the dark (Grout, 1995). Generally, the most suitable temperature for storage as well as whether dark or light storage is preferable, depends on genotype. Some crop species are more cold-tolerant than others thereby permitting the maintenance of cultures at very low temperatures. Shoots and

plantlets are usually stored at temperatures between 20-28°C *in vitro*, but successful storage at temperatures between 0-10°C for some species have been reported (Blakesley *et al.*, 1996; Bonnier *et al.*, 1997). Cold-tolerant species can successfully be maintained at temperatures in the range of 0-5°C, but tropical species that are often cold-sensitive demand higher temperatures. Studies by Roca *et al.* (1984) indicated that cassava shoots have to be stored at temperatures above 20°C. The somatic embryos of oil palm (*Elaeis guineensis*) cannot tolerate even short-term exposure to temperatures below 18°C (Corbineau *et al.*, 1990). Although sugarcane is a tropical grass, leaf sheath tissue was stored at 0-4°C for a period of six months (Withers, 1982).

Cultures of many plant species have been successfully stored by reducing the incubation temperature and also by a combination of this with modified growth media. In some cases the combined effect appears to be superior to each of the methods applied in isolation.

1.4.3 ‘Synthetic seeds’ (‘synseeds’)

The large-scale propagation of elite genotypes of many crop species is currently being achieved by means of somatic embryogenesis, resulting in the production of large numbers of synchronously developing embryos. The production of ‘synthetic seeds’ or ‘synseeds’ as they are also known, by encapsulating somatic embryos has been proposed as a “low-cost-high-volume” propagation system providing a convenient and effective means of storing valuable germplasm (Redenbergh, 1990). Synthetic seeds are described by Gray and Purohit (1991) as “encapsulated somatic embryos that have been specially processed for use in commercial propagation, the exact process depending on the plant species and application.” Although synseeds were originally developed for somatic embryos (Redenbaugh, 1993), the technique is presently applied to shoots, axillary buds and other tissues used for *in vitro* or *ex vitro* cultivation (Piccioni, 1997). The procedure involves the encapsulation of embryos (or other explants) in a hydrogel bead of sodium, potassium or calcium alginate, Gel-Rite®, guar gum, tragacanth gum or sodium pectate (Redenbaugh *et al.*, 1993). Synseeds can theoretically be sown directly into potting medium, as are true

seeds (Bajaj, 1995a; Gray *et al.*, 1995). As an additional advantage, nutrients, pesticides and fungicides may be incorporated into the gel mixture to maintain a contaminant-free micro-environment. Survival of encapsulated somatic embryos of alfalfa (Zhong and Wang, 1989); wheat (Deng *et al.*, 1990) and eggplant (Rao and Singh, 1991), however, have been reported to be lower than those that were unencapsulated (Zhong and Wang, 1989; Deng *et al.*, 1990; Rao and Singh, 1991).

Effective minimal growth of encapsulated somatic embryos is achieved by desiccation, often with pre-treatment with ABA and/or a reduction in light and temperature (Pliegoalfaro *et al.*, 1996) or a combination of treatments (Castillo *et al.*, 1998). Encapsulated somatic embryos of sandalwood (*Santalum album*) were successfully stored for 45 days at 4°C (Bapat and Rao, 1998) and somatic embryos of interior spruce (*Picea glauca*) for one month at the same temperature (Lulsdorf *et al.*, 1993).

Although effective apparently in the short-term only, this form of germplasm storage not only provides an efficient packaging and delivery system (Engelmann, 2000), but is extremely useful for propagation of valuable hand-pollinated hybrids, elite germplasm, and genetically engineered plants, specifically those with sterile unstable genotypes (Nieves *et al.*, 1998). It affords the opportunity to store the germplasm of cloned as well as genetically heterozygous species (Bajaj, 1995b; Gray *et al.*, 1995). However, encapsulated material will inevitably undergo slow growth in storage and the maintenance of genetic stability following the use of growth retardants may not be assured (Ashmore, 1997). While encapsulated embryos of some species (e.g. carrot) have been shown to survive for a brief period at room temperature, and not more than 60 days at 2°C (Liu *et al.*, 1990), such structures might be the vehicle for cryopreservation (ultra-low temperature storage).

1.5 Cryopreservation

Cryopreservation offers a method for long-term storage of viable germplasm at ultra-low temperatures (Gnanapragasam and Vasil, 1990; Bajaj and Jian, 1995; Engelmann, 1997). It involves the cooling and subsequent storage of biological material usually in liquid nitrogen (LN) at -196°C which is accompanied by the minimisation and subsequent cessation of all cellular division and metabolic processes. Currently, this is considered as the ideal method for long-term germplasm conservation (Kantha and Engelmann, 1994; Berjak *et al.*, 1999, Wesley-Smith *et al.*, 1999). Plant material can thus theoretically be stored without modification or alteration for an indefinite period, since it is maintained at temperatures that curtail all biochemical activity and consequently, all molecular movements necessary for reactions (Benson and Bremner, 2004). Moreover, frozen material should maintain genetic stability in addition to requiring very limited maintenance (Gonzalez-Arno *et al.*, 1999; reviewed by Harding, 2004).

Cryopreservation of partially hydrated plant tissue, however, has so far required optimisation of manipulations for individual species and culture types, the success of which is largely dependent on various factors that operate in combination (Berjak and Pammenter, 2004). These include the size and physiological state of the explant, water content and ability to withstand dehydration and the drying rate at which this is achieved, the nature and concentration of cryoprotectants, the method and rate of both cooling and thawing, and the rehydration and regeneration conditions (Mycock *et al.*, 1995). The methods and rates of cooling, thawing and rehydration will be described in detail later. As a result of the variability in plant tissue of different organs of the same species and among different species, responses to cooling also vary widely, thereby making it imperative that protocols for cryopreservation of plant germplasm are, at least presently, developed empirically for each species and explant used (Wesley-Smith *et al.*, 1995; Kioko *et al.*, 1998). Such requirements may also have to be met on a variety basis, as was shown to be the case for rice, for example (Fatima *et al.*, 2002).

1.5.1 Type of explant

Cryopreservation is the only available method for long-term storage of clonal germplasm (Engelmann, 2000), and, according to Kartha (1985), can be successfully applied to a wide variety of plant material including cells and protoplast cultures, meristems, callus, somatic and zygotic embryos, anthers, pollen (microspores) and even whole seeds. It should be noted, however, that in practice successful cryostorage is not invariably achieved (Kioko, 2003).

Already in the 1980s, callus cultures and cell suspensions were reported as successfully cryopreserved in liquid nitrogen (Withers, 1985), and survival rates as high as 50–80% were achieved, along with stability of desirable traits (Watanabe *et al.*, 1985). Shoot cultures and meristems supposedly offer a higher level of genetic stability than callus, but have proved to be more difficult to freeze effectively (Withers, 1988). For non-orthodox species, somatic embryos (Mycock *et al.*, 1995), excised embryonic axes (Normah *et al.*, 1986, Wesley-Smith *et al.*, 1992, 1999, 2001a, b; Sershen *et al.*, 2007), and occasionally whole seeds (Potts and Lumpkin, 1997) are more appropriate. Kioko *et al.* (2000) reported successful cryopreservation of whole non-orthodox seeds of *Warburgia salutaris*. However, excised zygotic axes are the most feasible alternative for cryopreservation since most recalcitrant seeds are too large to dry or cool successfully (Berjak, 2000). While zygotic embryonic axes fulfil the requirement for genetic diversity conservation, clonally-produced explants are required to conserve elite genotypes of vegetatively-produced crop species.

There has been extensive development of systems for *in vitro* induction of somatic embryos especially for species of economic importance (e.g. oil palm, date palm, coffee, etc.) since cryopreservation of somatic embryos has great potential for the long-term storage of selected genotypes (Tisserat, 1984; Hatanaka *et al.*, 1991). Somatic embryos of oil palm have been reported to be remarkably tolerant to different rates and methods of cooling (Engelmann, 1990; Dumet *et al.*, 1993a), and it has been suggested that the amenability of

somatic embryos to cooling may be due both to their physiology and morphology (Ford-Lloyd and Jackson, 1991). Mycock *et al.* (1995) identified four essential factors that operate in combination to determine the ability of somatic embryos to be cryopreserved without vigour or viability loss. These factors include size and developmental stage of the embryos, the water content and ability to withstand dehydration, qualitative and quantitative suitability of cryoprotectants, and the method and rates of both cooling and thawing.

1.5.2 Dehydration of plant material

The cryo-process is greatly impacted upon by two features viz. water content and size of explant. Water content is important as it determines the extent of intracellular ice crystal formation, and size of the explant regulates the cooling and thawing rates (Mycock *et al.*, 2004). Due to their highly hydrated nature, *in vitro* plant materials do not tolerate subzero temperatures (Meryman and Williams, 1985), which invariably impedes long-term preservation efforts using cryostorage (Vertucci *et al.*, 1991). Dehydration in some form is therefore required to protect from damage caused by the formation of lethal crystalline ice (Stanwood, 1985; Reed, 1996), arising from 'free' water (Crowe *et al.*, 1990), i.e. solution water in cells and tissues. Lethal ice-crystal formation can be limited by:

- (i) reducing or eliminating freezable water by dehydrating the tissue prior to cooling (Stanwood, 1985; Reed, 1996);
- (ii) increasing the cooling rate since it has been shown that water movement is minimised when the cooling rate is extremely rapid; the result is formation of only very small (micro) ice crystals (Mazur, 1984); and
- (iii) pretreating explants with cryoprotectants.

Although drying induces numerous stresses within cells, which, in turn, affect membrane and organellar integrity, when applied as a pre-treatment to cryopreservation, partial removal of cellular water reduces the likelihood of lethal ice crystal formation, which can

occur readily during cooling and storage at low temperatures (Meryman and Williams, 1985; Pritchard and Prendergast, 1986; Pammenter *et al.*, 1991, 1998; Vertucci *et al.*, 1991; Berjak *et al.*, 1992; Wesley-Smith *et al.*, 2001a, b). This is ideally achieved by promoting the glassy state (vitrification) within the tissue (Stanwood, 1985; Normah *et al.*, 1986; Pritchard and Prendergast, 1986; Wesley-Smith *et al.*, 1992, 1999, 2001a, b; Reed, 1996) by increasing cytoplasmic viscosity (Leprince and Hoekstra, 1998; Leprince *et al.*, 1999; Wesley-Smith, *et al.*, 2004b), which hinders ice crystal growth on exposure of explants to the cryogen (Luyet *et al.*, 1962; Wesley-Smith *et al.*, 2004a). In addition to being associated with less severe cooling damage (Meryman and Williams, 1980; Wesley Smith *et al.*, 1992; 2004a, b), lower water contents give rise to higher glass transition temperatures (Meryman and Williams, 1980; Leprince and Walters-Vertucci, 1995).

Desiccation minimises the amount of cooling damage incurred by the cells when exposed to low temperatures since most of the solution water has been removed. In addition, desiccation serves to decrease the thermal load of tissue thereby aiding the quick passage through the range of critical temperatures that encourages ice crystal growth (Wesley-Smith, 2002), while depressing the freezing point of tissue water. However, it is imperative that the water content of plant material to be cryopreserved be optimised to minimise damage by desiccation at too low water contents or by cooling at higher water contents (Normah *et al.*, 1986; Pence, 1990; Chaudhury *et al.*, 1991; Vertucci *et al.*, 1991; Wesley-Smith *et al.*, 1992, 2001a, b) and in order to increase the chances of successful cooling (Berjak *et al.*, 1990, 1999).

Previous research involving zygotic embryonic axes of a number of recalcitrant-seeded species has shown that the more rapidly water loss is achieved, the lower the water content before viability is affected (Normah *et al.*, 1986; Pritchard and Prendergast, 1986; Berjak *et al.*, 1989). Seeds generally, and more especially recalcitrant seeds, are too large to dry rapidly (Pammenter and Berjak, 1999; Wesley-Smith *et al.*, 2001b; Sershen *et al.*, 2007). However, when isolated embryonic axes are dehydrated using flash-drying (Berjak *et al.*, 1990), rapid dehydration rates can be achieved (e.g. Normah *et al.*, 1986; Pammenter and

Berjak, 1999; Perán *et al.*, 2004; Sershen *et al.*, 2007). The same would be true for other actively metabolic explants, e.g. somatic embryos or meristems.

The ability to withstand complete loss of solution water (i.e. to water contents $\leq 0.1 \text{ g g}^{-1}$ dry mass), or true desiccation-tolerance, as defined by Vertucci and Farrant (1995) and further explored by Berjak (2006), has been demonstrated in propagating structures like pollen, orthodox seeds and dormant buds. Even if a species produces orthodox seeds, the somatic embryos developed contain a larger proportion of water and appear to be less resistant to desiccation than the seeds (Shimonishi *et al.*, 2000). The degree of desiccation tolerance of somatic embryos varies depending on the species. Hatanaka *et al.* (1994) reported that 50-80% of *Coffea canephora* embryos withstood dehydration to a water content of 20% (0.25 g g^{-1} , dmb), but 80% loss of viability occurred when clumps of somatic embryos of oil palm were desiccated to water contents below 0.7 g g^{-1} (Dumet *et al.*, 1993a). Vertucci and Farrant (1995) hypothesised that it is the ability of a damaged structure to entrain repair, rather than protection from desiccation that determines the desiccation-tolerance of vegetative tissue. Therefore deleterious reactions induced by the removal of water must be prevented, controlled and counteracted in organisms that are naturally desiccation-tolerant.

1.5.3 Developmental stage

The success of the dehydration and cooling processes is significantly affected by the developmental stage of the explant (Wesley-Smith *et al.*, 1995; Berjak *et al.*, 1999). In general, more immature, but germinable, zygotic axes do not survive cryopreservation, while those that are more mature; survive (Vertucci *et al.*, 1991; Berjak *et al.*, 2000; Daws and Pritchard, 2008). For example, mature embryonic axes of *Landolphia kirkii* have been shown to be more tolerant of cryopreservation at -80°C than are immature axes at similar water content (Vertucci *et al.*, 1991). This has been attributed to the physiological status of seeds, which changes as development continues: germinating axes again becoming more sensitive (Berjak *et al.*, 2000), but showing a lesser vulnerability to excision damage

(Goveia *et al.*, 2004). A contributing factor to successful cryopreservation of zygotic embryos or axes could be associated with changes in the content of sucrose and other oligosaccharides with progressive development of seeds, as these may function as natural cryoprotectants (Wesley-Smith *et al.*, 1995).

The developmental stage of somatic embryos was identified as one of the key factors that determine their ability to be cryopreserved without loss of vigour or viability (Mycock *et al.*, 1995). For example, young somatic embryos were shown to be more tolerant to desiccation than those comprised of more differentiated cells, since the former possess fewer vacuoles and a denser cytomatrix (Engelmann, 2000). For plant cell suspension cultures, cells that are more tolerant to cooling are those that are exponentially growing rather than those harvested during the lag or stationary phases (Sugawara and Sakai, 1974; Withers, 1985; Yoshida *et al.*, 1993; Reinhoud *et al.*, 1995). The characteristics that make these cells the ideal starting material include the fact that they have the smallest volume in the culture period and that they possess small vacuoles, thus containing relatively less water (Reinhoud *et al.*, 2000a).

1.5.4 Cryoprotection

Kartha (1985) described cryoprotectants as a group of compounds, used in various combinations to lower the supercooling and freezing points of cellular solutes. When applied individually or in combination, these compounds act as desiccants by withdrawing water into the extracellular environment (Finkle and Ulrich, 1979; Benson, 1995), and increasing the sugar and overall solute concentrations within the cell (Dereuddre *et al.*, 1988). This is achieved by an optimal balance of electrolyte concentration proportional to water in the liquid state below 0°C, or by dehydration of the cells by osmosis, thereby effecting a reduction in the amount of free water available for ice crystal formation (Kartha and Engelmann, 1994). These properties are thought to confer a resistance to cooling and thawing injury (Dumet *et al.*, 1993b; Gonzalez-Arno *et al.*, 1995; Phunchindawan *et al.*,

1997). Additionally, by acting as free radical scavengers, certain molecules provide an element of protection against free radical effects during cooling (Benson, 1990).

Compounds belonging to this group include: the hydroxyl radical scavenger, dimethyl sulphoxide (DMSO), glycerol, sorbitol, polyethylene glycol, sugars and sugar alcohols (Kantha and Engelmann, 1994), and dextran and polyvinylpyrrolidone (PVP) (Meryman and Williams, 1985; Potts and Lumpkin, 1997). Sucrose has been suggested to afford protection against desiccation damage in plant tissues and organs (Crowe *et al.*, 1987, 1988; Carpenter and Crowe, 1988; Koster and Leopold, 1988; Leopold, 1990; Koster, 1991). Its cryoprotective abilities have been demonstrated in oil palm somatic embryos, which exhibited survival of liquid nitrogen immersion after pre-treatment on a medium containing 25% sucrose (Engelmann *et al.*, 1985; Dumet *et al.*, 1993a). It has also been shown that many sugars are involved in stabilisation of proteins in solution (Back *et al.*, 1979; Arakawa and Timasheff, 1982), while affording cryoprotection to isolated enzymes (Shikama and Yamazaki, 1961; Whittam and Rosano, 1973; Carpenter *et al.*, 1986). Glycerol is actively involved in the maintenance of structure of biological macromolecules and in promoting protein self-assembly through preferential hydration (Sousa, 1995; Davis-Searles *et al.*, 2001) as well as being associated with enhanced activity of anti-oxidants (Sershen and Varghese, pers. comm.²).

The beneficial effects of the use of a combination of cryoprotectants have been demonstrated with somatic embryos of date palm and coffee (Mycock *et al.*, 1995). These somatic embryos survived cryopreservation following treatment with mixtures of sucrose and glycerol (5% followed by 10%), while for immature zygotic embryos of coconut, combinations of 60% glucose and 10-15% glycerol were used as pre-treatments for cryopreservation (Assy-Bah and Engelmann, 1992). Other compounds found to possess cryoprotectant activity, but with lower efficacy, include small molecular weight solutes such as amino acids (alanine, glycine, proline), other sugars (glucose, lactose and ribose),

² Sershen, and Varghese, B. School of Biological and Conservation Sciences, Department of Biology, University of KwaZulu-Natal, Durban, South Africa.

and amides (acetamide and formamide) (Lovelock, 1954; Vos and Kaalen, 1965). Other soluble sugars, as well as proline, have successfully been used as conditioning agents to acclimatise and cold-harden tissue to be cooled (Koster and Lynch., 1992).

Cryoprotectants can be broadly classed as either permeating/penetrating (chemicals that are capable of diffusing through the plasma membrane and equilibrating in the cytoplasm), or non-permeating/non-penetrating (which do not enter the cytoplasm). The mode of action of penetrating cryoprotectants is generally held to be by lowering the freezing point of the intracellular solution through colligative action (Storey and Storey, 1996; Santarius and Franks, 1998). Non-penetrating cryoprotectants, in contrast, act by drawing water out of the cells by osmosis, thereby reducing the amount of freezable water available for ice crystal formation (Storey and Storey, 1996). Treatment with these compounds at concentrations between 5 and 20% is applied prior to cooling. The cryoprotection procedure generally involves immersing explants in the cryoprotectant for a given time (Meryman and Williams, 1985; Mycock *et al.*, 1995), or preculturing on a medium containing cryoprotectants, sometimes serially at increasing concentrations, over several days (Dumet *et al.*, 1994; Cho *et al.*, 2001).

Some cryoprotectants are believed to interact directly with cell membranes thus preventing deleterious effects by eliciting a safer or more damage-resistant membrane configuration and/or by preventing freeze induced uncoupling of photophosphorylation (Koster and Lynch, 1992; Kartha and Engelmann, 1994). DMSO is believed to interact electrostatically with phospholipid bilayers (Anchordoguy *et al.*, 1987) but also is a powerful antioxidant (Benson and Bremner, 2004). Disaccharide sugars, specifically sucrose and trehalose, have been suggested to stabilise membranes during hypertonic exposure as ice crystals grow, by interacting with polar head groups of phospholipids (originally suggested by Rudolph and Crowe, 1985). However, there is much scepticism that this is the mode of action of disaccharides (reviewed by Berjak, 2006). That author is of the opinion that the sugar(s) act synergistically with Late Embryogenic Accumulating Proteins (LEAs) in promoting intracellular vitrification and Halperin and colleagues (2006) further suggest that

concentrated sucrose solutions prevent lateral contact between closely-positioned intracellular membranes.

There have been reports that some, if not all, cryoprotectants, may have toxic effects on cells, especially when applied in high concentrations (Fahy, 1986; Withers, 1988, Steponkus *et al.*, 1992). The effects, however, appear to be concentration dependent. For example, glycerol is considered less toxic to plant cells than DMSO at equimolar concentrations, but DMSO has been shown to be the superior of the two (Kartha and Engelmann, 1994). Mycock *et al.* (1995) reported that DMSO concentrations between 5-10% (v/v) may be tolerated, but the cytotoxic nature and ability of cryoprotectants to cause dehydration injury and osmotic damage during cooling and thawing should be recognised. At higher concentrations, DMSO is thought to interfere with microfilament and microtubule structure and configuration (Withers, 1988). Rapid cooling rates with combined usage of a solution of ionised calcium and magnesium chlorides in cryoprotocols appear to assist in maintaining cytoskeletal integrity (Mycock, 1999). During the cooling process, solute concentrations fluctuate and alter the dynamic equilibrium that is associated with the assembly and disassembly of cytoskeletal elements. Calcium and magnesium ions serve to buffer the intracellular environment and reduce uncontrolled depolymerisation of microtubules and microfilaments (Mycock, 1999).

Cryoprotection, in general, is a time-consuming process and often, a simpler approach to reducing water content of explants, by drying in a stream of air, is favoured (Normah *et al.*, 1986; Pritchard and Prendergast, 1986; Pence, 1990, 1992, 1995; Wesley-Smith *et al.*, 1992; 1995; 2001a, b). However, explant type may emerge as the deciding factor when determining the optimum mode of dehydration.

1.6 Sugarcane and the rationale of this study

Sugarcane, (*Saccharum officinarum* L.), a member of the Poaceae, is a principal crop in many tropical developing countries because of its high sucrose content, vegetative method of propagation and capacity for multiple harvests from the same rootstock (Butterfield *et al.*, 2004). Sugarcane has become an increasingly important crop in recent years, not only because it is the raw material for sugar industries, but also because of its allied importance in industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed (Arencibia, 1998). South Africa is a prominent producer of sugarcane, and in doing so, has accounted for as much as 60% of the world's sugar supply (Ho and Vasil, 1983a). Sugarcane, cultivated by approximately 2 000 large-scale and over 48 000 small-scale cane growers in the Eastern Cape, KwaZulu-Natal and Mpumalanga, yielded 2.76 million tonnes of sucrose in the 2002-2003 season alone (Butterfield *et al.*, 2004). According to information furnished by the South African Sugar Association (SASA), the sugar industry earns the country about R1.7 billion in foreign exchange while providing direct or indirect employment to approximately 350 000 people. This earns South Africa a sixth position in the ranking as a global sugar exporter (Anonymous, 2001). The demand for sugar, however, has been described as "greatly outpacing the ability to produce sugarcane" (Lorenzo and Gonzalez, 1998). Its importance in the agricultural industry provides strong motivation for ongoing sugarcane research in order to create more desirable cane with respect to increased sucrose content and disease-resistance; however, this selection leads to narrowing or loss of genetic diversity.

Desirable and advantageous novel characteristics in sugarcane may be achieved by different breeding techniques, or by means of genetic modification. The South African Sugarcane Research Institute (SASRI) at Mount Edgecombe, KwaZulu-Natal, is responsible for such breeding programmes and produces the commercial varieties grown in South Africa. The selection process, which takes between 12-15 years for completion, targets clones for their sucrose content, sucrose yield per hectare, and resistance to various diseases and pests that are common to the industry (Butterfield and Thomas, 1996). Sexual

propagation by seed is undesirable when attempting to retain the elite characteristics of out-breeding populations, and vegetative or clonal propagation by somatic embryos is the preferred method of propagation (Nieves *et al.*, 2001). In addition, the maintenance of large numbers of sugarcane clones as growing plants demands a large facility and adequate staff and resources. Plants are also prone to damage by environmental factors such as drought and infestation/infection by pests and diseases respectively.

The use of *in vitro* techniques for germplasm preservation, therefore, provides an alternative for conservation of delicate or disease-susceptible clones, and for genetically-modified material. *In vitro* techniques have successfully been applied to mass propagation of sugarcane via the use of axillary buds (Sauvaire and Galzy, 1978), shoot tip culture (Hendre *et al.*, 1983; Lee, 1986), and callus culture (Barba *et al.*, 1978; Liu, 1984). These techniques, as applied to sugarcane, offer a convenient and effective means for international exchange of germplasm, since pest and disease-free plants are produced after thermotherapy and chemotherapy of meristem tissue, the mass of plant material transferred is reduced, and the transferred material is contained (Hendre *et al.*, 1975; Waterworth and Kahn, 1978).

Several sugarcane varieties exhibiting desirable characteristics have successfully been developed and need to be stored or banked for future use. As discussed above, although several *in vitro* methods are available for the storage of germplasm, cryopreservation is potentially the best, since it affords long-term conservation and avoids environmental hazards. Different types of explants of sugarcane can be used for cryopreservation, including shoots from axillary buds or cell suspension cultures, somatic embryos etc. Somatic embryos were the explants of choice for this study since large numbers of units representing the germplasm are produced, limited manipulation steps are required, the induction of dormancy and long-term storage is possible, and because they afford the possibility of encapsulation as a packaging and delivery system. Furthermore, direct somatic embryogenesis from leaf rolls is easily achieved (Ho and Vasil, 1983a; Chen *et al.*,

1988; Grisham and Bourg, 1989; Snyman *et al.*, 1996, 2000a) thus avoiding callus and the risk of somaclonal variation.

The South African Sugarcane Research Institute is involved in ongoing studies which focus, in part, on the optimisation of *in vitro* culture conditions for production and germination of somatic embryos derived from several varieties of sugarcane. Already in the 1970s several breeding and propagation programmes achieved marked success with plantlet regeneration from tissue cultures of sugarcane (Heinz *et al.*, 1977; Koga and Kudo, 1977; Nadar and Heinz, 1977). Later, plants were regenerated from tissue, cell and protoplast cultures of several graminaceous species via somatic embryogenesis (Vasil, 1982a, b, 1983; Vasil *et al.*, 1982). A relatively new avenue of research at SASRI is that of microprojectile bombardment of embryogenic callus in genetic engineering programmes to introduce characteristics such as disease resistance and improved sucrose content (Snyman *et al.*, 2000b).

Transformed material, however, ultimately requires being stored under conditions maintaining vigour, viability (Teixeira da Silva, 2005) and integrity of the now-novel genome (Harding, 2004). Also, during periods of experimentation, effective cryopreservation, which offers a suitable means of storing transformed material for ultimate assessment and potential release, would be extremely useful.

The current investigation was carried out on a variety of sugarcane (88H0019) supplied by SASRI, which had been the subject of earlier, unsuccessful cryopreservation trials (O'Brien, 2001; Cheruiyot, 2002). The work closely examined the effects of pre-growth, dehydration and various cryoprotectant treatments on the survival of somatic embryos. The different methods and rates of cooling and the effects of various plant growth regulators on regeneration of cryopreserved embryos were also investigated. Finally, cryopreservation of an alternative explant in the form of fragments of leaf tissue was investigated. Cryopreservation protocols for sugarcane have been developed by others for various explants. For example, successful cryopreservation protocols have been developed for

apices of *in vitro* plantlets using the encapsulation-dehydration technique (González-Arno *et al.*, 1993), cell suspensions (Finkle and Ulrich, 1979; Gnanapragasam and Vasil, 1990) and embryogenic callus (Eksomtramage *et al.*, 1992; Gnanapragasam and Vasil, 1992; Martínez-Montero *et al.*, 1998). Jian *et al.* (1987) reported high survival rates and recovery of whole plants using embryogenic callus of a single clone of sugarcane following cryopreservation. Studies performed by Eksomtramage *et al.* (1992) reported the development of a cryopreservation process for embryogenic calli of a commercial hybrid of sugarcane, which was successfully applied to calli of 10 varieties out of the 11 tested. Successful cryopreservation techniques were also reported by Martínez-Montero *et al.* (2000, 2002, 2008). However, somatic embryos of the local variety, 88H0019, proved intractable to cryopreservation, as had been the case in previous studies (O'Brien, 2001; Cheruiyot, 2002).

This account provides possible explanations for the difficulties associated with cryopreserving sugarcane germplasm of this variety and offers recommendations for future attempts.

Chapter 2: Induction of Somatic Embryos

2.1 Introduction

Sugarcane is a commercial crop that remains vegetatively propagated, which is successfully achieved by means of stalk cuttings with axillary buds. This method of propagation is of particular importance for the maintenance of favourable genotypes, but is not ideal for preservation of genetic resources since it demands that plants from the stalks be grown in nurseries (Vasil and Vasil, 1994; Bajaj and Jian, 1995). The potential of *in vitro* culture systems, has long been investigated for the purposes of *in vitro* micropropagation (Lee, 1987), the production of disease-free plants (Irvine and Benda, 1985), somatic cell improvement through culture-induced mutations (Heinz and Mee, 1969; Larkin and Scowcroft, 1981) and genetic transformation (Bower *et al.*, 1996; Gallo-Meagher and Irvine, 1996; Arencibia *et al.*, 1997). Cryopreservation offers the means for long-term conservation and, when successful, is far more cost-effective and less labour-intensive than the use of culture systems. However, implicit to the success of cryopreservation is the establishment of appropriate *in vitro* growth conditions prior to the cooling process. This involves the optimisation of tissue culture protocols including the nutrient media; prior surface decontamination, since *in vitro* germination is frequently impeded by micro-organism presence and proliferation; and the smallest possible explant size (Engelmann, 2000).

In vitro growth media provide nutrition to developing explants and contain a carbon source generally in the form of sucrose and/or glucose, as well as vitamins and essential nutrients (Gamborg and Shyluk, 1981). Some of the macronutrients that are required by developing explants include nitrogen, phosphorus, potassium, calcium, sulphur and magnesium, together with the micronutrients, iron, manganese, zinc, boron and molybdenum (Bhojwani and Razdan, 1983). These macro- and micronutrients are available as prepared standard media formulations, such as that of Murashige and Skoog (1962) or Gamborg *et al.* (1968), while the nutrient requirements of woody plant species that are sensitive to high salt

concentrations are generally satisfied by a 'woody plant medium' (WPM) developed by Lloyd and McCown (1981). In some instances, plant growth regulators are necessary to promote root and/or shoot formation in non-responsive explants (e.g. Haissig, 1972; Strömquist and Hansen, 1980).

This chapter reports on: the successful induction of somatic embryos via both the direct and indirect routes of micropropagation; a suitable regeneration medium for plantlet establishment pre- and post-cooling; viability assays to determine the germinability of both directly and indirectly derived somatic embryos; the determination of yield in terms of the number of plantlets that were produced as a result of direct and indirect somatic embryogenesis, both at the SASRI and UKZN laboratories; and the assessment of the water content of individual clumps of directly and indirectly derived somatic embryos.

2.2 Materials and Methods

2.2.1 Plant material

The apical region of the stem of field-grown sugarcane, (*Saccharum officinarum* L.) of the 88H0019 variety, were harvested as stalks on a weekly basis at SASRI, Mount Edgecombe. The stalks were trimmed and the older remaining outer leaves removed, before being packed in black plastic bags to minimise water loss. While some of the initial experiments were performed at SASRI, mostly the stalks were promptly transported by road to the laboratory at the University of KwaZulu-Natal (UKZN), Durban, where they were processed within 24 h of harvesting.

2.2.2 Induction of somatic embryos

The apical regions of the stem were trimmed to 200-300 mm. The remaining outer leaves were removed and the leaf roll bearing the apical meristem was swabbed with 70% (v/v) ethanol. In a laminar air flow, the leaf rolls were sprayed with 70% (v/v) ethanol and a further two or three leaf layers were peeled away under aseptic conditions. The innermost leaves were sliced into transverse sections approximately 3 mm thick, 20 leaf roll discs (explants) per stalk being taken specifically from the region directly above the uppermost node in the apical region. These leaf roll discs were placed in 90 mm Petri dishes (10 explants per Petri dish) containing 30 ml embryo induction medium comprising 4.4 g l⁻¹ Murashige and Skoog (MS) basal salts and vitamins, 20 g l⁻¹ sucrose, 0.5 g l⁻¹ casein hydrolysate and 8 g l⁻¹ agar (Agar-agar; Biolab, South Africa), pH 5.6-5.8. For direct and indirect embryogenesis, 0.6 mg l⁻¹ and 3 mg l⁻¹ 2,4-dichloro-phenoxyacetic acid (2,4-D), respectively, were added to the embryo induction medium. Leaf sections were placed with the adaxial surface in contact with the culture medium. Petri dishes were sealed with Parafilm® and incubated in the dark at 25±1 °C for 6 weeks. Explants were subcultured onto fresh medium at two-week intervals. For plantlet regeneration (indirect embryogenesis approach), embryogenic callus was transferred to the same medium but lacking 2,4-D, solidified with 2 g l⁻¹ Gelrite®, pH 5.6-5.8, and placed in a growth room with 100 µmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD), 16 h light/8 h dark photoperiod and 24°C day/21°C night temperatures. For plantlet regeneration (direct embryogenesis approach), embryo clumps (clumps of a minimum of three embryos) were excised and transferred to the regeneration medium described above. The initial somatic embryo induction was performed at the SASRI laboratory and all subsequent inductions were carried out at the UKZN laboratory.

2.2.3 Regeneration medium

The basic regeneration or growth medium for untreated somatic embryos was previously established (O'Brien, 2001) comprising 4.4 g l⁻¹ MS basal salts and vitamins, 20 g l⁻¹ sucrose, 0.5 g l⁻¹ casein hydrolysate and 8 g l⁻¹ agar, and not containing any plant growth regulators (PGRs), pH 5.6-5.8. This medium, however, proved unsuccessful for the establishment of plantlets after rapid dehydration, cryoprotection or cooling the explants (see later). Consequently, the effect of incorporating plant growth regulators into the basal regeneration medium on plantlet establishment from directly-induced somatic embryos following cryopreservation was tested. The plant growth regulators tested were 2,4-D at concentrations of 0.6 mg l⁻¹ and 3.0 mg l⁻¹, or benzylaminopurine (BAP) at concentrations of 0.5 mg l⁻¹, 1.0 mg l⁻¹, 1.5 mg l⁻¹ and 2.0 mg l⁻¹.

2.2.4 Initial viability assays

Freshly excised embryo clumps were placed on the basic regeneration medium and cultured under growth room conditions (see 2.2.2) to assess initial viability. Survival of at least one embryo per clump was assessed on the basis of root and shoot development. The protrusion of a radicle coupled with shoot formation was scored as survival, whereas those not exhibiting such development after 2 months were scored as non-surviving. Non-surviving embryo clumps were tested with 2,3,5-triphenyl-tetrazolium chloride (TTZ), as employed by the ISTA (1999) for viability retention.

2.2.5 Determination of yield

Induction of embryos via direct and indirect embryogenesis was carried out using stalks of the 88H0019 variety of sugarcane (n=30) at the SASRI laboratory. Stalks were harvested from the field, prepared according to the protocol outlined (2.2), and cultured such that exactly half the number of stalks should have yielded embryos via direct embryogenesis and the other half via indirect embryogenesis. Twenty explants were obtained from each

stalk and these were plated at 10 explants per Petri dish thereby yielding a total of 60 plates (30 for direct and 30 for indirect embryogenesis). These were incubated in the dark at SASRI at 28°C for 6 weeks to induce somatic embryo production. The same experiment was simultaneously performed at the University of KwaZulu-Natal (UKZN) laboratory such that the plantlet yield at the two venues could be compared. Cultures at the UKZN were incubated in the dark, but at 25°C, also for 6 weeks. Explants at the two venues were subcultured onto fresh media at two-week intervals and levels and types of residual microbial contamination were recorded. Following incubation, the white, nodular embryos produced in all cases were excised and cultured on regeneration medium. These were transferred to light culture conditions at both venues (16 h light/8 h dark photoperiod 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD and 24°C day/21°C night temperature) and subcultured onto fresh regeneration medium at two-week intervals until plantlets could be separated from each other and counted individually. The presence of both a root and shoot was scored as a functional plantlet.

2.2.6 Water content determination

Somatic embryos at the late globular stage of development were excised from the explant in clumps of three (Fransz and Schel, 1991; Dumet *et al.*, 1993a; Mycock, 1999). The water content per clump of fresh embryos was determined gravimetrically by weighing on a Mettler MT5 six-place balance before and after drying in an oven at 80°C for 48 h. Ten embryo clumps were used per experiment and water content was determined on a dry mass basis (dmb), expressed as $\text{g H}_2\text{O g}^{-1}$ dry mass (g g^{-1}). This experiment aimed to ascertain the water content of freshly excised embryos produced both directly and indirectly, and the associated ability for conversion into plantlets.

2.2.7 Statistical analyses

Each variable was tested for normal distribution as described by Fowler *et al.* (1999). Percentage variables were transformed (for statistical analysis only and not for presentation of data) in accordance with $x=2 \arcsin ((x/100)^{0.5})$. Thereafter analysis of variance (ANOVA) and Tukey's post-hoc tests were performed. The same approach was applied to data presented in subsequent chapters.

2.3 Results and Discussion

2.3.1 Somatic embryo induction

Somatic embryos were successfully generated via both the direct and indirect routes of micropropagation; however, the time taken for somatic embryo production via the two routes differed. Somatic embryos produced via the direct route of micropropagation were at the late globular stage of development (stage at which embryos were excised for cryopreservation studies) after 5-6 weeks whereas those produced via the indirect route reached this stage of development only after 7-8 weeks (Figure 2.1a and b respectively).

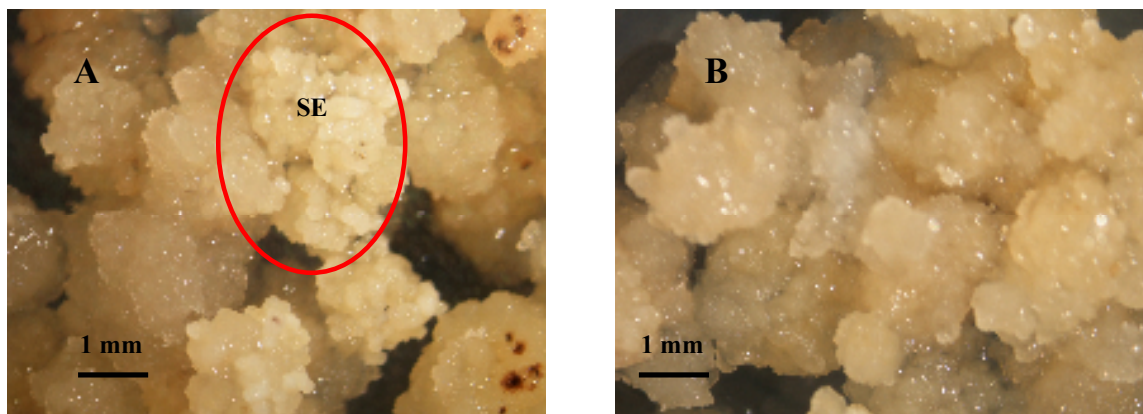


Figure 2.1 Somatic embryos (SE) at the late globular developmental stage (A), and callus (B), following 6 weeks culture on direct and indirect embryo induction media containing 0.6 and 3.0 mg l⁻¹ 2,4-D, respectively

2.3.2 Regeneration media

The regeneration of plantlets from sugarcane callus has been reported by several workers (Nadar *et al.*, 1978; Ho and Vasil, 1983a; Liu, 1984; Chen *et al.*, 1988; Snyman *et al.*, 1996) and histological studies have demonstrated that plantlet regeneration occurs by either organogenesis (Liu and Chen, 1974) or somatic embryogenesis (Ho and Vasil, 1983a; Chen *et al.*, 1988). Ho and Vasil (1983a, b) further suggested that previous reports of shoot formation via organogenesis may be re-interpreted as ‘abnormal somatic embryo development’. Later studies, however, have demonstrated that shoot production from callus tissue of barley (Weigel and Hughes, 1985) and maize (Lowe *et al.*, 1985) is the result of both somatic embryogenesis and organogenesis. It is very possible that both processes may occur simultaneously in sugarcane as well (Chen *et al.*, 1988).

Presently, whether the cultures were exposed to light or held in the dark, somatic embryos (derived directly and indirectly) on medium containing no PGRs developed shoots and roots after 14 d and exhibited the highest percentage of direct embryo conversion in

comparison to the other media tested, which contained 2,4-D or BAP (Figure 2.2). This supports findings of a study performed by Nadar and co-workers (1978) which demonstrated that omission of the auxin analogue, 2,4-D, from media promoted plantlet differentiation from sugarcane callus; however, this does not necessarily apply to all subcultures. In the present study, inclusion of 2,4-D or BAP in media for embryo conversion was also assessed, as subsequent experiments had shown that the PGR-free medium was not conducive to plantlet formation after further processing of embryos for cryopreservation (see later). For cultures presently maintained in the dark inclusion of 2,4-D did not appear to have any adverse effect on the percentage of embryo conversion, irrespective of the concentration used. However, in these cases there was an intervening callus phase (see below). Significantly less conversion occurred on media containing BAP whether calli were held in the dark or not (Figure 2.2).

The auxin, 2,4-D, is reported the most effective for induction of callus and formation of somatic embryos in cell and tissue cultures of grasses (Vasil *et al.*, 1982), including sugarcane (Ho and Vasil, 1983b). In the present study the development of plantlets occurred directly from the somatic embryos without the formation of callus tissue only on the PGR-free medium, which may have important implications for the maintenance of genetic integrity (Taylor and Dukic, 1993). Formation of callus with potential tissue culture instability, may, in turn lead to genetic changes in regenerated plants (Scowcroft, 1984), regardless of whether regeneration occurs through somatic embryogenesis or by adventitious shoot formation (Larkin and Scowcroft, 1981; Scowcroft, 1984).

Somatic embryos cultured in the dark on medium containing 2,4-D (0.6 mg l^{-1} and 3.0 mg l^{-1}) exhibited high embryo conversion (Figure 2.2); however, the formation of shoots and roots was preceded by callus formation, as has also been found in the Poaceae when immature zygotic embryos are used as explants in indirect somatic embryogenesis (Vasil and Vasil, 1994). It has been suggested that callus originates from peripheral cells on specific regions of the scutellum (Vasil, 1987), and that 2,4-D functions to perpetuate embryogenic callus by facilitating continued division of embryogenic cells which ensures

that cell division in meristematic zones formed by proliferating tissue remains ongoing (Vasil, 1987). Somatic embryos are effectively formed as a result of the organisation of cells at the periphery of embryogenic callus in response to lowered levels of 2,4-D (Ho and Vasil, 1983a; Fransz and Schel, 1991). Very low levels of 2,4-D, however, are associated with irreversible differentiation of embryogenic cells, characterised by their enlargement and increased degree of vacuolation, loss of basophilicity and cytoplasm-rich character, cell wall thickening, and disappearance of starch, all of which culminate in either the formation of friable non-embryogenic callus, or root formation (Vasil, 1987). This could provide an explanation, in part, for the formation of roots by somatic embryos when embryos were presently regenerated on medium without PGRs.

The high yields of indirect somatic embryogenesis recorded on medium containing 2,4-D correlate well with the findings of a study conducted by Cheruiyot (2002) who reported best results when sugarcane (variety N12) somatic embryos were regenerated on medium containing 0.6 mg l^{-1} 2,4-D. This is further supported by reports of high survival of sucrose-desiccated (0.75 M) oil palm somatic embryos following transfer onto medium containing 0.2 mg l^{-1} 2,4-D (Dumet *et al.*, 1993a), and by the results of Snyman *et al.* (2000a, b) who reported successful regeneration of late globular sugarcane somatic embryos on medium containing 0.3 mg l^{-1} 2,4-D.

The results of this experiment also indicate that dark culture conditions are more favourable for indirect regeneration of somatic embryos on media containing 2,4-D (Figure 2.2). This is in general agreement with a study conducted by O'Brien (2001) on the N12 variety, who reported successful regeneration when somatic embryos induced on medium containing 0.6 mg l^{-1} 2,4-D and cultured in the dark for 5 weeks, were transferred to the light for conversion.

Hendre *et al.* (1983) reported successful shoot multiplication of *in vitro* plantlets following culture on medium containing kinetin and BAP. Regeneration media containing BAP at the concentrations presently used did not prove as successful as the medium containing no

PGRs (as depicted in Figure 2.2). Statistical analyses show that there was a significant difference in the conversion capacity of somatic embryos cultured on the different regeneration media (Table 2.1). The reduced percentage conversion of somatic embryos cultured on media containing BAP could be explained by reports of inhibitory effects of cytokinins on sugarcane callus growth, as has been reported for other gramineous species (Dudits *et al.*, 1975; Dale and Deambrogia, 1979). However, those authors also identified the role of cytokinins in initiation of multiple shoot meristems and precocious germination following the formation of pro-embryoids.

Although culture on the presently used regeneration medium containing no PGRs resulted in the highest embryo conversion, this medium did not prove to be useful for the regeneration of somatic embryos after cryopreservation, as there was no shoot and/or root formation (see Chapter 4).

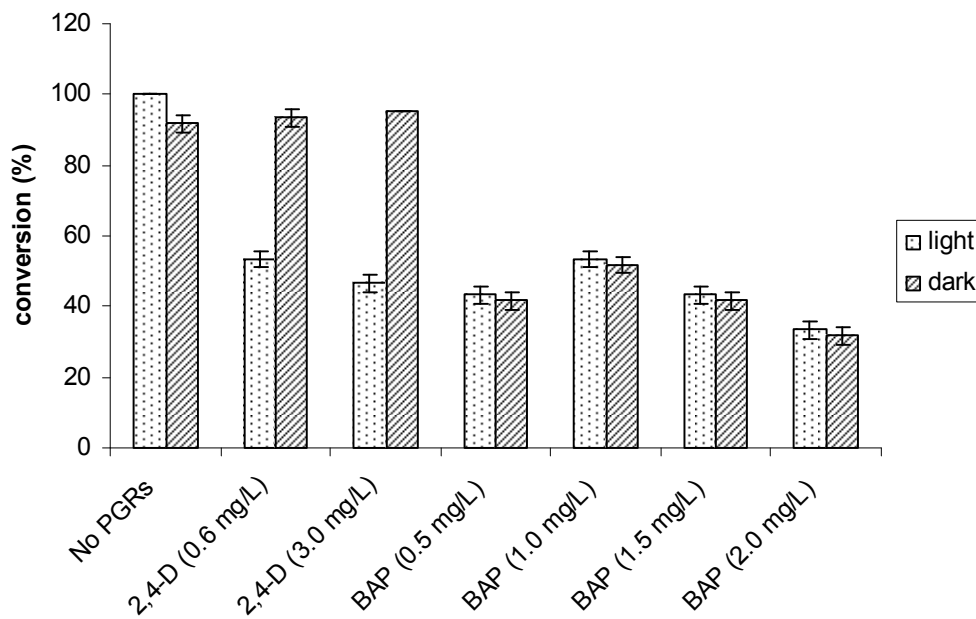


Figure 2.2 The percentage somatic embryo conversion of directly-induced somatic embryos on different regeneration media after 8 weeks *in vitro* cultured under either light and dark conditions (n=60). Bars indicate standard deviation. Note that callus-free conversion occurred only in the absence of any PGR

Table 2.1 Effects of 2,4-D and BAP on the conversion capacity (%) of sugarcane somatic embryos

Treatment	Mean (%)
No PGRs	95.9 (81.7) ^{* a}
2,4-D (0.6 mg l ⁻¹)	73.3 (61.1) ^b
2,4-D (3.0 mg l ⁻¹)	70.9 (60.1) ^b
BAP (0.5 mg l ⁻¹)	42.5 (40.7) ^d
BAP (1.0 mg l ⁻¹)	52.5 (46.5) ^c
BAP (1.5 mg l ⁻¹)	42.5 (40.7) ^d
BAP (2.0 mg l ⁻¹)	32.5 (34.8) ^e

*The values in parentheses indicate the arcsine transformation of conversion (%).

The same letters indicate no significant difference between the means according to the Tukey test at 5% level of significance.

The interactive effect of plant growth regulators (PGRs) and light/dark regime on the conversion capacity (%) of sugarcane somatic embryos is shown in Table 2.2 and indicates that the conversion of somatic embryos is significantly affected by light and dark conditions, as well as type and concentrations of PGRs. This has also been reported for other species, e.g. *Rauvolfia micrantha* (Sudha and Seeni, 2006). The effect of light on *in vitro* cultures has been demonstrated in different plant species (Arumugam and Bhojwani, 1990), where it is believed effectively to alter the endogenous level of PGRs and sensitivity of cells to hormones, thereby either suppressing or promoting embryogenesis. It has been suggested that darkness most likely preserves light-sensitive endogenous components (Evans *et al.*, 1981b). While the induction of somatic embryos is favoured by dark conditions, and is well documented (Hutchinson *et al.*, 2000), embryo maturation and development of plants is enhanced by light conditions as a result of light-enhanced metabolism of auxin (Sudha and Seeni, 2006).

Table 2.2 The interactive effect of the plant growth regulators (PGRs), 2,4-D or BAP, and light/dark regime on the conversion capacity (%) of sugarcane somatic embryos

Treatment	Light	Dark
No PGRs	100 (90.0) ^{* a}	91.7 (73.4) ^b
2,4-D (0.6 mg l ⁻¹)	53.3 (46.9) ^{cd}	93.3 (75.3) ^b
2,4-D (3.0 mg l ⁻¹)	46.7 (43.1) ^{cd}	95.0 (77.1) ^b
BAP (0.5 mg l ⁻¹)	43.3 (41.1) ^d	41.7 (40.2) ^{de}
BAP (1.0 mg l ⁻¹)	53.3 (46.9) ^c	51.7 (46.0) ^{cd}
BAP (1.5 mg l ⁻¹)	43.3 (41.1) ^d	41.7 (40.2) ^{de}
BAP (2.0 mg l ⁻¹)	33.3 (35.3) ^e	31.7 (34.2) ^e

*The values in parentheses indicate the arcsine transformation of conversion (%).

The same letters indicate no significant difference between the means according to the Tukey test at 5% level of significance.

2.3.3 Determination of yield

Somatic embryos were generated via the direct and indirect routes of micropropagation with yields at UKZN labs being lower than those at SASRI laboratories for both routes. The best yield at SASRI was seven plantlets/explant by the direct route, whereas five plantlets/explant developed at the UKZN (Table 2.3). Embryos also appeared to develop more slowly at the UKZN than at SASRI. These differences are ascribed to slight variation in the growth room conditions between the two venues, or perhaps that there was no delay in initiating procedures at SASRI, as was imposed by transport of the stalks to the UKZN laboratory. In addition to the difference between plantlet yields at the two venues, more plantlets resulted from the direct route of micropropagation than the indirect (Table 2.3).

Theoretically, it is expected that callus should produce more plantlets; however, a study performed by Lee (1987) showed that this is not necessarily true.

Table 2.3 Plantlet yield at the two culture venues, SASRI and UKZN laboratories

Culture venue	Yield of plantlets per explant	
	Direct	Indirect
SASRI	7	4
UKZN	5	4

2.3.4 Initial water content

Cryopreservation is influenced by several critical factors that act either in isolation, or in combination with others to determine success or failure of the cryo-process. Water content is one such factor in its implication in the intracellular formation of potentially lethal ice crystals during cooling (Mazur, 1970; 1984; Wesley-Smith *et al.*, 2001a, b; Özkavukcu and Erdemli, 2002). Essentially, the lower the water content at the liquid-solid transition temperature during cooling, the less solution (freezable) water will be available for ice crystal formation. Additionally, lower water content translates into reduced thermal load thereby facilitating the quick passage of plant material through the critical temperature range that encourages ice crystal formation (Wesley-Smith *et al.*, 2001a, 2004b; Li and Sun, 2002; Wesley-Smith, 2002). Furthermore, lowering the water content promotes freezing point depression of cellular contents, which in turn reduces the critical temperature range at which crystallisation can occur (reviewed by Wesley-Smith, 2002). It was therefore important to determine the water content of freshly excised directly- and indirectly-induced somatic embryos, as a starting point.

The water content of freshly excised somatic embryo clumps was gravimetrically determined according to the protocol described in section 2.2.6. A comparison was made between the water contents of directly- and indirectly-induced somatic embryos to ascertain whether there were marked differences that might affect their suitability for cryopreservation. The mean water content ($\text{g g}^{-1} \pm$ standard deviation) of somatic embryo clumps derived via the direct and indirect routes of micropropagation was $8.38 \pm 0.19 \text{ g g}^{-1}$ and $8.45 \pm 0.33 \text{ g g}^{-1}$, respectively. The mean percentage conversion at these water contents was 97% for directly and 98% for indirectly derived somatic embryos (Figure 2.3). The data show no significant difference in the water contents, or conversion capacity, between directly- and indirectly-induced somatic embryos.

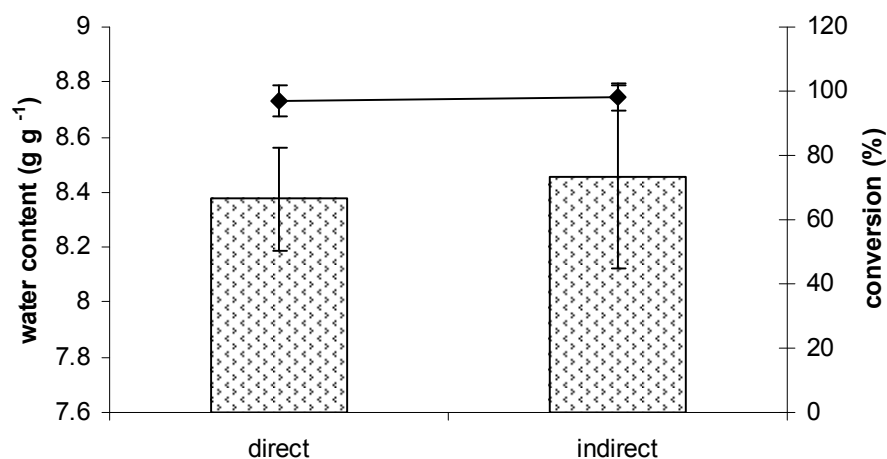


Figure 2.3 Mean water contents of freshly excised clumps of somatic embryos generated via the direct and indirect routes of micropropagation ($n=30$), and the corresponding mean percentage conversion ($-\blacklozenge-$) of the somatic embryos on PGR-free medium ($n=60$). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

2.3.5 Concluding remarks

Somatic embryos of sugarcane variety 88H0019 were successfully generated via both the direct and indirect routes of micropropagation, with directly-induced embryos forming in a shorter time than indirectly-induced embryos (5-6 weeks for directly- and 7-8 weeks for indirectly-induced embryos, respectively). Both directly- and indirectly-induced embryos were successfully converted on medium without PGRs with a higher plantlet yield via the direct route of micropropagation compared with the indirect route. The incorporation of 2,4-D and BAP in the regeneration medium did not result in increased survival levels of freshly excised somatic embryos. In fact, inclusion of these PGRs had negative effects, with BAP being more deleterious than 2,4-D. A comparison between the water contents of directly- and indirectly-induced embryos showed no significant difference between the two, with mean water contents of $8.38 \pm 0.19 \text{ g g}^{-1}$ and $8.45 \pm 0.33 \text{ g g}^{-1}$, respectively. The percentage conversion at these water contents were 97% for directly- and 98% for indirectly-induced embryos. Initial water content is a critical factor affecting the success or failure of the cryo-process as explants must be capable of surviving considerable dehydration to obviate the intracellular formation of potentially lethal ice crystals during cooling (Wesley-Smith *et al.*, 2001a, b). These data provided a starting point for manipulations leading to the possible cryopreservation of sugarcane somatic embryos of variety 88H0019.

Chapter 3: Responses of Directly-Induced Somatic Embryos to Dehydration

3.1 Introduction

Many studies have shown that the water content of explants has a major effect on the success (or otherwise) of the cryo-process, as it determines the extent of intracellular ice crystal formation (e.g. for somatic embryos of *Elaeis guineensis*, Dumet *et al.*, 1993a; somatic embryoids of *Phoenix dactylifera*, Mycock *et al.*, 1997; axillary buds of *Vitis vinifera*, Miaja *et al.*, 2000; and embryogenic cultures of *Magnifera indica*, Wu *et al.*, 2003). Except in the case of already-dry, desiccation-tolerant specimens, partial dehydration of plant material is an essential prerequisite for cryopreservation. This can be achieved by physical drying by several means, including relatively rapid dehydration in the sterile airstream of a laminar hood, e.g. for zygotic axes of rubber (Normah *et al.*, 1986), tea (Chaudhury *et al.*, 1991), coffee (Abdelnour-Esquivel *et al.*, 1992), and oil palm (Engelmann *et al.*, 1995a), as well as for somatic embryos of oil palm (Dumet *et al.*, 1993a), coffee, and date palm (Mycock *et al.*, 1995; Mycock, 1999, respectively). Alternatively, relatively fast drying in airtight containers containing activated silica gel can be carried out, as for zygotic embryonic axes of *Euphoria longan* (Fu *et al.*, 1993) and somatic embryos of oil palm (Dumet *et al.*, 1993a, b). The duration of desiccation generally varies with the size and initial water content of the explant (Withers and Engelmann, 1998). Survival after dehydration to a water content range of 10-20% fresh mass basis (fmb) *i.e.* 0.09-0.17 g g⁻¹ dry mass, has been reported not to be compromised by subsequent cryopreservation, for zygotic embryos of tea (*Camellia sinensis*) (Chaudhury *et al.*, 1991); hazel (*Corylus avellana*) (Gonzalez-Benito and Perez, 1994a) and coffee (Abdelnour-Esquivel *et al.*, 1992).

Generally, the most rapid dehydration rate is achieved via flash-drying, which circulates a dry air over explants that are suspended on a mesh above a fan in a sealed vessel containing a bed of activated silica gel (Berjak *et al.*, 1990; Pammenter *et al.*, 1991, 2002). The efficacy of this method for the non-injurious, rapid dehydration of recalcitrant zygotic axes

is supported by a number of studies (Berjak *et al.*, 1996; Kioko *et al.*, 1998; Wesley-Smith *et al.*, 1999; Pammenter *et al.*, 2002). For example, flash-drying has successfully been employed for dehydration of zygotic axes of *Landolphia kirkii* (Pammenter *et al.*, 1991, Vertucci *et al.*, 1991) and *Camellia sinensis* (Wesley-Smith *et al.*, 1992; Berjak *et al.*, 1993) and those of a variety of other recalcitrant-seeded species (Berjak and Pammenter, 2004). Flash-dried zygotic axes of *Trichilia dregeana* maintained viability to a water content of $\pm 0.16 \text{ g g}^{-1}$, whereas axes that were slowly dried exhibited a loss of viability at considerably higher water contents (Kioko *et al.*, 1998). As a further example of the effects of drying rate on recalcitrant material, Wesley-Smith *et al.* (2001b) showed that axes of jackfruit that were flash-dried to 0.4 g g^{-1} maintained 100% survival, whereas no axes that were dried slowly to the same water content survived. These observations are supported by other findings which showed that drying rate affects the degree of desiccation transiently tolerated by recalcitrant seeds or excised axes, with faster drying rates permitting dehydration to lower water contents before desiccation-induced damage becomes evident (Berjak *et al.*, 1990, 1993; Pammenter *et al.*, 1991, 1998; Kundu and Kachari, 2000; Liang and Sun, 2000; Wesley-Smith *et al.*, 2001a). This has also been shown to be the case with somatic embryos of carrot (*Daucus carota*), where rapid drying facilitated higher survival than did slow drying, although there was little or no difference in overall protein secondary structure irrespective of drying rate (Wolkers *et al.*, 1999). Those authors explain this absence of protein denaturation by the transformation of the cytoplasm into a glassy state in both drying regimes, which prevented changes in protein conformation.

Excised embryonic axes of *Hevea brasiliensis* (Normah *et al.*, 1986) and *Araucaria hunsteinii* (Pritchard and Prendergast, 1986; Poulsen, 1992) were successfully dried in a laminar flow cabinet; however, this method of dehydration is often not rapid enough to minimise metabolism-linked damage occurring at intermediate hydration levels (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998, 2002; Berjak and Pammenter, 2001; Walters *et al.*, 2001). Although (as explained below) slow drying favours desiccation-tolerance in orthodox seeds (Bewley and Black, 1994; Corbineau *et al.*, 2000), it has been found to be deleterious in recalcitrant seeds (Côme and Corbineau, 1996; Walters *et al.*, 2002;

Pammenter *et al.*, 1998, 2002). In summary, the faster the drying rate, the lower the water content that can be tolerated by recalcitrant seeds or excised axes (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999), whereas the opposite effect pertains in orthodox seeds before the onset of (natural) maturation drying. Those authors suggest that this is because slow drying allows sufficient time for the induction and operation of inherent protection mechanisms in orthodox seeds. The absence or inefficiency of these mechanisms in recalcitrant tissue results in unbalanced metabolism during dehydration, which is exacerbated when tissue is exposed to intermediate hydration levels for extended periods (Berjak and Pammenter, 1997; Pammenter *et al.*, 1998; Walters *et al.*, 2001). While damage associated with slow dehydration of recalcitrant tissue may be avoided or limited by increased physical dehydration rates (Wesley-Smith, 2002), there are other methods by which non-injurious dehydration of explants may be achieved. These include pregrowth, preculture and encapsulation-dehydration which are more commonly employed for explants other than zygotic embryonic axes, in cryopreservation trials (e.g. Shibli and Al-Juboory, 2000; Hornung *et al.*, 2001; Wu *et al.*, 2003).

3.1.1 Pregrowth, preculture and encapsulation-dehydration

Pregrowth refers to the culture of explants for one to several days on medium containing cryoprotectants such as sucrose, DMSO, glycerol, etc. (Cho *et al.*, 2001). Preculture involves a similar procedure but for shorter times (minutes to a few hours). Pregrowth is associated with an osmotic decrease in the water content of explants, and where penetrating cryoprotectants are employed, an overall increase in intracellular concentration (Cho *et al.*, 2001). Pregrowth and preculture are considered to be critical steps in cryopreservation protocols for zygotic and somatic embryos of various species (Engelmann *et al.*, 1995a, b; Dumet *et al.*, 1997; Engelmann, 1997). This approach can be further refined by employing the encapsulation-dehydration technique, as described by Fabre and Dereuddre (1990). This involves encapsulation of explants in alginate beads, subsequent exposure to a medium enriched in sucrose, further partial dehydration, usually in a laminar airflow or with activated silica gel, followed by plunging into liquid nitrogen. Both osmotic and

physical dehydration effect an increase of sucrose concentration within the beads until the saturation point of the sucrose solution is reached or exceeded, apparently facilitating glass transition during cooling to -196°C (Dereuddre *et al.*, 1991). This procedure appears to be effective in precluding lethal intracellular ice crystallisation (Engelmann, 1997), and has permitted successful cryopreservation of organised tissues such as shoot tips and somatic embryos of different plant species (Dereuddre, 1992; Shibli and Al-Juboory, 2000; Hornung *et al.*, 2001; González *et al.*, 1995; Wu *et al.*, 2003).

3.1.2 Desiccation damage

For desiccation-sensitive seeds and their component parts dehydration damage can be either metabolism derived, which is characterised by unbalanced metabolism during drying (Pammenter and Berjak, 1999; Walters *et al.*, 2001), and/or related to removal of non-freezable water (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998; Walters *et al.*, 2001). Slow drying damage is associated with continuing, but unbalanced metabolism, resulting in the uncontrolled generation of free radicals (Henry *et al.*, 1992; Chaitanya and Naithini, 1994; Smith and Berjak, 1995; Finch-Savage *et al.*, 1996; Leprince *et al.*, 2000; Walters *et al.*, 2001). It has, however, been shown to be less injurious to embryos of occasional recalcitrant-seeded species than more rapid dehydration (Liang and Sun, 2000), but this may have been a consequence of the volume of tissue undergoing dehydration and the *actual* rate of dehydration of critical regions, that was achieved.

Actively metabolic axes from recalcitrant seeds respond better to rapid dehydration since this allows low water contents to be reached with minimal accumulation of metabolism-linked damage (Wesley-Smith, 2002) by curtailing the time available for damaging aqueous-based reactions to occur (Berjak and Pammenter, 1997; Walters *et al.*, 2001, 2002). There is, however, considerable variation in the optimal water contents appropriate for cryopreservation of zygotic, recalcitrant axes between and within species. Should the hydration level decline to, or below, the level where all water is non-freezable, then desiccation damage *sensu stricto* may occur (Pammenter and Berjak, 1999; Walters *et al.*,

2001). Here too, the value apparently differs from one type of explant to the next. Since non-freezable water is associated with the maintenance of membrane and macromolecule integrity, the removal of this water is lethal to tissues not having the inherent capacity for intracellular protection (Pammenter and Berjak, 1999).

The present chapter reports on investigations of the responses of somatic embryos of sugarcane (variety 88H0019) to different dehydration techniques. These investigations were aimed at identifying the minimum water content to which excised embryo clumps could be dehydrated with 50% or more still maintaining viability. The development of a successful cryopreservation protocol is pivotally dependent on the reduction of the water content in cells, prior to exposure to LN, while avoiding extensive dehydration that would result in desiccation damage. It was therefore imperative that the range of water contents supporting survival during and subsequent to cooling, be defined experimentally.

3.2 Materials and Methods

3.2.1 Flash-drying

Directly-induced somatic embryos at the late globular stage of development (n=210) were excised from the mother explant in clumps of three and accumulated within Petri dishes on filter paper moistened with deionised water. The embryos were then dehydrated in a flash-dryer (for design, see Pammenter *et al.*, 2002) and sampled at regular intervals after increasing drying times. The water content of ten individual embryo clumps was gravimetrically determined at each drying interval and the germinability of twenty embryo clumps was tested following rehydration by direct immersion in sterile calcium-magnesium (Ca/Mg) solution containing 1 μ M CaCl₂.2H₂O and 1 mM MgCl₂.6H₂O (Mycock, 1999) in the dark for 30 min at 25°C. After rehydration the embryo clumps were surface decontaminated in 1% calcium hypochlorite [Ca(OCl)₂] for 5 min, rinsed three times with the sterile Ca/Mg solution, and germinated on regeneration medium under standard growth room conditions (as described previously; section 2.1). Somatic embryos were confirmed

dead by means of a negative result for the TTZ test (ISTA, 1999). The experiment was replicated three times; the results presented representing the pooled data.

3.2.2 Osmotic dehydration

Somatic embryos generated by both direct and indirect embryogenesis (n=90, in both cases) were excised in clumps of three at the late globular stage and dehydrated osmotically. This was achieved by culturing the embryo clumps on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h on each medium. Ten explants were sampled for water content, and twenty for germinability and plantlet establishment after incubation on media containing 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M and 1.2 M sucrose, in order to establish the best pre-growth treatment (i.e. sufficiently reduced water content coupled with maintenance of high viability of embryos). The control (fully hydrated) embryos were subcultured on a medium with standard sucrose concentration (0.056 M) for 48 h, and sampled simultaneously with the experimental explants for water content, germinability and plantlet establishment. The experiments were replicated three times; the results presented representing the pooled data.

3.3 Results and discussion

3.3.1 Response of somatic embryos to flash-drying

Somatic embryos were flash-dried and sampled at 5 min intervals over a period of 30 min during which there was a rapid decline of the mean water content from $7.07 \pm 0.94 \text{ g g}^{-1}$ to $0.38 \pm 0.17 \text{ g g}^{-1}$ (Figure 3.1). Control somatic embryos (not flash-dried) exhibited 100% conversion characterised by normal root and shoot formation; however none of those subjected to flash-drying - even for 5 min - survived. This experiment was repeated three times (Figure 3.1 showing the pooled data) yielding 0% survival of flash-dried embryos each time. The flash-drying technique was developed for rapid dehydration of excised zygotic embryonic axes (Berjak *et al.*, 1990) such that relatively low water contents could

be achieved rapidly, in several minutes to hours (depending on the explant), with minimal injury to explants (Wesley-Smith *et al.*, 1995, 2001b; Berjak and Pammenter, 2001; Pammenter *et al.*, 2002). Studies on axes excised from recalcitrant seeds have revealed that the degree of desiccation tolerated is linked to drying rate, with less damage inflicted on explants at any one water when drying rates are more rapid (Pammenter *et al.*, 1998; Berjak *et al.*, 1999; Walters *et al.*, 2001). Flash-drying, however, does not confer desiccation-tolerance on explants from recalcitrant seeds, which die if left at ambient temperature even for a few hours (Walters *et al.*, 2001). In the present study, however, despite being immediately rehydrated, the somatic embryos of sugarcane did not tolerate such rapid dehydration, even to relatively high water content ($2.06 \pm 1.08 \text{ g g}^{-1}$) achieved in 5 min.

Water within cells occurs in different states depending on the hydration status of the tissue (Vertucci and Farrant, 1995; Walters *et al.*, 2005; Berjak, 2006). Water present in tissue at a concentration above $\sim 0.3 \text{ g g}^{-1}$ is generally considered freezable and below this level water is mostly associated with structures and surfaces within cells and is therefore considered non-freezable (Vertucci, 1990). While metabolism-linked damage may be avoided, or minimised by rapid drying, dehydration of desiccation-sensitive material to/near the level of non-freezable water is associated with generally lethal desiccation damage *sensu stricto* (Pammenter *et al.*, 1998; Walters *et al.*, 2001). Those authors suggest that in an attempt to reach intracellular equilibrium, a proportion of the structure-associated water may be lost to the freezable fraction. This could possibly explain the inability of sugarcane somatic embryos of variety 88H0019 to survive any dehydration by flash-drying, which resulted in complete viability loss within 5 min. However, in this short time, considerable metabolism-linked damage may well have occurred, as the mean water content had not yet declined below 2.0 g g^{-1} . Alternatively, it is possible that these somatic embryos could survive *slow* physical dehydration to low water contents. As sugarcane seeds are apparently orthodox, given sufficient time during dehydration, it is possible that the genetic mechanisms facilitating seed desiccation tolerance (reviewed by Berjak *et al.*, 2007), might be capable of being re-activated in somatic embryos.

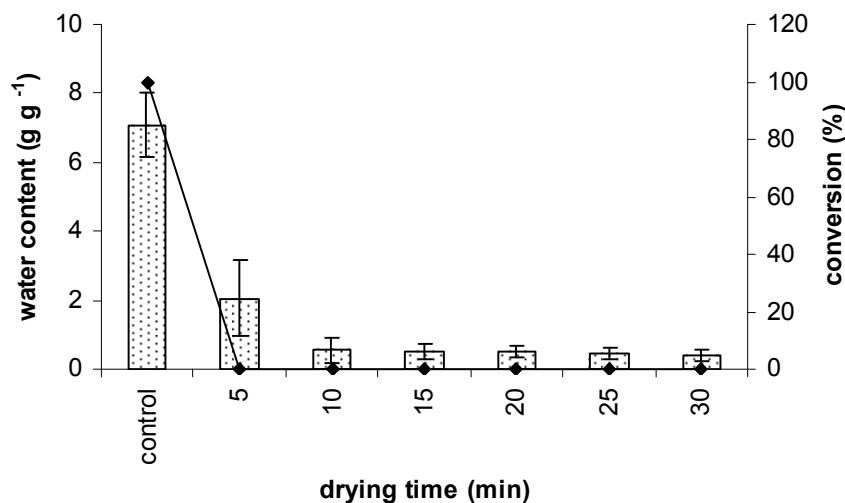


Figure 3.1 Change in percentage conversion of somatic embryos with water content over drying time. The histogram indicates the water content after different drying times (n=30); line graph (-♦-) represents the conversion response at those water contents (n=60). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

3.3.2 Osmotic dehydration

Directly- and indirectly-induced somatic embryos were osmotically dehydrated by culturing sequentially on media containing increasing concentrations of sucrose for 48 h at each concentration. The mean water content of freshly excised control embryos was $8.38 \pm 0.19 \text{ g g}^{-1}$ (Figure 3.2) and $8.45 \pm 0.33 \text{ g g}^{-1}$ (Figure 3.3) for embryos generated via the direct and indirect routes of micropropagation, respectively. Both directly and indirectly propagated embryos exhibited similar responses to osmotic dehydration with mean water content being reduced to $0.94 \pm 0.03 \text{ g g}^{-1}$ and $0.95 \pm 0.02 \text{ g g}^{-1}$ after dehydration on media containing 1.0 M sucrose, while maintaining between 98 and 100% conversion, respectively (Figures 3.2 and 3.3). These mean water content values were considered still too high to render the embryos amenable to cooling to cryogenic temperatures. Further

dehydration of embryos on media containing 1.2 M sucrose resulted in mean water content being reduced to $0.65 \pm 0.02 \text{ g g}^{-1}$ and $0.72 \pm 0.05 \text{ g g}^{-1}$ for directly and indirectly propagated embryos, respectively. However, this was coupled with a significant decline in percentage conversion to 55 and 53%, respectively.

The results indicated a decrease in embryo water content as the concentration of sucrose contained in the medium was increased (Figures 3.2 & 3.3) in line with the steeper chemical gradients created between intracellular water and sucrose, which would draw water out of the cells until the point of equilibrium. The accumulation of sucrose and other oligosaccharides has been identified as one of the key events occurring in tissues of orthodox seeds and resurrection plants in preparation for desiccation tolerance (Leprince *et al.*, 1993; Horbowicz and Obendorf, 1994; Blackman *et al.*, 1995; Obendorf, 1997; Black *et al.*, 1999; Farrant, 2007) along with the production of late embryogenesis abundant (LEA) proteins (e.g. Galau and Hughes, 1987; Blackman *et al.*, 1995; Wolkers *et al.*, 1998), and the appearance of novel antioxidants that are supposedly exclusive to desiccation-tolerant organisms (Aalen, 1999; Illing *et al.*, 2005). Sucrose is considered to be a non-penetrating cryoprotectant; hence its use to create an osmoticum in growth media presents a different scenario from the intracellular developmental accumulation of this disaccharide during the natural acquisition of desiccation tolerance. Here, the primary effect is to cause osmotic movement (i.e. of water only) out of cells/tissues and not to effect increasing sucrose concentrations intracellularly. However, it is also possible that during the relatively slow dehydration accompanying exposure of somatic embryos to increasing sucrose concentrations, a measure of inherent desiccation tolerance might be induced, which could include an increase in intracellular sucrose concentration.

Dehydration by culturing the somatic embryos on medium containing 1.0 M sucrose appears to be the threshold for the somatic embryos of the variety of sugarcane presently used since total conversion declined considerably upon further dehydration (Figures 3.2 & 3.3). The water contents after dehydration through exposure to increasing sucrose concentrations to 1.0 M sucrose for 48 h were $0.94 \pm 0.03 \text{ g g}^{-1}$ and $0.95 \pm 0.02 \text{ g g}^{-1}$ for

directly- and indirectly-induced embryos, respectively. Results obtained from a study performed with yam (*Discorea rotundata*) shoot tips showed a similar trend, with explants excised from cultures, pregrown for 5 weeks and precultured on MS medium with 1.0 M sucrose exhibiting the lowest survival in comparison with those precultured on media containing 0.09, 0.30, 0.50 or 0.70 M sucrose for 1, 3, 5 or 7 d (Quain, pers. comm³). That study showed a significantly greater survival of explants when exposed to 0.09, 0.30 or 0.50 M sucrose, than at higher concentrations, suggesting that “both concentration and duration of exposure impose thresholds beyond which deleterious effects might be inevitable” (Quain, pers. comm³). It could therefore be suggested that the time of incubation in the present study should be prolonged in order to minimise the deleterious concentration effects of sucrose preculture.

At increasing water contents above 0.3 g g^{-1} , an increasing proportion of the water will be freezable, but below this level, all or most, of the water is associated with structures and surfaces within cells and is therefore considered non-freezable (Vertucci, 1990). The water contents commensurate with viability retention indicated by an undiminished capacity for embryo conversion (i.e. plantlet formation), which were presently achieved are still considered too high to allow explant cooling at rates precluding lethal ice crystal formation (Wesley-Smith, pers. comm.⁴). However, the associated decline in percentage conversion below a water content of $\sim 0.94 \text{ g g}^{-1}$, demanded attempts to cryopreserve these embryos at these water contents.

³ Quain, M.D. CSIR-Crops Research Institute, P.O.Box 3785, Kumasi, Ghana.

⁴ Wesley-Smith, J. University of KwaZulu-Natal, Durban, South Africa

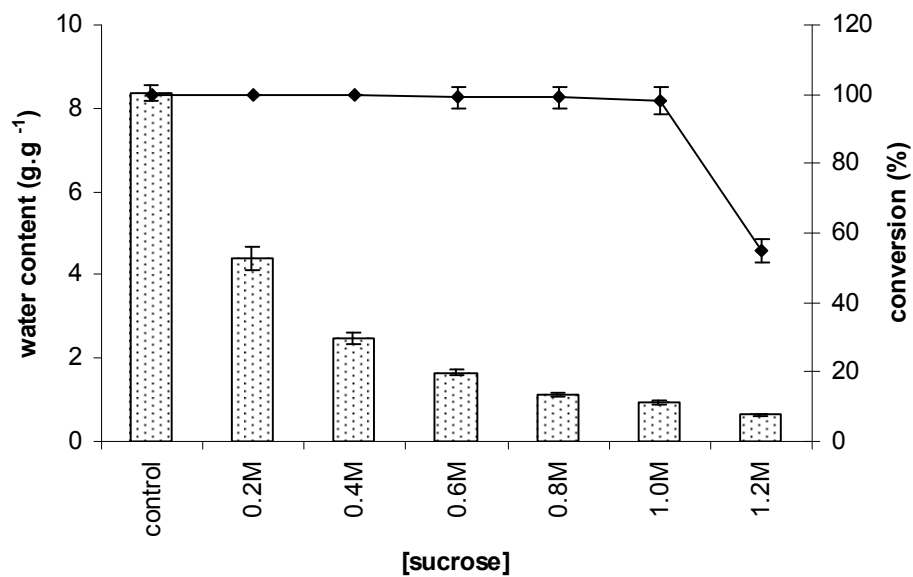


Figure 3.2 Change in water content and percentage conversion of directly-induced somatic embryos subjected to osmotic dehydration on basal MS medium containing sucrose at 0.056 M (control) and at 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M and 1.2 M. The histogram indicates the change in water content after incubation in relation to sucrose concentration (n=30); line graph (-♦-) represents the conversion response at those water contents (n=60). Bars indicate standard deviation. Data shown were derived from pooled results of three replicated experiments

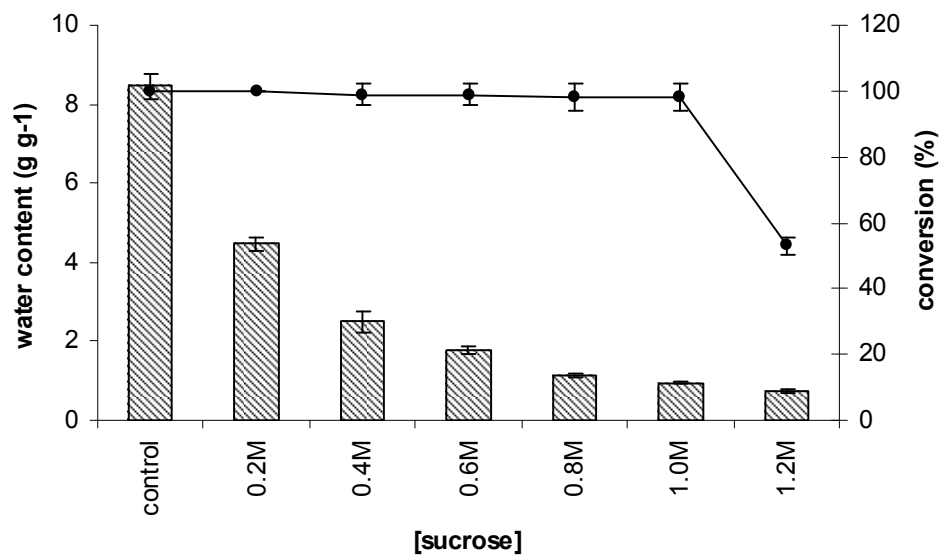


Figure 3.3 Change in water content and percentage conversion of indirectly-induced somatic embryos subjected to osmotic dehydration on basal MS medium containing sucrose at 0.056 M (control) and at 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1.0 M and 1.2 M. The histogram indicates the change in water content after incubation in relation to sucrose concentration (n=30); line graph (-●-) represents the conversion response at those water contents (n=60). Bars indicate standard deviation. Data shown were derived from pooled results of three replicated experiments

Chapter 4: Cryopreservation Studies

4.1 Introduction

Cryopreservation is fast becoming an essential tool for long term conservation of plant germplasm since, most importantly, to quote Sakai (2004), it represents a method for the “non-injurious reduction and subsequent interruption of metabolic functions of biological materials by temperature reduction to the level of LN (-196°C).” It also offers a very useful method for the management of large scale production of elite genotypes by means of somatic embryogenesis (Engelmann, 1997; Withers and Engelmann, 1998), since the maintenance of these genotypes demands a considerable investment in human resource, space and material, and is time consuming (Eksomtramage *et al.*, 1992). Additionally, cryopreservation serves to minimise the risk of undesired somaclonal variation and loss of embryogenic potential that can occur during long term maintenance of actively growing embryogenic cultures (Corredoira *et al.*, 2004), while also reducing the risk of contamination (Häggman *et al.*, 2000). It has successfully been adopted for the storage of base collections of clonal material to ensure future availability of plant germplasm (Towill, 1996; Reed *et al.*, 1998).

Cryopreservation has been successfully applied to more than 80 different plant species in various forms, such as cell suspensions, calli, meristems, zygotic and somatic embryos (Kantha & Engelmann, 1994; Towill, 1996; Engelmann, 1997). It has also been applied to preserve the unique biosynthetic properties of medicinal plants (Yoshimatsu *et al.*, 2000) and their biotechnological derivatives (Benson and Hamill, 1991).

The dynamics of the cooling process are particularly important, and successful cryopreservation is largely dependent on manipulation, and subsequent optimisation, of several factors before any survival after cooling can be achieved. These include: the type and physiological state of the explant, nature and rate of dehydration, cryoprotection, cooling rate, cooling temperature, thawing, rehydration, and regeneration conditions.

Chapters 2 and 3 provided a detailed account of how the physiological state of the explant was determined, and how the water content was optimised for cryopreservation with the least effect on viability. This chapter therefore follows on to describe the attempts at establishing a successful cryopreservation protocol for partially dehydrated somatic embryos of the variety 88H0019 of *Saccharum officinarum*.

Since cryopreservation protocols are still largely empirical, and far from optimized or universal, the underlying theory and the various approaches commonly used, are presented in some detail below.

4.1.1 Cryopreservation-induced injury

The cooling process either partially, or completely, interrupts the biochemical and biophysical pathways in tissue, and is accompanied by cryo-injury, which may arise at any stage during cryopreservation (Harding, 2004). Biochemical injury occurs as a result of changes in the intracellular pH as water is drawn out of the cells in response to the formation of ice in the extracellular spaces during the cooling process (Ring and Danks, 1994; Fleck *et al.*, 1999). Physical injury results from shrinkage as water leaves the cell, as well as by destruction of cell membranes by growing ice crystals (Wesley-Smith *et al.*, 1992; Wesley-Smith, 2002). Biochemical damage, coupled with the actual physical damage of cells during cooling and subsequent thawing, is believed to be the primary causes of post-cryo death and is indirectly related to tissue water content (Kantha, 1985). Oxidative damage may also occur as a result of the production of damaging free radicals during cooling (Fleck *et al.*, 1999; Dumet and Benson, 2000; Benson and Bremner, 2004). Free radicals target (among other intracellular components) the lipids that comprise cell membranes, forming lipid peroxides that are further converted to toxic secondary oxidation products (Henry, 1993; Dumet and Benson, 2000), which are free to cross-link with other macromolecules, such as DNA and proteins, leading to impaired cell function (Dumet and Benson, 2000; Benson and Bremner, 2004). Although it is uncertain whether the genome is impacted upon by the damage induced by the cooling process, somaclonal variation, with

the potential to produce distinct differences in the genotype/phenotype of the explant, may be induced (Harding, 2004).

It has been suggested that the formation of intracellular ice during cooling is the primary mode of cellular damage (Stanwood, 1985) and that the extent of cryo-injury is determined by the rate at which cells and tissues are cooled (Mazur, 1990). Several authors have suggested that since the cooling rate of wetter tissue is generally slower, there is a prolonged period for passage through the temperature range that is associated with ice crystal formation, making this the primary reason that most recalcitrant material cannot be cryopreserved without prior manipulation (Walters *et al.*, 2001).

Due to the high initial intracellular water contents and inherently cooling-intolerant nature of most explants to be cryopreserved, injury upon immersion in the cryogen is almost inevitable. Attempts to avoid this involve manipulations, either to reduce the water content of the explant to levels amenable to cooling without lethal ice formation (Mazur, 1984; Berjak *et al.*, 2000), and/or to achieve the desired rate of cooling throughout the tissue (Wesley-Smith *et al.*, 1995). Alternative to dehydration by flash drying followed by rapidly plunging explants into the cryogen (e.g. Berjak *et al.*, 1999), are two other approaches, viz. cooling-induced dehydration (slow cooling) or vitrification techniques (Dumet and Benson, 2000; Engelmann, 2000).

4.1.2 Cooling rates

It has been well documented that physical or biochemical damage to cells may be caused by the transition of extra- and intra-cellular water into ice (Meryman, 1960; Mazur, 1970, 1977, 1984; Withers and Davey, 1978). Freezing of extracellular water occurs first, effecting a flow of water from the cytoplasm and vacuole to the extracellular space where it freezes (Farrant, 1977, 1980; Taylor, 1986). The amount of water leaving the cell before the intracellular contents solidify is dependent on the rate of cooling (Pitt, 1992). The existence of an optimum cooling rate in the region of 1°C or 2°C per min has been

demonstrated in studies on survival of cryopreserved cell suspension culture systems (e.g. Kartha, 1985; Withers, 1980). However, as disassociated cells afford the simplest of systems, parameters optimised may not apply – or may not be uniformly achievable – for more complex multicellular explants.

4.1.2.1 Slow cooling/Cooling-induced dehydration

Explants are generally slowly cooled in a programmable cooling apparatus at a constant rate of between 0.5 and 2°C per minute to -40°C and subsequently directly immersed in liquid nitrogen, or slow cooling may be continued to temperatures down to -60°C to -70°C before immersion (Mazur, 1984; Kartha and Engelmann, 1994). Cooling rates of less than 10°C per min are considered fast enough to prevent slow cooling damage but slow enough to restrict ice formation to the external *milieu* (i.e. outside the plasmalemma) based on the premise that the extracellular medium initially supercools as temperature is progressively reduced (Mazur, 1984; Mazur *et al.*, 1972; Kartha and Engelmann, 1994; Wesley-Smith *et al.*, 2001b). Ice is suggested to be prevented from penetrating and freezing the interior of the cell by the wall and plasmalemma in plant cells (Yamada *et al.*, 2002). Concurrent with the progressive lowering of temperature is the concentration of extracellular solutes as the fraction of extracellular solution being converted to ice increases; which, in turn, draws water out of the cells resulting in protoplast shrinkage (Mazur, 1977). The resultant increasing cytoplasmic solute concentrations may facilitate a glass transition (ice-free vitrification) upon immersion of the explant in the cryogen (Kartha, 1985; Mazur, 1990; Wesley-Smith *et al.*, 1995; Dumet and Benson, 2000; Engelmann, 2000). From work on protoplasts, it has also been suggested that shrinkage and loss of surface area of the plasmalemma could render the cell incapable of resuming its original volume and surface area after thawing, therefore resulting in lethal rupture (Wiest and Steponkus, 1978; Wolfe and Steponkus, 1983; Steponkus, 1984; Steponkus and Lynch, 1989). The nature of damage under different cooling regimens has been clarified to an extent by light and electron microscopic studies of cell suspension cultures and isolated protoplast systems (Withers and Davey, 1978; Grout and Henshaw, 1980; Gazeau *et al.*, 1992).

Slow cooling, usually in combination with other pre-treatments, has successfully been employed for cryopreservation of zygotic germplasm of several species that produce non-orthodox seeds (e.g. by Fu *et al.*, 1990; Runthala *et al.*, 1993; Dussert *et al.*, 2000), but has proven to be significantly more successful for meristems (Hirai *et al.*, 1998; Hirai and Sakai, 1999; Scocchi *et al.*, 2004), cell cultures (Huang *et al.*, 1995; Ishikawa *et al.*, 1996; Kobayashi *et al.*, 2005; 2006) and somatic embryos (Withers, 1985; Kobayashi *et al.*, 1990; Tessereau, 1993). Other techniques with cooling rates that are also considered as being slow, include droplet cooling and vitrification-based cooling, both of which ideally should function to avoid intracellular ice formation (Sakai *et al.*, 1990; Blakesley *et al.*, 1996; Phunchindawan *et al.*, 1997).

4.1.2.1a Vitrification

Current cryopreservation techniques for plant materials have been developed using a range of methodologies that include rapid and slow cooling rates. Vitrification is the direct transition of water from the liquid to an amorphous glass state and thereby offers practical solutions to limiting ice crystallisation in frozen and thawed tissue (Fahy *et al.*, 1984; Phunchindawan *et al.*, 1997), and encapsulation-vitrification (Tessereau *et al.*, 1994). It is believed that vitrification “may be the only freeze-avoidance mechanism that enables hydrated cells, tissues and organs to survive at the temperature of liquid nitrogen” (Sakai, 1960, 1965, 1995).

Procedures aimed at achieving, and then reversing, vitrification generally include a pre-treatment (‘loading’), where samples are treated with cryoprotective substances, a dehydration step using highly concentrated vitrification solutions, rapid cooling and thawing steps, a procedure to remove (‘unload’) cryoprotectants, and a recovery step (Engelmann, 1997). The pre-treatment, which has successfully been applied to protoplasts, cell suspensions, apices or somatic embryos from more than 20 different species (Engelmann, 1997), is generally followed by rapid cooling and employing one or other of the four recognized procedures to achieve vitrification: encapsulation-dehydration,

encapsulation-vitrification, encapsulation-desiccation and pregrowth-desiccation (Blakesley, 1997). Vitrification with or without encapsulation, and the encapsulation-dehydration techniques are presently viewed as potentially valuable cryogenic procedures for cryopreservation of apical meristems and somatic embryos (Sakai, 2000).

All of the above-mentioned methods involve the treatment of explants with vitrification solutions such as Plant Vitrification Solution 2 (PVS2) [30% (v/v) glycerol, 15% (v/v) ethylene glycol, and 15% (v/v) DMSO together with 15% sucrose] prior to cooling (Sakai *et al.*, 1991; Sakai *et al.*, 1992) to reduce the intracellular water content osmotically and promote the transition to an amorphous metastable glass phase (Dumet and Benson, 2000; Engelmann, 2000) thereby limiting ice crystal formation during the cooling and thawing processes (Sakai *et al.*, 1990; Blakesley *et al.*, 1996; Phunchindawan *et al.*, 1997). The resultant intensive dehydration, however, can result in very strong plasmolysis, phase transitions of membrane lipids, and plasmalemma rupture leading to cell death (Wiest and Steponkus, 1978; Wolfe and Steponkus, 1983; Steponkus, 1984; Steponkus and Lynch, 1989; Popov, 1993). It has been suggested that viability loss associated with vitrification methods can primarily be attributed to osmotically induced damage (Shaw *et al.*, 1991). Coupled with the reduced risk of intracellular ice formation as a result of using high concentrations of cryoprotectants, are also increased adverse (toxic) effects, especially during long-term exposure (Fahy *et al.*, 1984; Valdez *et al.*, 1992). It is therefore essential that parameters such as exposure times and conditions of exposure be optimised to provide adequate protection from ice formation without damaging or even killing cells by osmotic or chemical stress (Engelmann, 1997; Benson, 1999). To circumvent such toxic effects, rapid vitrification techniques, which require only a brief exposure to cryoprotective agents, were developed (Kasai *et al.*, 1990; Shaw *et al.*, 1992).

The toxic effects of cryoprotectant vitrification solutions are reduced when dehydration is performed at 0°C instead of at room temperature, with the added advantage of “broadening the window of exposure durations ensuring survival of samples” (Engelmann, 1997). A study performed by Nishizawa and co-workers (1993) provided evidence of a rapid decline

in survival of asparagus embryogenic cell suspensions after 5 min of dehydration at 25°C but high survival following dehydration periods between 5 and 60 min when performed at 0°C (Nishizawa *et al.*, 1993). The duration of dehydration (by osmotic or other means), however, is largely dependent on the size of the explant and generally needs to be increased for larger explants (Engelmann, 1997). As an example of contrasts, Langis and Steponkus (1990) reported an optimal dehydration time of 60 s for rye protoplasts, whereas, apices of pear and apple require dehydration for 80 min (Niino *et al.*, 1992).

Following dehydration, specimens are rapidly cooled by direct immersion in LN in order to promote vitrification of the internal solutions, and rewarming by direct immersion in a water-bath held at 20-40°C is performed as rapidly as possible to avoid ice crystallisation during devitrification (Engelmann, 1997). Although there have been reports of callusing/abnormal plant development, recovery after vitrification procedures appears usually to be direct and rapid, with high survival rates (Towill, 1990; Gonzalez-Benito and Perez, 1994b). The use of vitrification procedures for cryopreservation eliminates the need for slow cooling, and permits the direct transfer of explants into LN while reducing potentially damaging effects of intra- and extracellular crystallisation (Sakai, 1997).

Although the mechanisms by which vitrification protects cells are not well understood, it has been suggested that they function as elaborate desiccants and decrease the amount of water available for lethal ice crystal formation (Sakai *et al.*, 1991), or stabilise cell structures during desiccation and cooling processes (Crowe *et al.*, 1998; Bryant *et al.*, 2001). Several authors have reported successful cryopreservation of cell cultures, callus, somatic embryos and shoot apices of various species using this technique (e.g. grape, Matsumoto *et al.*, 1998; cassava, Charoensub *et al.*, 1999; almond, Shatnawi *et al.*, 1999; olive, Shibli and Al-Juboory, 2000; citrus, Hao *et al.*, 2002; pineapple, Gámez-Pastrana *et al.*, 2004; garlic, Kim *et al.*, 2005; horse chestnut, Lambardi *et al.*, 2005; yams, Leunufna and Keller, 2005).

4.1.2.1b Encapsulation-vitrification

For this procedure, explants are initially encapsulated in a hydrogel bead of sodium, potassium or calcium alginate, Gel-Rite®, guar gum, tragacanth gum or sodium pectate (Redenbaugh *et al.*, 1993). Encapsulation-vitrification is operationally less complex than vitrification alone (Ashmore 1997; Engelmann, 1997), since the procedures for dehydration and cryoprotectant permeation are more carefully controlled, and injury by chemical toxicity or excessive osmotic stresses during dehydration are reduced (Sakai, 1997). Other advantages associated with this cryogenic protocol include the ease with which plant material can be handled, and consequently the increased rate at which explants can be treated, compared with vitrification alone (Hirai *et al.*, 1998; Hirai and Sakai, 1999), so affording potential for large-scale cryopreservation (Shibli and Al-Juboory, 2000). In a study that compared shoot formation by wasabi meristems cooled to -196°C by three different cryogenic protocols, Matsumoto and Sakai (1995) reported that vitrification and encapsulation-vitrification procedures facilitated far higher levels of shoot formation than did encapsulation-dehydration (see below). This procedure has been used for cryopreservation of e.g. strawberry meristems (Hirai *et al.*, 1998) and axillary buds of mint (Hirai and Sakai, 1999).

4.1.2.1c Encapsulation-dehydration

The encapsulation-dehydration technique was derived from the technology developed for the production of synthetic seeds, by which somatic embryos were encapsulated in a bead of calcium alginate gel (Redenbaugh *et al.*, 1993). This technique has successfully been employed for cryopreservation of relatively large explants, for example 2-3 mm-long heart or torpedo stage somatic embryos (de Boucaud *et al.*, 1994; Hatanaka *et al.*, 1994). Following encapsulation, explants are subjected to what is termed pregrowth, in liquid medium enriched with sucrose or other sugars for periods between 16 h (Niino and Sakai, 1992) and 7 d (Fabre and Dereuddre, 1990). This is followed by desiccation either in the air current of a laminar flow cabinet or by exposure to silica gel until the appropriate water

content is reached, and ultimately rapid cooling by direct immersion of samples in liquid nitrogen is employed (Withers and Engelmann, 1998). The success of this technique was demonstrated for sugarcane (*Saccharum* spp.) apices, which exhibited higher survival rates after rapid than slow cooling (Gonzalez-Arno *et al.*, 1993). In contrast, however, encapsulated grape apices showed better survival following controlled slow cooling down to -100°C (Plessis *et al.*, 1991).

4.1.2.2 Rapid cooling

Rapid cooling rates, usually in the order of several hundred degrees C per second (Wesley-Smith *et al.*, 2004a), are generally achieved by direct rapid immersion of naked explants in nitrogen slush (LN subcooled to -210°C) (e.g. Wesley-Smith *et al.*, 1992). Lower cooling rates are achieved by enclosing explants in polypropylene plastic vials prior to cooling (e.g. *Cocos nucifera*, [Assy-Bah and Engelmann, 1992]; *Trichilia dregeana*, [Kioko *et al.*, 1998]) or in aluminium foil jackets (Dumet *et al.*, 1997). Somatic embryos of oil palm, coffee, and date palm have been successfully cryopreserved using such procedures (Engelmann *et al.*, 1985; Dumet *et al.*, 1993a, b; Mycock *et al.*, 1995).

The cooling rate achieved by direct immersion of a specimen in LN will vary in accordance with its volume and mode of introduction into the cryogen (Wesley-Smith *et al.*, 1992). Hydrated zygotic embryonic axes of pea (Mycock *et al.*, 1991) and *Camellia sinensis* (Wesley-Smith *et al.*, 1992) have successfully been cryopreserved at cooling rates of 1 200°C and 550°C s⁻¹, respectively. A more rapid cooling rate, 1 700°C s⁻¹, achieved by the use of a spring-loaded device that plunges axes into nitrogen slush, was employed in the successful cryopreservation of zygotic embryonic axes of *Poncirus trifoliata* (Wesley-Smith *et al.*, 2004a). Other explants that have been successfully cryopreserved using rapid cooling rates include mainly shoot tips, of e.g. tomato, chickpea, cassava and potato (Kantha, 1985).

Rapid cooling does not allow the time for efflux of cellular water to equilibrate with external ice or vapour-pressure deficit, forcing instead intracellular cooling, which is potentially lethal (Grout, 1995; Benson, 1995). However, rapid cooling of germplasm facilitates swift traversing over the temperature range associated with ice crystal formation, such that these either do not form, or the ice crystals formed are too small to inflict significant damage (Wesley-Smith *et al.*, 1992, 2001b). The axes of several recalcitrant-seeded species have been successfully cryopreserved using rapid dehydration (flash-drying) in combination with rapid cooling (e.g. Berjak *et al.*, 1999; Wesley-Smith *et al.*, 2004a, b).

4.1.3 Thawing

Cryopreservation by storage of explants in LN, serves to curtail biochemical processes in cells, and forfeits any energy input (Benson and Bremner, 2004). However, survival of material that has been frozen is influenced by other factors and processes, including thawing following their removal from cryostorage. As with cooling of explants, thawing/warming also needs to be performed in a manner that avoids ice crystal formation. The types of damage caused during thawing and rehydration have been identified as deplasmolysis injury, and realisation of the consequences of damaging events that may have occurred during cooling and storage (Withers, 1988). It has been shown that freshly thawed plant cells may be characteristically leaky, presumably as result of the physically weakened plasmalemma, and exhibit depleted pools of key metabolites (Cella *et al.*, 1982) which, together with a respiratory lesion, is also indicative of mitochondrial damage associated with oxidative stress and further serious metabolic injury (Benson, 1990). The measurement of volatile hydrocarbons evolved by recovering cells provided evidence indicating that membrane degeneration can continue for several days, as does free radical scavenging by cryoprotectants such as DMSO (Withers, 1988), which according to that author, also provides strong motivation for avoiding post-thaw washing.

It is believed that the transition of ice structures from the vitreous to crystalline state can occur even at around -130°C , which poses a severe threat to cellular integrity (Bowers, 1990; George, 1993). Further, that author suggests that the formation of ice at temperatures of -70°C and higher is potentially more damaging, since ice crystals form and grow on the surface of a pre-existing lattice via recrystallisation. Similar to the cooling process, the fast movement of cells through the temperature range at which ice crystal formation occurs, should minimise crystallisation and the associated damage (Mazur, 1984; Wesley-Smith, 2002). In fact, rapid thawing is considered the only way to limit the extent of recrystallisation and ice crystal growth as intracellular water melts during the thawing process (Kartha, 1985). The potential mechanical and biochemical damage incurred during the thawing process may be avoided by previously having manipulated and subsequently optimized dehydration and cooling rates, as well as by having used cryoprotectants (Wesley-Smith *et al.*, 2001a, b).

Earlier methods of rapid thawing included warming in a microwave oven (Meryman and Williams, 1985), but more recent techniques commonly used involve rapid immersion of frozen material for 1 to 2 min either in water held at 35 to 40°C (Kartha and Engelmann, 1994; Berjak *et al.* 1999; Wesley-Smith, 2002), or in liquid growth medium held at the same temperature (Kartha and Engelmann, 1994; Grout, 1995; Kioko *et al.*, 1998; Wesley-Smith *et al.*, 1992, 2001a). The method of simultaneous thawing and rehydration by plunging specimens directly into liquid culture medium, either warmed to 37°C or maintained at ambient temperature has successfully been applied to zygotic axes of tea (Wesley-Smith *et al.*, 1992) and also somatic embryos of coffee and date palm (Mycock *et al.*, 1995). Alternatively, thawing may be achieved by immersion in a solution containing calcium and magnesium cations ($1\mu\text{M CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $1\text{mM MgCl}_2 \cdot 6\text{H}_2\text{O}$) held to promote the re-assembly of cyto- and nucleo-skeletal elements (Berjak *et al.*, 1999; Mycock, 1999; Berjak and Mycock, 2004). Those authors reported significantly increased number of embryonic axes forming normal seedlings after dehydration and/or cryopreservation following thawing (and rehydration) in the Ca/Mg solution, compared with effecting these processes in water.

4.1.4 Rehydration

Rehydration refers to the direct immersion of material in distilled water, and more recently, in Ca/Mg solution immediately following thawing (e.g. Wesley-Smith *et al.*, 2001a, b), or simply after dehydration (Berjak and Mycock, 2004). Alternatively, slow rehydration may be achieved by placing material on moistened filter paper (e.g. Leprince *et al.*, 1998). As with most, if not all, processes involved in cryopreservation protocols for plant material, there is considerable damage associated with the rehydration process as well. Imbibitional damage occurs even in desiccation-tolerant material imbibed from the dry state in water, leading to leakage of solutes (Hoekstra *et al.*, 1999), and such damage, in combination with low temperatures, can result in loss of vigour (Bramlage *et al.*, 1978).

4.1.5 Regeneration

After thawing, it is imperative that both zygotic axes and somatic embryos be transferred on to a previously developed and tested culture medium for further development (Dumet *et al.*, 1997). Also, culture in the dark or under minimal lighting is considered a likely means of reducing photo-oxidation (Withers, 1988; Touchell and Walters, 2000). Thus *in vitro* regrowth/regeneration of frozen-thawed plant material is another challenge to the successful cryopreservation of germplasm, and the ability of this plant tissue to regrow is a cardinal means of assessing its viability. The regrowth of plant material after thawing/warming has been described by Withers and Engelmann (1998) as being direct, but with occasional observations of modified regrowth patterns. Cryopreserved embryos of *Veitchia merrillii* and *Howea fosteriana* exhibited non-development of the haustorium and more rapid leaf expansion in comparison with unfrozen controls (Chin *et al.*, 1988). Other forms of abnormal regrowth in the form of callusing or incomplete development were reported for a fraction of cryopreserved embryos of *Castanea* and *Quercus* (Pence, 1992), *Hevea brasiliensis* (Normah *et al.*, 1986), and oil palm (Engelmann *et al.*, 1995a). It is possible that such abnormalities, as well as reduced vigour following cryopreservation, may

be the consequence of epigenetic events (Harding, 2004). This, however, opens up new avenues of previously unconsidered investigation.

The survival of cryopreserved material can be significantly improved by modification of the recovery conditions, e.g. by adjusting the balance of plant growth regulators in the culture medium to ensure normal post-thaw development (Dumet *et al.*, 1997), as reported for zygotic axes of *Coffea liberica* embryos (Abdelnour-Esquivel *et al.*, 1992; Normah and Vengadasalam, 1992). A similar situation was reported for oil palm somatic embryos, which required short-term addition of 2,4-D (0.2 mg l^{-1}) for increased recovery following cryopreservation (Engelmann *et al.*, 1985; Dumet *et al.*, 1993a).

Most of the procedures described above, were applied in an attempt to achieve survival after cryopreservation of somatic embryos of the sugarcane variety, 88H0019.

4.2 Materials and Methods

4.2.1 Chemical cryoprotection

Directly-induced somatic embryos at the late globular stage of development (Fransz and Schel, 1991; Mycock, 1999), generated as described in Chapter 2, were excised from the mother explant in clumps of three in a laminar air flow and accumulated within closed Petri dishes on filter paper moistened with deionised water, prior to chemical cryoprotection. The cryoprotectants tested in this experiment were sucrose (S), glycerol (G), DMSO (D), sucrose and glycerol combined (S+G), glycerol and DMSO combined (G+D), sucrose and DMSO combined (S+D) and sucrose, glycerol and DMSO in combination (S+G+D). The somatic embryo clumps were immersed in 5% aqueous cryoprotectant solutions for 1 h and thereafter transferred to 10% solutions for another hour. A separate experiment was carried out in the exact same manner using aqueous solutions of the same cryoprotectants but at concentrations of 5 M and 10 M. Later, cryoprotection was performed using amino acids viz. proline or casamino acid separately at concentrations of 0.5 and 1% and 5 and 10%

(v/v), freshly excised somatic embryo clumps were immersed in a 0.5% aqueous solution for 30 min and thereafter transferred to a 1% solution for a further 30 min, and similarly in 5% and 10% aqueous solutions.

Immediately after cryoprotection, the water content of ten individual embryo clumps per treatment was determined gravimetrically (see Chapter 2 for details), and twenty embryo clumps per treatment were decontaminated in 1% calcium hypochlorite for 5 min, rinsed three times with a sterile solution of Ca/Mg (see Chapter 3), and plated on regeneration medium devoid of plant growth regulators. The medium comprising 4.4 g l⁻¹ Murashige and Skoog (MS) basal salts and vitamins, 20 g l⁻¹ sucrose, 0.5 g l⁻¹ casein hydrolysate and 8 g l⁻¹ agar, at pH 5.6-5.8. Embryo clumps were cultured in growth room conditions as described in Chapter 2, to assess the effect of cryoprotection on embryo conversion, which was scored as positive upon normal shoot and root production without callus formation. Controls (embryos that were not cryoprotected) were plated out at the same time for conversion assessment under the same growth room conditions. Both cryoprotected and control embryos were also plated on the five growth media containing plant growth regulators previously used, viz. 2,4-D at concentrations of 0.6 mg l⁻¹ and 3.0 mg l⁻¹, or BAP at concentrations of 0.5 mg l⁻¹, 1.0 mg l⁻¹, 1.5 mg l⁻¹ and 2.0 mg l⁻¹.

4.2.2 Cooling, thawing and rehydration

4.2.2.1 Slow cooling

4.2.2.1a Cooling in cryovials and foil jackets

Excised embryo clumps were dehydrated by culture on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h on each medium. Subsequently, 20 embryo clumps were enclosed in sterile propylene 2 ml cryovials (Greiner™, five embryo clumps per vial), mounted on cryocanes, and immersed in LN in a Dewar cryovial (Schorn Cryogenics, Gauteng, South Africa) for 48 h, while the water

content of a further ten embryo clumps was determined gravimetrically. Cryovials containing the embryo clumps were thereafter removed from LN storage and samples were thawed by immediately plunging the vials into a waterbath and held for 5 min at 40°C. Cooling in sterile foil jackets was carried out in the exact same manner as described for cooling in cryovials. Thawed embryo clumps were then directly immersed in aqueous Ca/Mg solution at $25\pm 2^\circ\text{C}$ for 30 min in the dark to rehydrate. Following rehydration, embryo clumps were decontaminated and processed for conversion as described in section 4.2.1. Embryo conversion was assessed on the six different growth media described above, as detailed below (4.2.3).

4.2.2.1b Cooling in Mr Frosty

After dehydration by culture on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h on each medium, twenty excised embryo clumps were enclosed in cryovials (Greiner™, 5 embryo clumps per vial) and then inserted into Mr Frosty (Nalgene™, New York, U.S.A) while the water content of a further ten embryo clumps was determined gravimetrically. Mr Frosty is a plastic vessel containing isopropanol in the outer chamber that cools at an approximate rate of 1°C per min when placed in a -70°C freezer. Once the apparatus was retrieved from the -70°C freezer, the cryovials were removed, and the embryo clumps thawed and rehydrated as described above. For 2-stage cooling, cryovials retrieved from Mr Frosty were rapidly ‘clipped’ into aluminium cryocanes and immediately plunged into LN [cooling rate of 10°C per min (Vertucci, 1989)]. After at least 2 h, cryovials were retrieved from LN storage, thawed by immediate plunging into a waterbath held for 5 min at 40°C, rehydrated in the Ca/Mg solution for 30 min in the dark, processed for conversion as described in section 4.2.1 and cultured as described in 4.2.3, below.

4.2.2.1c Vitrification

The vitrification procedure employed was a modification of that described by Matsumoto *et al.* (1995). Somatic embryos were excised in twenty clumps of three, transferred to sterile 2 ml cryovials (total of 210 embryo clumps with 5 embryo clumps per vial) containing 1 ml of a filter-sterilised loading solution, which comprised 2 M glycerol and 0.4 M sucrose dissolved in MS medium. Embryo clumps were maintained in the loading solution for 20 min at 25±2°C. Following removal of the loading solution from the cryovials with a sterile Pasteur pipette, embryo clumps were infiltrated and dehydrated at 0°C for various intervals (0, 5, 10, 15, 20, 25, 30 min) with 1 ml PVS2 solution (Sakai *et al.*, 1990) comprising 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethylsulphoxide in MS medium with 0.4 M sucrose (pH 5.8). At each sampling interval, the water content of ten individual embryo clumps was determined gravimetrically. Cryovials containing the samples were thereafter plunged into LN, and held at this temperature for a minimum of 15 h before thawing, which was achieved by immediately plunging the vials into a 40°C waterbath for 2 min. Thawed embryo clumps were then transferred to a Petri dish, completely immersed in the Ca/Mg solution at 25±2°C and then placed in the dark for 30 min to rehydrate. Following rehydration, 20 embryo clumps per dehydration treatment were processed for conversion, as described below (4.2.3).

4.2.2.1d Encapsulation-vitrification

The encapsulation-vitrification procedure employed was a modification of that described by Sakai *et al.* (2000). Somatic embryos were excised in clumps of three (n=210) and placed on filter paper moistened with deionised water before suspension in a solution of MS medium with 3% (w/v) Na-alginate (low viscosity) and loading solution (2 M glycerol plus 0.4 M sucrose). The mixture including clumps of somatic embryos was dispensed in a drop-wise manner from a sterile Pasteur pipette into a 0.1 M CaCl₂ solution. The beads formed (each containing a single embryo clump) were allowed to polymerise for 30 min, after

which they were placed in 2 ml cryovials (5 embryo clumps per vial) and infiltrated and dehydrated at 0°C for various time intervals (0, 5, 10, 15, 20, 25, 30 min) with 1 ml PVS2 solution. Thereafter, cryovials containing the samples were clipped into cryocanes, plunged directly into LN and held at this temperature for 48 h before warming. Warming was achieved by plunging the cryovials in water held at 40°C for 2 min immediately after retrieval from LN storage. Thawed embryo clumps were then transferred to a Petri dish and completely immersed in the Ca/Mg solution at 25±2°C and placed in the dark for 30 min to rehydrate. Following rehydration, 20 embryo clumps per dehydration treatment were processed for conversion (see section 4.2.3). Embryo clumps that had produced callus on medium containing 3 mg l⁻¹ 2,4-D were subsequently transferred to medium without PGRs.

4.2.2.1e Slow controlled cooling

This protocol was modified from that reported by Martínez-Montero and co-workers (1998) as a simplified cooling process for cryopreservation of sugarcane embryogenic callus. Embryos were generated as described in section 2.2 of Chapter 2, excised in clumps of three (n=360) and introduced into sterile 1 ml cryovials (GreinerTM, 5 embryo clumps per vial). Embryo clumps were then pretreated in liquid medium with various sucrose concentrations (0.25 M; 0.5 M; 0.75 M and 1 M) for 1 h at 0°C. Thereafter DMSO was progressively added to the liquid medium over a period of 30 min until the final concentrations (5, 10, 15%, v/v) were reached. Three replicate cryovials containing ten embryo clumps per vial were used for each experimental condition. Cooling was carried out in a home-made ethanol bath, consisting of a propylene ice box containing 700 ml of ethanol precooled at 0°C. Cryovials were inserted in holes pierced in a polypropylene sheet floating on top of the ethanol, such that the bottoms of the cryovials were immersed in the coolant. The protocol of Martínez-Montero *et al.* (1998) stipulated that the ethanol bath be placed in a -40°C freezer, which facilitated an average cooling rate of 0.4-0.6°C min⁻¹ between 0°C and -40°C. As a -40°C freezer was unavailable, cryovials were then placed in coolant [ethanol bath precooled to -40°C by controlled addition of dry ice (solid CO₂) into the ethanol] in the freezer (-20°C) for 2 h. Cryovials were thereafter removed, mounted on

aluminium cryocanes and rapidly plunged into LN for a further 2 h at -196°C . This was followed by thawing in a water bath held at 40°C until melting of ice was complete. Embryos were then removed from the cryovials in a laminar air flow and plated, without washing, on the six different growth media described in section 2.2 of Chapter 2, and also on MS medium containing 2,4-D (1 mg l^{-1}), arginine (50 mg l^{-1}) and proline (500 mg l^{-1}) as outlined in the protocol of Martínez-Montero *et al.* (1998). Embryos were cultured as described in section 4.2.3.

4.2.2.2 Rapid cooling

Liquid nitrogen in a polystyrene cup was supercooled to -210°C by placing in a desiccator and lowering the pressure by means of a vacuum pump, thus forming nitrogen slush. The cooling rates obtained with nitrogen slush vary depending on the size of the explant, the water content at cooling, and the immersion procedure. Nitrogen slush was then poured into another polystyrene cup containing twenty excised embryo clumps previously dehydrated by culture on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h on each medium. After 30 min the LN was decanted and replaced with the aqueous Ca/Mg solution at 40°C for 2 min. Thawed embryo clumps were then transferred to a Petri dish and completely immersed in Ca/Mg at $25\pm 2^{\circ}\text{C}$ and placed in the dark for 30 min to rehydrate. Following rehydration, all 20 embryo clumps were processed for conversion as described below.

4.2.3 Embryo conversion (regeneration)

The procedure used for embryo conversion common to all the methods of cooling employed involved decontamination in 1% calcium hypochlorite for 5 min, followed by three rinses with a sterile solution of calcium magnesium ions (Ca/Mg). Decontaminated embryo clumps were thereafter plated on regeneration medium devoid of PGRs, and on media containing 2,4-D and BAP at the concentrations given above in section 4.2.1.

Embryo clumps were cultured in a growth room with $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, 16 h light/8 h dark photoperiod at 24°C day/ 21°C night temperatures, or incubated in the dark at $25 \pm 1^\circ\text{C}$.

4.3 Results and Discussion

4.3.1 Chemical cryoprotection

4.3.1.1 'Traditional' cryoprotectants

The effects of the various cryoprotectants on water content and percentage conversion are illustrated in Figure 4.1. The water content of control explants not subjected to any cryoprotectant treatment was $7.7 \pm 1.19 \text{ g g}^{-1}$ and these exhibited 100% embryo conversion. Treatment with sucrose only, glycerol only, and DMSO only reduced the water content to $1.46 \pm 0.29 \text{ g g}^{-1}$, $4.26 \pm 1.07 \text{ g g}^{-1}$ and $5.36 \pm 0.93 \text{ g g}^{-1}$, respectively. Treatment with sucrose+glycerol, glycerol+DMSO, sucrose+DMSO, and sucrose+glycerol+DMSO also reduced the water content of the embryos to $1.66 \pm 0.29 \text{ g g}^{-1}$, $5.25 \pm 0.84 \text{ g g}^{-1}$, $2.31 \pm 1.17 \text{ g g}^{-1}$ and $1.64 \pm 0.22 \text{ g g}^{-1}$, respectively. Results obtained in the present study indicated that cryoprotection had a dehydrating effect on the test tissue, which was also observed for carrot somatic embryos treated with sucrose (Thierry *et al.*, 1997) and embryonic axes of *Juglans regia* (de Boucaud *et al.*, 1991).

Although the various cryoprotectants, and combinations thereof, all lowered the water content to a greater or lesser extent, all treatments resulted in 0% embryos conversion, regardless of whether conversion was attempted in the dark or light. Concentrations of cryoprotectants between 5 and 10%, such as those employed in this study, appear to be well tolerated by certain explants (e.g. Mycock *et al.*, 1995); however, these treatments clearly had adverse effects on embryo conversion in the present study, resulting in death of embryos. This was further confirmed by a negative tetrazolium test (ISTA, 1999), and by the fact that this experiment yielded similar results when repeated twice more.

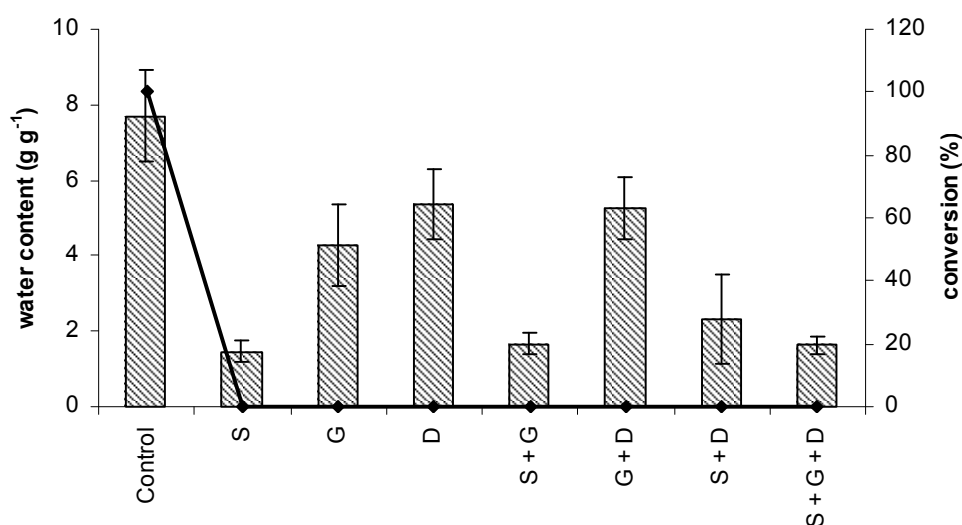


Figure 4.1 Change in water content and percentage conversion of directly-induced somatic embryos after treatment with various cryoprotectants. The histogram indicates the change in water content following cryoprotection (n=30); line graph (-♦-) represents the percentage conversion response at those water contents (n=60). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

4.3.1.2 Amino acids as cryoprotectants

In view of the lethal effects of the ‘traditional’ cryoprotectants, it was decided to experiment with amino acids (Withers and King, 1979; Rudolph and Crowe, 1985; Heszky *et al.*, 1990). Thus the effects of proline (Pro) and casamino acid (CAc) as potential cryoprotectants were assessed in terms of explant dehydration, and viability retention. Conversion of cryoprotected embryos was assessed under light and dark culture conditions at the standard growth room conditions. The effects of proline treatment at concentrations of 0.5 and then 1% and 5 and then 10% (v/v) on water content and percentage conversion are illustrated in Figures 4.2 and 4.3, respectively. Results indicated a reduction in water content from $9.69 \pm 0.89 \text{ g g}^{-1}$ to $3.94 \pm 0.55 \text{ g g}^{-1}$ at the lower concentrations, with percentage conversion of embryos ranging between 100 and 83% under light culture conditions and 73 and 45% for embryos cultured in the dark (Figure 4.2). Embryos treated with proline at 5

and then 10% exhibited a reduction in water content from $7.06 \pm 0.99 \text{ g g}^{-1}$ to $1.63 \pm 0.54 \text{ g g}^{-1}$ with percentage conversion ranging between 100 and 87% under light culture conditions and 72% and 47% for embryos cultured in the dark. The decline in embryo conversion for treatments with both the lower (0.5 and then 1%) and higher (5 and then 10%) concentrations of proline was related to the time of immersion. While water stress accompanying dehydration could account for the decline, the possible effects of increasing oxygen depletion cannot be ignored. It is noteworthy that treatment with the higher concentration of proline produced the greater reduction in embryo water content. This is extremely useful from the objective of dehydration prior to cryopreservation of these embryos.

Light culture conditions favoured good shoot and root development (see Figure 4.4).

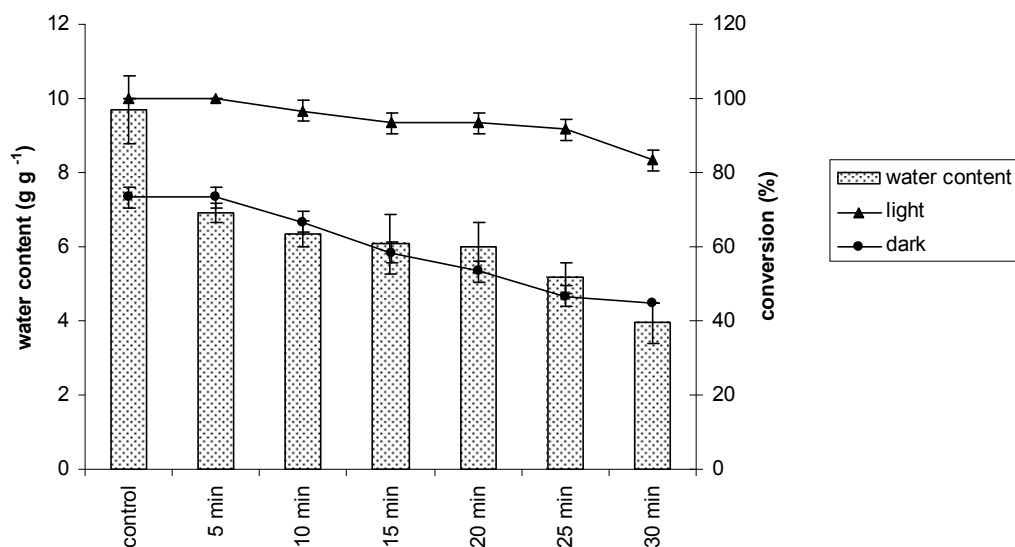


Figure 4.2 Change in water content and percentage conversion of directly-induced somatic embryos following treatment with proline (0.5 and then 1%). The histogram indicates the change in water content following cryoprotection (n=30); line graphs represent the percentage conversion on PGR-free medium at those water contents under light (-▲-) and dark (-◆) culture conditions (n=120). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

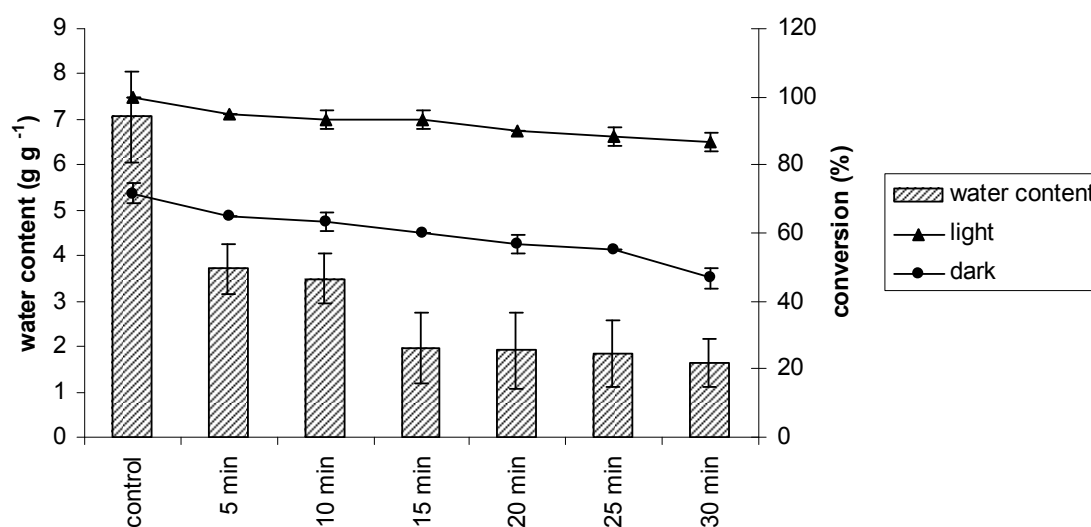


Figure 4.3 Change in water content and percentage conversion of directly-induced somatic embryos following treatment with proline (5 and then 10%). The histogram indicates the change in water content following cryoprotection (n=30); line graphs represent the percentage conversion on PGR-free medium at those water contents under light (-▲-) and dark (-◆) culture conditions (n=120). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

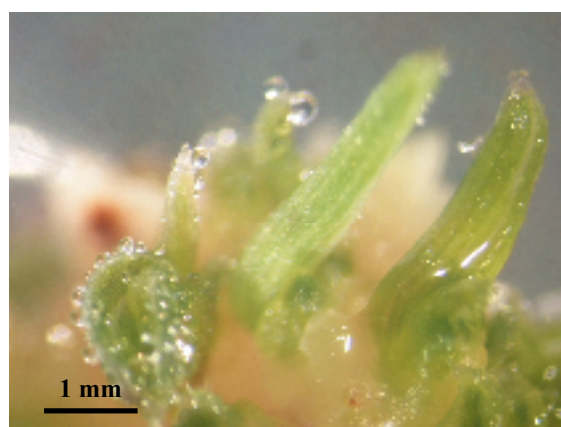


Figure 4.4 Direct shoot formation from embryo clumps pretreated with proline at 5 and then 10% for 30 min at each concentration

Statistical analysis indicated that there was a significant difference between the conversion capacity of embryos regenerated under light and dark culture conditions, and that percentage conversion of embryos was significantly affected by the different proline treatments (indicated by the different letters assigned to treatments in Table 4.1).

Table 4.1 Effects of lower (0.5 and then 1%) and higher (5 and then 10%) proline immersion for different times on the conversion capacity (%) of sugarcane variety 88H0019 somatic embryos

Treatment	Mean (%) Conversion
Control 1	85.8 ^{ab}
Proline (0.5 & 1%) 5 min	82.5 ^{ab}
Proline (0.5 & 1%) 10 min	75.0 ^c
Proline (0.5 & 1%) 15 min	73.3 ^c
Proline (0.5 & 1%) 20 min	71.7 ^c
Proline (0.5 & 1%) 25 min	67.5 ^{cde}
Proline (0.5 & 1%) 30 min	62.5 ^{efg}
Control 2	86.7 ^a
Proline (5 & 10%) 5 min	86.7 ^a
Proline (5 & 10%) 10 min	81.7 ^b
Proline (5 & 10%) 15 min	75.8 ^c
Proline (5 & 10%) 20 min	73.3 ^c
Proline (5 & 10%) 25 min	69.2 ^{cd}
Proline (5 & 10%) 30 min	64.2 ^{ef}

The same letters indicate no significant difference between the means according to the Tukey post-hoc test at 5% level of significance.

Similar trends were observed for embryos treated with casamino acid at concentrations of 0.5 and then 1%, and 5 and then 10% (v/v). Results indicated a reduction in water content from $9.69 \pm 0.89 \text{ g g}^{-1}$ to $3.47 \pm 0.62 \text{ g g}^{-1}$ following treatment with casamino acid (0.5 and then 1%) for 30 min, with percentage conversion of embryos ranging between 100 and 78% under light culture conditions and 73 and 47% for embryos cultured in the dark (Figure 4.5). As for the proline treatments, there was a decline in embryo conversion capacity with increasing immersion time, which could have been the outcome of water stress and/or increasingly anoxic conditions during immersion. Following casamino acid treatment too, light culture conditions favoured good shoot and root production (see Figure 4.7). Embryos treated with the higher concentrations of casamino acid exhibited a reduction in water content from $9.69 \pm 0.89 \text{ g g}^{-1}$ to $2.01 \pm 0.79 \text{ g g}^{-1}$ with percentage conversion ranging between 100 and 78% under light culture conditions and 75 and 47% for embryos cultured in the dark (Figure 4.6). Here too, treatment with the higher concentration of casamino acid produced the greater reduction in embryo water content. However, treatment with proline appeared to be more effective as a dehydration treatment of these somatic embryos.

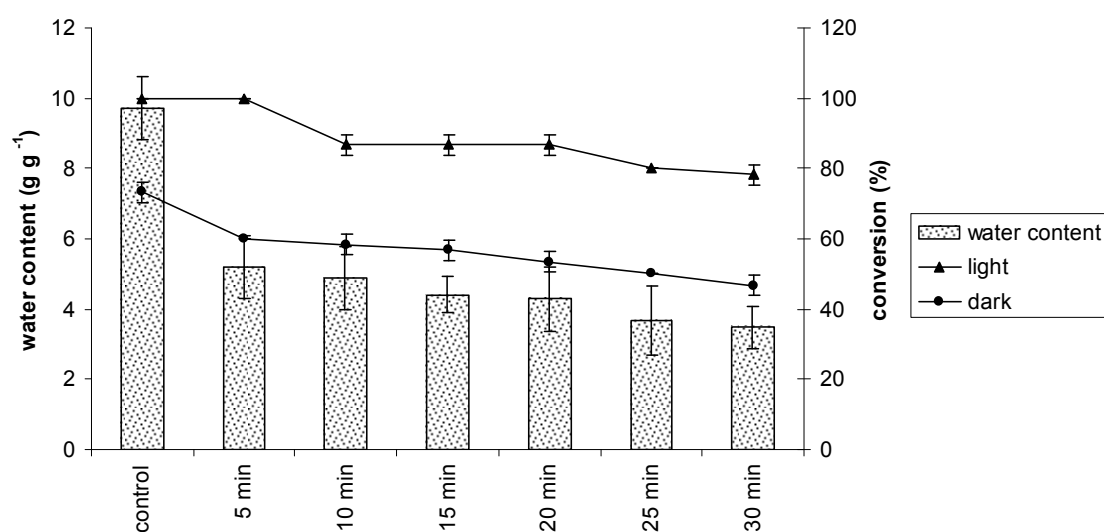


Figure 4.5 Change in water content and percentage conversion of directly-induced somatic embryos following treatment with casamino acid (0.5 and then 1%). The histogram indicates the change in water content following cryoprotection (n=30); line graphs represent the percentage conversion on PGR-free medium at those water contents under light (-▲-) and dark (-◆) culture conditions (n=120). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

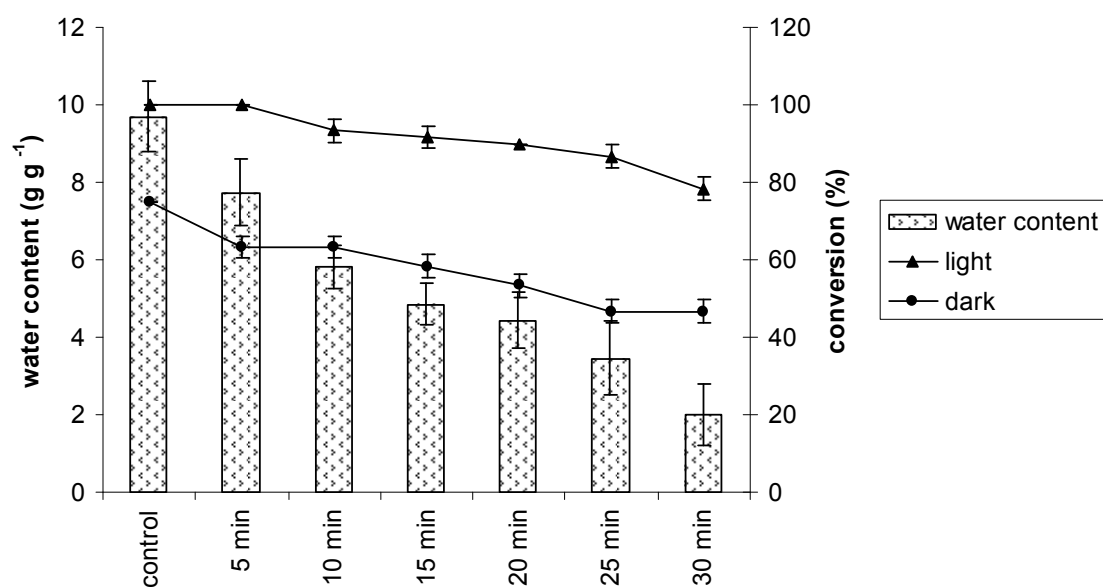


Figure 4.6 Change in water content and percentage conversion of directly-induced somatic embryos following treatment with casamino acid (5 and then 10%). The histogram indicates the change in water content following cryoprotection (n=30); line graphs represent the percentage conversion on PGR-free medium at those water contents under light (-▲-) and dark (-◆-) culture conditions (n=120). Bars indicate standard deviation. Data shown were derived from pooled results from three replicated experiments

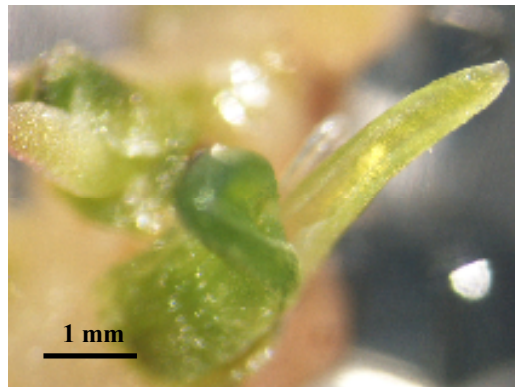


Figure 4.7 Direct shoot formation from embryo clumps pretreated with casamino acid at 5 and then 10% for 30 min at each concentration

Statistical analysis indicated that there was a significant difference in the conversion capacity of embryos regenerated under light and dark culture conditions, and that percentage conversion of embryos was also significantly affected by the different casamino acid treatments (indicated by the different letters assigned to treatments in Table 4.2).

Table 4.2 Effects of casamino acid treatment at two different concentrations and for different times on the conversion capacity (%) of sugarcane variety 88H0019 somatic embryos

Treatment	Mean (%) Conversion
Control 1	87.5 ^a
Casamino acid (0.5 & 1%) 5 min	81.7 ^{ab}
Casamino acid (0.5 & 1%) 10 min	78.3 ^b
Casamino acid (0.5 & 1%) 15 min	75.0 ^{bc}
Casamino acid (0.5 & 1%) 20 min	71.7 ^{cd}
Casamino acid (0.5 & 1%) 25 min	66.7 ^{de}
Casamino acid (0.5 & 1%) 30 min	62.5 ^{efg}
Control 2	86.7 ^a
Casamino acid (5 & 10%) 5 min	80.0 ^{bc}
Casamino acid (5 & 10%) 10 min	72.5 ^{cd}
Casamino acid (5 & 10%) 15 min	71.7 ^{cd}
Casamino acid (5 & 10%) 20 min	70.0 ^{cd}
Casamino acid (5 & 10%) 25 min	65.0 ^{ef}
Casamino acid (5 & 10%) 30 min	62.5 ^{efg}

The same letters indicate no significant difference between the means according to the Tukey post-hoc test at 5% level of significance.

Studies have shown that some single amino acids have an inhibitory effect on the growth of cultured cells (Gamborg, 1970; Heimer and Filner, 1970). However, others have reported that some amino acids supplied in certain concentrations induce good production of embryogenic callus (Claparols *et al.*, 1993). The positive role of proline in maize

embryogenesis was demonstrated by several authors (Green and Rhodes, 1982; Armstrong and Green, 1985; Duncan *et al.*, 1985; Tomes and Smith, 1985). In addition to its role in embryogenesis, proline accumulation in plant cells has been associated with the development of tolerance to vitrification *sensu in vitro* cultures (Reinhold *et al.*, 2000b). Desiccation tolerance by treatment with ABA, which induces proline accumulation, was achieved with somatic embryos of *Funaria* (Werner *et al.*, 1991) and carrot (Tetteroo *et al.*, 1995). It has been suggested that proline may play a significant role in conferring protection on cellular components during stress (Le Rudulier *et al.*, 1984), and increased tolerance to osmotic stress was observed in transgenic plants that overproduced proline (Kishor *et al.*, 1995). Although not much is known about the mechanisms by which proline confers protection (Naidu *et al.*, 1992), it is thought that it, being a non-perturbing osmolyte, is able to balance osmotic potentials (Morgan, 1984) and counteract the intracellular concentration of electrolytes during conditions of water stress (Kwon and Handler, 1995). Its possible role as an osmo- and cryoprotectant by conferring protection to membranes and proteins during the cooling process has also been suggested from *in vitro* studies (Rudolph and Crowe, 1985; Carpenter and Crowe, 1988), by preferential exclusion (Carpenter *et al.*, 1990) and by hydrophobic interaction (Schobert and Tschesche, 1978; Anchordoguy *et al.*, 1987).

These suggested protective abilities of proline provide possible explanations for the tolerance of sugarcane somatic embryos to amino acid treatment compared with treatment with the 'traditional cryoprotectants.'

4.3.2 Cooling, rehydration and regeneration

4.3.2.1 Slow cooling

4.3.2.1a Cooling in cryovials and foil jackets

Excised embryo clumps previously dehydrated by culture on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h each, and with resultant mean water contents of $0.94 \pm 0.03 \text{ g g}^{-1}$ were cooled in cryovials and foil jackets, thawed, rehydrated and decontaminated following the protocol described in section 4.2.2.1. This was followed by regeneration on the different media (see section 4.2.1) under the standard growth room conditions and culture under light and dark conditions. Cooled embryo clumps showed no signs of survival on any of the regeneration media tested, regardless of whether maintained under light or dark culture conditions, despite being subcultured on to fresh regeneration medium every 2 weeks. Embryos also tested negative with TTZ indicating their inability to survive the cryo-process. This experiment was repeated and yielded similar results.

The relatively slow cooling rates associated with this conventional means of cryopreservation encourage the formation of a few, large, extracellular ice crystals, and the method has, therefore, been favoured in a number of studies (e.g. de Boucaud *et al.*, 1994; Demeulemeester *et al.*, 1993; Kioko *et al.*, 1998). Although the success of this method of cooling has been reported for cryopreserving axes from non-orthodox seeds such as *Araucaria hunstenii*, (Pritchard and Prendergast, 1986), *Camellia sinensis* (Chaudhury *et al.*, 1991) and *Coffea* spp. (Abdelnour-Esquivel *et al.*, 1992), the water contents of the explants successfully cryopreserved in those studies ranged between 0.3 and 0.4 g g^{-1} . Explants in the present study exhibited a significant decline in percentage conversion at water contents of $\sim 0.94 \text{ g g}^{-1}$ even before cooling. It is therefore conjectured that it was the intracellular ice crystal formation at this cooling rate (Mazur, 1984) that might have proved lethal to the test explants. In addition, slow cooling rates are believed to allow the

continuation of a variety of physicochemical events during the cooling process (Kantha, 1985), which may have brought about lethal damage to the test explants.

4.3.2.1b Cooling in Mr Frosty

Embryo clumps dehydrated to mean water contents of $0.94 \pm 0.03 \text{ g g}^{-1}$ and enclosed in cryovials were cooled in Mr Frosty, thawed, rehydrated and decontaminated following the established protocol (section 4.2.2.1.). This was followed by regeneration on the different media (section 4.2.1 & 4.2.3) and cultured in the dark or exposed to the standard growth room conditions. Cooled embryo clumps exhibited no signs of survival on any of the regeneration media tested, regardless of the culture conditions (i.e. whether set to regenerate under light or dark culture conditions), although subcultured on fresh regeneration medium every 2 weeks. Embryos also tested negative for TTZ reactivity, thereby indicating their inability to survive the cryo-process. This experiment was repeated and yielded similar results.

It has been suggested that slow cooling in this manner facilitates ice nucleation in the extracellular medium (i.e. medium outside the plasmalemma) thereby causing the withdrawal of water from cells; this results in increased cytoplasmic concentrations, which should, theoretically, promote glass transitions in the intracellular *milieu* when tissues are plunged into liquid nitrogen (Mazur, 1990; Wesley-Smith *et al.*, 1995; Dumet and Benson, 2000; Engelmann, 2000). Slow cooling has been successful for cryopreserving plant cell suspension cultures (e.g. Withers and Engelmann, 1998; Swan *et al.*, 1999) and shoot apices (e.g. Brison *et al.*, 1995; Escobar *et al.*, 1997) but proved unsuccessful for the cryopreservation of the test explants in the present study. The most likely reason for this is that the cooling rate may not have been appropriate for the test explants, which were still at unfavourably high water contents ($0.94 \pm 0.03 \text{ g g}^{-1}$). Control of the rate of slow cooling is essential, as cooling rates that are too slow may be conducive to associated lethal dehydration, while rates that are too fast may induce supercooling, and thus the occurrence of detrimental intracellular ice nucleation (Wesley-Smith *et al.*, 1995).

4.3.2.1c Vitrification

Freshly excised embryos were pretreated and cooled following the protocol described in section 4.2.2.1 and thawed, rehydrated and decontaminated following the procedure described in section 4.2.2. This was followed by regeneration on the different media (section 4.2.1 & 4.2.3) under the standard growth room conditions, and cultured under light and dark conditions.

Cooled embryo clumps exhibited no signs of survival on any of the regeneration media tested, regardless of the culture conditions (i.e. whether set to regenerate under light or dark culture conditions), and subcultured on to fresh regeneration medium every 2 weeks. Embryos also tested negative for TTZ reactivity, indicating their inability to survive the cryo-process. This experiment was repeated twice, and yielded similar results.

Although vitrification solutions or permeating cryoprotectants are associated with limitation of ice crystal growth during cryogenic cooling by increasing cytoplasmic concentration (Finkle *et al.*, 1985), results obtained in this study suggest that the use of vitrification solutions may not offer a suitable means of cryopreserving the sugarcane explants of variety 88H0019. This may possibly be attributed to the highly hydrated nature of the somatic embryos in the present study (mean water content of $1.12 \pm 0.33 \text{ g g}^{-1}$ after exposure to PVS2 for 30 min). Also, it has been shown that prolonged exposure of *Arachis* spp. embryonic axes to PVS2 is associated with abnormal callus formation in response to compromised membrane integrity (Gagliardi *et al.*, 2002). It is possible that a similar phenomenon occurred in the present explants, where membranes may have been damaged by detrimental processes similar to those affecting the *Arachis* embryonic axes.

4.3.2.1d Encapsulation-vitrification

Freshly excised embryo clumps were encapsulated (Figure 4.8), immersed in PVS2 for various times up to 30 min, cooled, thawed, rehydrated and decontaminated following the protocol described in section 4.2.2.1. This was followed by regeneration on the different media (4.2.1 & 4.2.3) under the standard growth room conditions and cultured under light and dark conditions. The mean water content of ten freshly excised encapsulated embryo clumps was $7.29 \pm 0.42 \text{ g g}^{-1}$, and this was reduced to $1.67 \pm 0.51 \text{ g g}^{-1}$ following exposure to the PVS2 solution for 30 min before being cryopreserved. After retrieval from cryostorage, 30% (6/20) encapsulated embryo clumps that were regenerated on medium containing 3 mg l^{-1} 2,4-D and cultured in the dark formed callus following 10 weeks in culture. Transfer of callused embryo clumps to regeneration medium without PGRs resulted in the production of shoots and roots (Figures 4.9A and 4.9B, respectively). There was no direct conversion of embryos in any of the cases.

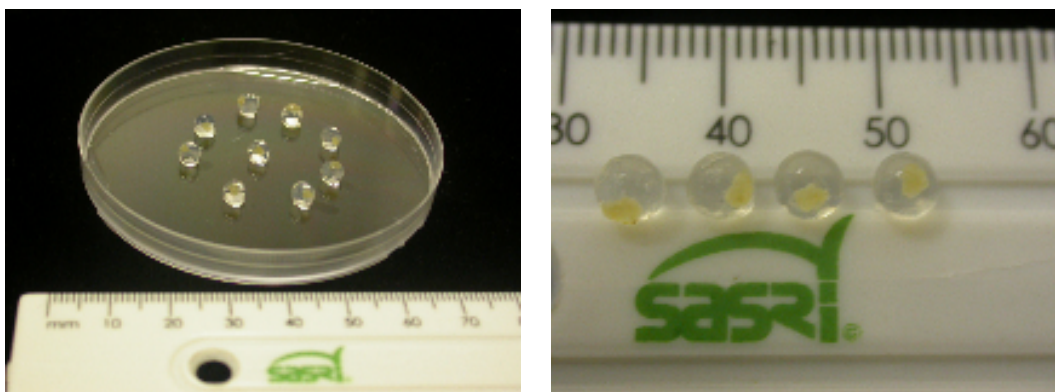


Figure 4.8 Individually encapsulated somatic embryo clumps

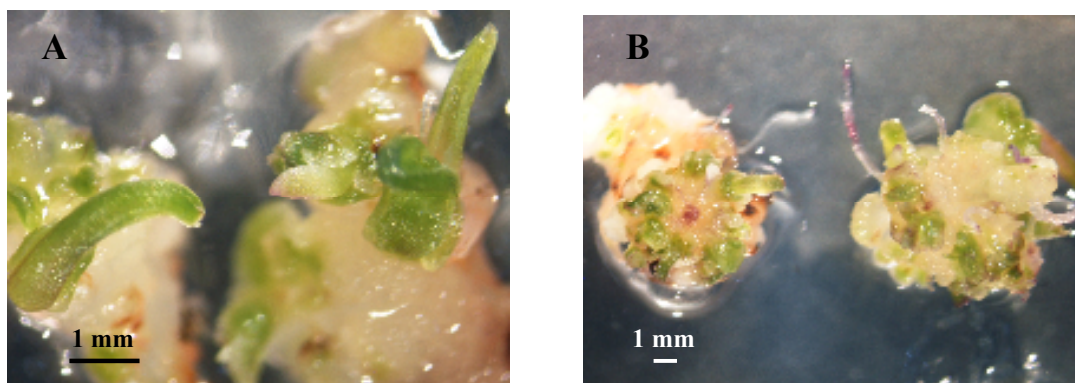


Figure 4.9 Shoot (A) and root (B) formation from callus formed by embryo clumps cooled by encapsulation-vitrification protocol and converted on medium containing 3 mg l^{-1} 2,4-D in the dark for 8 weeks and subsequently transferred to medium without PGRs

Statistical analysis of the data was obtained from an experiment examining the effects of the different treatments within the encapsulation-vitrification protocol on the conversion capacity of embryo clumps regenerated on medium containing 3 mg l^{-1} 2,4-D. The analysis indicated that there was a significant difference between the conversion capacity of embryo clumps allowed to be regenerated under light and dark conditions and between different treatments within the encapsulation-vitrification protocol (illustrated by the different letters in Table 4.3).

The limited success in terms of conversion capacity obtained with embryo clumps following encapsulation-vitrification indicated that cells of somatic embryos of sugarcane variety 88H0019 could withstand cryopreservation. However, formation of an intervening callus phase on retrieval from the cryogen, is not ideal. Nevertheless, improvement on the 30% survival – and the possibility of direct embryo conversion – may be achievable by meticulous refinement of the procedures, as well as optimisation and then standardising embryo clump size. The latter parameter could be critical in optimising not only thermal mass, but also achieving uniform water content across explants.

Table 4.3 Interactive effects of treatment with loading solution (L), vitrification solution (V) or unloading solution (U) within the encapsulation-vitrification protocol, and light and dark culture conditions on the conversion capacity (%) of sugarcane variety 88H0019 somatic embryos

Treatment	Embryo Conversion		Mean
	Light	Dark	
Control	56.7 (48.9)*	58.3 (49.8)	57.5 (49.4) ^a
20 min L	51.7 (46.0)	53.3 (46.9)	52.5 (46.5) ^{ab}
15 min V	46.7 (43.1)	51.7 (46.0)	49.2 (44.6) ^{bc}
30 min V	45.0 (42.1)	48.3 (44.0)	46.7 (43.1) ^{bcd}
45 min V	43.3 (41.1)	46.7 (43.1)	45.0 (42.1) ^{cde}
60 min V	43.3 (41.1)	46.7 (43.1)	45.0 (42.1) ^{cde}
15 min U	43.3 (41.1)	46.7 (43.1)	45.0 (42.1) ^{cde}
30 min U	41.7 (40.2)	45.0 (42.1)	43.4 (41.2) ^{cde}
45 min U	36.7 (37.3)	45.0 (42.1)	40.9 (39.7) ^{de}
60 min U	33.3 (35.3)	43.3 (43.1)	38.3 (38.2) ^e
	44.7 (41.7) ^a	48.5 (44.1) ^b	

*The values within parentheses indicate the arcsine transformation of conversion (%).

The same letters indicate no significant difference between the means according to the Tukey post-hoc test at 5% level of significance.

The encapsulation-vitrification method has been successfully implemented for cryopreservation of shoot apices of yam (Malaurie *et al.*, 1998) and pineapple (Gámez-

Pastrana *et al.*, 2004). The cryopreservation of sugar beet shoot tips, however, has been achieved by the encapsulation-dehydration method (Vandenbussche and De Proft, 1996).

4.3.2.1e Slow controlled cooling

Freshly excised embryo clumps were pretreated, cooled, thawed and rehydrated following the protocol described in section 4.2.2.1. This was followed by regeneration on the different media described in section 4.2.1 & 4.2.3, as well as on MS medium containing 2,4-D (1 mg l^{-1}), arginine (50 mg l^{-1}) and proline (500 mg l^{-1}) as outlined in the protocol (Martínez-Montero *et al.*, 1998) under the standard growth room conditions and cultured under light and dark conditions. Embryo clumps retrieved from LN showed no signs of survival on any of the regeneration media tested, regardless of the culture conditions (i.e. whether set to regenerate under light or dark culture conditions), and subcultured on to fresh regeneration medium every 2 weeks. Embryos also tested negative with TTZ, indicating their inability to survive the cryo-process. This experiment was repeated twice and yielded similar results.

Although this cooling protocol has been successfully employed for cryopreservation of sugarcane calli (Jian *et al.*, 1987; Gnanapragasam and Vasil, 1990; Eksomtramage *et al.*, 1992), it proved unsuccessful for cryopreservation of the explants of variety 88H0019 in the present study. This could be due to differences in the varieties of the test material used in the fore-mentioned studies and the present study. As a result of the variability in plant tissue of different organs of the same species, and among different species, responses to cooling also vary widely, thereby making it imperative that protocols for cryopreservation of plant germplasm are, at least presently, developed empirically for each species and explant used (Wesley-Smith *et al.*, 1995; Kioko *et al.*, 1998). Such requirements may also have to be met on a variety basis, as was shown to be the case for rice, for example (Fatima *et al.*, 2002), and could possibly be the case for sugarcane as well. Another possible explanation for the lack of success could be the non-availability of a -40°C freezer, as was stipulated in the protocol. The use of dry ice to keep the temperature of the ethanol bath at

-40°C does not guarantee a cooling rate of 0.4-0.6°C min⁻¹ between 0°C and -40°C, as would presumably have been the case if a -40°C freezer had been available.

4.3.2.2 Rapid cooling

Excised embryo clumps previously dehydrated by culture on a series of media with increasing concentrations of sucrose (from 0.2 M to 1.2 M) for a period of 48 h on each, and with mean water contents of 0.94±0.03 g g⁻¹ were tumbled naked in nitrogen slush, thawed, rehydrated and decontaminated following the protocol described in section 4.2.2.1. This was followed by regeneration on the different media (4.2.1. & 4.2.3) under the standard growth room conditions and cultured under light and dark conditions. No embryo clumps that had been cooled showed signs of survival on any of the regeneration media tested, regardless of the culture conditions (i.e. whether allowed to regenerate under light or dark culture conditions), despite subculture on to fresh regeneration medium every 2 weeks. Embryos also tested negative for TTZ reactivity, indicating their inability to survive the cryo-process. This experiment was repeated twice and yielded similar results.

Rapid cooling rates, usually in the order of several hundred °C s⁻¹ (Wesley-Smith *et al.*, 2001a; 2004a), are generally achieved by direct rapid immersion of naked explants into nitrogen slush (LN subcooled to -210°C) (e.g. Wesley-Smith *et al.*, 1992). It has been suggested that these cooling rates limit ice crystal formation and facilitate either intracellular vitrification (James, 1983), or non-injurious microcrystalline ice formation (Wesley-Smith *et al.*, 1992). The success of rapid cooling rates for cryopreservation has been reported for non-orthodox zygotic embryonic axes of several species, e.g. *Aesculus hippocastanum* (Wesley-Smith *et al.*, 2001a), *Acer saccharinum* (Wesley-Smith, 2002), *Camellia sinensis* (Wesley-Smith *et al.*, 1992), *Quercus robur* (Berjak *et al.*, 1999) and *Poncirus trifoliata* (Wesley-Smith *et al.*, 2004a, b). Furthermore, *in vitro* cultures appear to achieve tolerance to cooling after being acclimated to cold conditions by exposure to low temperatures (Reed and Chang, 1997). Explants in the present study were not subjected to any cold acclimation, which perhaps partly accounts for their inability to survive rapid

cooling. However, the size of explants in the present study (less than 1 mg dry mass), theoretically render them better suited to cooling than larger explants according to Wesley-Smith (2002), who reported that cooling rates above $1\ 200^{\circ}\text{C s}^{-1}$ are possible for material with a dry mass between 1 and 2 mg. Larger specimens present greater difficulty in achieving rapid cooling rates, but this improves substantially when the sample is partially dehydrated (Wesley-Smith *et al.*, 1992, 1999, 2004b).

4.3.2.2a Cryoprotection followed by rapid cooling

Cryoprotection trials using both ‘traditional’ cryoprotectant solutions and amino acid solutions showed a positive response of embryos to treatment with the amino acids only (see section 4.2.2.1). Therefore embryos pretreated with amino acids (proline and casamino acid separately) were rapidly cooled described in section 4.2.2.2. Ten pretreated embryo clumps per amino acid treatment with mean water contents of $3.94\pm 0.55\ \text{g g}^{-1}$ (0.5 and 1 % proline), $1.63\pm 0.54\ \text{g g}^{-1}$ (5 and 10 % proline), $3.47\pm 0.62\ \text{g g}^{-1}$ (0.5 and 1 % casamino acid) and $2.01\pm 0.79\ \text{g g}^{-1}$ (5 and 10 % casamino acid) were tumbled naked in nitrogen slush, thawed, rehydrated and decontaminated following the protocol described in section 4.2.2.1. This was followed by regeneration on the different media described in sections 4.2.1 & 4.2.3 under the standard growth room conditions and cultured under light and dark conditions. Cooled embryo clumps showed no signs of survival on any of the regeneration media tested regardless of the culture conditions (i.e. whether allowed to regenerate under light or dark culture conditions), and subcultured on to fresh regeneration medium every 2 weeks. Embryos also tested negative for TTZ reactivity, thereby indicating their inability to survive the cryo-process. This experiment was repeated twice and yielded similar results.

Several studies have shown that the stresses imposed by individual pre-cooling manipulations during the cryopreservation protocol are cumulative (Kioko *et al.*, 1998; Berjak *et al.*, 1999, 2000), and the collective stresses can prove lethal to the explant. Therefore, although most explants survived pre-treatment with proline and casamino acid at

the concentrations used prior to cooling, it may be that the additional stress imposed by the actual cryogenic cooling was lethal.

5: Concluding Comments

The results presented in this study demonstrate the intractability of sugarcane somatic embryos of variety 88H0019 to survive cryopreservation. Minimal success was achieved by the encapsulation-vitrification technique, which involved encapsulation of embryo clumps in a solution of MS medium with 3% (w/v) Na-alginate and loading solution containing 2 M glycerol plus 0.4 M sucrose, followed by infiltration and dehydration at 0°C for various time intervals (0, 5, 10, 15, 20, 25, 30 min) with 1 ml PVS2 solution and thereafter, rapid immersion in liquid nitrogen. Under such conditions, 30% of cryopreserved somatic embryos retained viability, which is not considered a significant level of survival, but does indicate that cells of somatic embryos of sugarcane variety 88H0019 can withstand cryopreservation. However, considerable refinement of the procedures involved, as well as optimisation of embryo clump size, may result in the improved capacity for survival of cryo-storage, albeit involving callus formation. The developmental stage, as identified by Mycock *et al.* (1995), is also one of the key factors that may have determined the ability of sugarcane somatic embryos of the 88H0019 variety to be cryopreserved without loss of vigour or viability.

As a result of the variability in plant tissue of different organs of the same species and among different species, responses to cooling also vary widely, therefore making it imperative that protocols for cryopreservation of plant germplasm are, at least presently, developed empirically for each species and explant used (Wesley-Smith *et al.*, 1995; Kioko *et al.*, 1998). Such requirements may also have to be met on a variety basis, as was shown to be the case for rice (Fatima *et al.*, 2002), and could also be the case for sugarcane somatic embryos of variety 88H0019 in the present study. Previous attempts to cryopreserve sugarcane somatic embryos of the N12 variety (also from SASRI) also proved unsuccessful (O'Brien, 2001; Cheruiyot, 2002), further suggesting that the lack of success may be variety-linked. This possibility is reinforced by results of other investigators (e.g. Martínez-Montero *et al.*, 1998) who have successfully cryopreserved embryogenic calli of different varieties of sugarcane.

The success of a cryopreservation protocol is largely influenced by the tissue culture processes employed since the two are often inextricably linked (Benson, 2008). Tissue culture recalcitrance, described by Benson (2000a, b) as “any form of *in vitro* culture which is non-responsive to manipulation, or to a culture that has lost responsiveness and totipotency with time in culture,” could therefore be a significant factor determining the outcome of a cryopreservation protocol. That author has listed suboptimal culture and growth regimes, endophytic bacteria, lack or loss of totipotency, juvenility-maturation status, oxidative stress, *in vitro* ageing, neoplastic progression and deterioration associated with genetic instability including deleterious epigenetic changes and possibly somaclonal variation, as some of the possible causes of tissue culture recalcitrance. Any of these phenomena may provide possible explanations for the limited success achieved with cryopreservation of sugarcane somatic embryos of the 88H0019 variety.

It is suggested that for sugarcane somatic embryos of this particular variety, and possibly other varieties that might prove essentially intractable to cryopreservation, *in vitro* storage by the method(s) described in Chapter 1, may be the only solution for conservation of the germplasm.

6: Literature Cited

- Aalen, R.B. 1999. Peroxiredoxin antioxidants in seed physiology. *Seed Science Research* 9: 285-295.
- Abdelnour-Esquivel, A., Villalobos, V. & Engelmann, F. 1992. Cryopreservation of zygotic embryos of *Coffea* spp. *CryoLetters* 13: 297-302.
- Allan, E. 1991. Plant cell culture. In: A. Stafford & G. Warren (eds), *Plant Cell and Tissue Culture*, pp. 1-23. Open University Press, Milton Keynes.
- Ammirato, P.V. 1983. The regulation of somatic embryo development in plant cell cultures: suspension culture techniques and hormone requirements. *Biotechnology* 1: 68-74.
- Ammirato, P.V. 1985. Patterns of development in culture. In: R.R. Henke, K.W. Hughes, M.J. Constantine & A. Hollaender (eds), *Tissue Culture in Forestry and Agriculture*, pp. 9-29. Plenum Press, New York.
- Ammirato, P.V. 1986. Control and expression of morphogenesis in culture. In: L.A. Withers & P.G. Alderson (eds), *Plant Tissue Culture and its Agricultural Application*, pp. 23-45. Butterworth, London.
- Ammirato, P.V. 1987. Organisational events during somatic embryogenesis. In: C.E. Green, D.A. Somers, W.P. Hackett & D.D. Biesboer (eds), *Plant Tissue and Cell Cultures*, pp. 57-81. Alan R. Liss. Inc., New York.
- Anchordoguy, T.J., Carpenter, J.F., Loomis, S.H., & Crowe, J.H. 1987. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* 24: 324-331.

- Anonymous. 2001. The sugar industry – Africa's unsung hero. *Traders*. Issue 8: October 2001-February 2002.
- Arakawa, T. & Timasheff, S. N. 1982. Stabilisation of protein structure by sugars. *Biochemistry* 21: 6536-6544.
- Arencibia, A. 1998. Gene transfer in sugarcane. *In*: T. Hohn & K.M. Leisinger (eds), *Biotechnology of Food Crops in Developing Countries*, pp. 79-104. Springer-Verlag, New York.
- Arencibia, A., Vázquez, R.I., Prieto, D., Téllez, P., Carmona, E.R., Coego, A., Hernández, L., De la Riva, G.A. & Selman-Housein, G. 1997. Transgenic sugarcane plants resistant to stem borer attack. *Molecular Breeding* 3: 247-255.
- Armstrong, C.L. & Green, C.E. 1985. Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. *Planta* 164: 207-214.
- Arumugam, N. & Bhojwani, S.S. 1990. Somatic embryogenesis in tissue culture of *Podophyllum hexandrum*. *Canadian Journal of Botany* 68: 487-491.
- Ashmore, S.E. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome.
- Asiedu, R., Ng, S.Y.C., Vuylsteke, D., Terauchi, R.T. & Hahn, S.K. 1992. Analysis of the need for biotechnology research on cassava, yam and plantain. *In*: G. Thottapillay, L.M. Monti, D.R. Mohan & A.W. Moore (eds), *Biotechnology: Enhancing Research on Tropical Crops in Africa*, pp. 27-32. International Institute of Tropical Agriculture.

- Assy-Bah, B. & Engelmann, F. 1992. Cryopreservation of immature embryos of coconut (*Cocos nucifera* L.). *CryoLetters* 13: 67-74.
- Back, J.F., Oakenfull, D. & Smith, M. 1979. Thermal stability of proteins. *Biochemistry* 18: 5191-5196.
- Bailey, R.A. & Bechet, G.R. 1989. A comparison of seedcane derived from tissue culture with conventional seedcane. *Proceedings of the South African Sugar Technologists' Association* 63: 125-129.
- Bajaj, Y.P.S. 1995a. Somatic embryogenesis and its applications for crop improvement. In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry 30. Somatic Embryogenesis and Synthetic Seed I*, pp. 105-119. Springer-Verlag, Berlin.
- Bajaj, Y.P.S. 1995b. *Biotechnology in Agriculture and Forestry 32: Cryopreservation of Plant Germplasm I*, pp. 512. Springer-Verlag, Berlin.
- Bajaj, Y.P.S. & Jian, L.C. 1995. Cryopreservation of germplasm of sugarcane (*Saccharum* spp.) In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry 32*, pp. 256-265. Springer-Verlag, Berlin.
- Bapat, V.A. & Rao, P.S. 1998. Sandalwood plantlets from synthetic seeds. *Plant Cell Reports* 7: 434-436.
- Barba, R.C., Zamora, A.B., Mallion, A.K. & Linga, C.K. 1978. Sugarcane tissue culture research. *Proceedings of the South African Sugar Technologists' Association* 16: 1843-1863.
- Benson, E.E. 1990. *Free Radical Damage in Stored Plant Germplasm*, pp. 37-68. International Board for Plant Genetic Resources, Rome, Italy.

- Benson, E. E. 1995. Cryopreservation of shoot-tips and meristems. *In*: J.G. Day & M.R. McLellan (eds), *Methods in Molecular Biology: Cryopreservation and Freeze-drying Protocols*, pp. 121-132. Humana Press Inc., Totowa, New York.
- Benson, E.E. 1999. Cryopreservation. *In*: E.E. Benson (ed.), *Plant Conservation Biotechnology*, pp. 83-95. Taylor & Francis, London.
- Benson, E.E. 2000a. Special symposium: *In vitro* plant recalcitrance: an introduction. *In Vitro Cellular and Developmental Biology – Plant* 36: 141-148.
- Benson, E.E. 2000b. Special symposium: *in vitro* plant recalcitrance: do free radicals have a role in plant tissue culture recalcitrance? *In Vitro Cellular and Developmental Biology – Plant* 36: 163-170.
- Benson, E.E. 2008. Cryopreservation of phytodiversity: A critical appraisal of theory and practice. *Critical Reviews in Plant Sciences* 27: 141-219.
- Benson, E.E. & Bremner, D. 2004. Oxidative stress in the frozen plant: A free radical point of view. *In*: B.J. Fuller, N. Lane & E.E. Benson, (eds), *Life in the Frozen State*, pp. 205-241. CRC Press, Boca Raton, London.
- Benson, E.E. & Hamill, J.D. 1991. Cryopreservation and post-freeze molecular and biosynthetic stability in transformed roots of *Beta vulgaris* and *Nicotiana rustica*. *Plant Cell, Tissue and Organ Culture* 24: 163-172.
- Berjak, P. 2000. Current status of cryopreservation research and future perspectives of its applications in South Africa. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Germplasm: Current Research Progress and Application*, pp. 315-319. International Plant Genetic Resources Institute, Rome.

- Berjak, P. 2006. Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Science Research* 16: 1-15.
- Berjak, P. & Mycock, D.J. 2004. Calcium, with magnesium, is essential for normal seedling development from partially-dehydrated recalcitrant axes: a study on *Trichilia dregeana* Sond. *Seed Science Research* 14: 217-231.
- Berjak, P. & Pammenter, N.W. 1997. Progress in the understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. In: R.H. Ellis, M. Black, A.J. Murdoch & T.D. Hong (eds), *Basic and Applied Aspects of Seed Biology*, pp. 689-703. Academic Publishers, Dordrecht, Kluwer.
- Berjak, P. & Pammenter, N.W. 2001. Seed Recalcitrance – Current perspectives. *South African Journal of Botany* 67: 79-89.
- Berjak, P. & Pammenter, N.W. 2004. Recalcitrant seeds. In: R.L. Benech-Arnold & R.A. Sánchez (eds), *Handbook of Seed Physiology. Applications to Agriculture*, pp. 305-317. Food Product Press and Haworth Reference Press, New York.
- Berjak, P., Farrant, J.M. & Pammenter, N.W. 1989. The basis of recalcitrant seed behaviour. Cell biology of the homoiohydrous seed condition. In: R.B. Taylorson (ed.), *Recent Advances in Development and Germination of Seeds*, pp. 89-108. Plenum Press, New York.
- Berjak, P., Farrant, J.M. & Pammenter, N.W. 2007. Seed desiccation-tolerance mechanisms. In: M.A. Jenks & A.J. Wood (eds), *Plant Desiccation Tolerance*, pp. 151-184. Blackwell Publishing, Iowa, U.S.A.

- Berjak, P., Pammenter, N.W. & Vertucci, C.W. 1992. Homoiohydrous (recalcitrant) seeds: developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. *Planta* 186: 249-261.
- Berjak, P., Vertucci, C.W. & Pammenter, N.W. 1993. Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitivity in recalcitrant seeds of *Camellia sinensis*. *Seed Science Research* 3: 155-166.
- Berjak, P., Farrant, J.M., Mycock, D.J. & Pammenter, N.W. 1990. Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation-sensitivity. *Seed Science Technology* 18: 297-310.
- Berjak, P., Walker, M., Watt, M.P. & Mycock, D.J. 1999. Experimental parameters underlying failure or success in plant germplasm conservation: A case study on zygotic axes of *Quercus robur* L. *CryoLetters* 20: 251-262.
- Berjak, P., Mycock, D.J., Wesley-Smith, J., Dumet, D. & Watt, M.P. 1996. Strategies of *in vitro* conservation of hydrated germplasm. In: M.N. Normah, M.K. Narimah & M.M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 18-28. Percetakan Watan, Kuala Lumpur.
- Berjak, P., Walker, M., Mycock, D.J., Wesley-Smith, J., Watt, P. & Pammenter, N.W. 2000. Cryopreservation of recalcitrant zygotic embryos. In: F. Engelmann & H. Takagi (eds.), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 140-155. Japan International Research Centre for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy.
- Bewley, J.D. & Black, M. 1994. *Physiology of development and germination of seeds*, pp. 140-143. Plenum Press, New York.

- Bhojwani, S.S. & Razdan, M.K. 1983. Clonal propagation. *In*: S.S Bhojwani & M.K. Razdan (eds), *Plant Tissue Culture: Theory and Practice*, pp. 313-372. Elsevier Science Publishers, Amsterdam.
- Bibby, C.J., Collar, N.J., Crosby, M.J., Heath, M.F., Imboden, C., Johnson, T.H., Long, A.J., Statterfield, A.J. & Thirgood, S.J. 1992. *Putting biodiversity on the map: Priority areas for Global Conservation*. International Council for Bird Preservation, Cambridge, U.K.
- Black, M., Corbineau, F., Gee, H. & Côme, D. 1999. Water content, raffinose, and dehydrins in the induction of desiccation tolerance in immature wheat embryos. *Plant Physiology* 120: 463-471.
- Blackman, S.A., Obendorf, R.L. & Leopold, A.C. 1995. Desiccation tolerance in developing soybean seeds: the role of stress proteins. *Physiologia Plantarum* 93: 630-638.
- Blakesley, D., Pask, N., Henshaw, G.G. & Fay, M.F. 1996. Biotechnology and the conservation of forest genetic resources: *in vitro* strategies and cryopreservation. *Plant Growth Regulation* 20: 11-16.
- Blakesley, D., Percival, T., Bhatti, M.H. & Henshaw, G.G. 1997. A simplified protocol for cryopreservation of embryogenic tissue of sweet potato [*Ipomoea batatas* (L.) Lam.] utilising sucrose preculture only. *CryoLetters* 18: 77-80.
- Bögre, L., Stefanov, I., Abraham, M., Somogyi, I. & Dudits, D. 1990. Differences in response to 2,4-dichlorophenoxyacetic acid (2,4-D) treatment between embryogenic lines of alfalfa. *In*: H.J.J. Nijkamp, L.H.W. Van der Plas & J. Van Aartrijk (eds), *Progress in Plant Cellular and Molecular Biology*, pp. 427-436. Kluwer Academic Publishers, Dordrecht.

- Bonnier, J.M.F., Hoekstra, A.F., Vos, R. & Vantuyl, J.M. 1997. Viability loss and oxidative stress in lily bulbs during long-term cold storage. *Plant Science* 122: 133-140.
- Bower, R., Elliott, A.R., Potier, B.A.M. & Birch, R.G. 1996. High efficiency, microprojectile-mediated co-transformation of sugarcane using visible or selectable markers. *Molecular Breeding* 2: 239-249.
- Bowers, S.A. 1990. Long-term storage of *Narcissus* anthers and pollen in liquid nitrogen. *Euphytica* 48: 275-278.
- Bramlage, W.J. Leopold, A.C. & Parrish, D.J. 1978. Chilling stress to soybeans during imbibition. *Plant Physiology* 61: 525-529.
- Brisibe, E.A., Miyake, H., Taniguchi, T. & Maeda, E. 1994. Regulation of somatic embryogenesis in long-term callus cultures of sugarcane (*Saccharum officinarum* L.) *New Phytology* 126: 301-301.
- Brison, M., de Boucaud, M.T. & Dosba, F. 1995. Cryopreservation of *in vitro* grown shoot tips of two interspecific *Prunus* rootstocks. *Plant Science* 105: 235-242.
- Brown, D.C.W. & Thorpe, T.A. 1995. Crop improvement through tissue culture. *World Journal of Microbiology and Biotechnology* 11: 409-415.
- Bryant, G., Koster, K.I. & Wolfe, J. 2001. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research* 11: 17-25.
- Butterfield, M.K. & Thomas, D.W. 1996. Sucrose, yield and disease resistance characteristics of sugarcane varieties under test in the SASEX selection programme. *Proceedings of the South African Sugar Technologists' Association* 70: 103-105.

- Butterfield, M.K., Rutherford, R.S., Carson, D.L. & Hockett, B.I. 2004. Application of gene discovery to varietal improvement in sugarcane. *South African Journal of Botany* 70(1): 167-172.
- Carlson, P.S. & Polacco, J.C. 1975. Plant cell cultures: genetic aspects of crop improvement. *Science* 188: 622-625.
- Carpenter, J.F. & Crowe, J.H. 1988. Modes of stabilisation of a protein by organic solutes during desiccation, *Cryobiology* 25: 459-470.
- Carpenter, J.F., Crowe, J.H. & Arakawa, T. 1990. Comparison of solute-induced protein stabilisation in aqueous solution and the frozen and dried states. *Journal of Dairy Science* 73: 3627-3636.
- Carpenter, J.F., Hand, S.C., Crowe, L.M. & Crowe, J.H. 1986. Cryoprotection of phosphofructokinase with organic solutes: characterisation of enhanced protection in the presence of deviated cations. *Archives of Biochemistry and Biophysics* 250: 505-512.
- Castillo, B., Smith, M.A.L. & Yadava, U.L. 1998. Plant regeneration from encapsulated somatic embryos of *Carica papaya* L. *Plant Cell Reports* 17: 172-176.
- Cella, R., Colombo, M., Gaili, G., Nielsen, E., Rollo, F. & Sala, F. 1982. Freeze-preservation of rice cells: A physiological study of freeze-thawed cells. *Physiologia Plantarum* 55: 279-284.
- Chaitanya, K.S.K. & Naithini, S.C. 1994. Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn.f. *New Phytologist* 126: 623-627.

- Chang, Y.F. & Wong, J.R. 1994. Regeneration of plants from protoplasts of *Triticum aestivum* L. (wheat). In: Y.P.S. Bajaj (ed.), *Plant Protoplasts and Genetic Engineering. V. Biotechnology in Agriculture and Forestry*, pp. 161-171. Springer-Verlag, Berlin.
- Charoensub, R., Phansiri, S., Sakai, A. & Yongmanitchai, W. 1999. Cryopreservation of cassava *in vitro* grown shoot tips cooled to -196°C by vitrification. *CryoLetters* 20: 89-94.
- Chaudhury, R., Radhamani, J. & Chandel, K.P.S. 1991. Preliminary observations on the cryopreservation of desiccated embryonic axes of tea (*Camellia sinensis* [L.] O. Kuntze) seeds for genetic conservation. *CryoLetters* 12: 31-36.
- Chawla, H.S. 2002. Micropropagation. In: H.S. Chawla (ed.), *Introduction to Plant Biotechnology*, pp. 39-56. Elsevier Science Publishers, Amsterdam.
- Chen, W.H., Davey, M.R., Power, J.B. & Cocking, E.C. 1988. Control and maintenance of plant regeneration in sugarcane callus cultures. *Journal of Experimental Botany* 199: 251-261.
- Cheruiyot, A.C. 2002. Towards the cryopreservation of sugarcane somatic embryos. Honours Thesis. University of Natal, Durban, South Africa.
- Chin, H.F., Krishnapillay, B. & Alang, Z.C 1988. Cryopreservation of *Veitchia* and *Howea* palm embryos: non-development of the haustorium. *CryoLetters* 9: 372-379.
- Cho, E.G., Hor, Y.L., Kim, H.H., Rao, V.R., & Engelmann, F. 2001. Cryopreservation of *Citrus madurensis* zygotic embryonic axes by vitrification: importance of pre-growth and pre-culture conditions. *CryoLetters* 22: 391-396.

- Christianson, M.L. 1987. Casual events in morphogenesis. *In*: C.E. Green, D.A. Somers, W.P. Hackett & D.D. Biesboer (eds), *Plant Tissue and Cell Culture*, pp. 44-55. Alan R. Liss, New York.
- Claparols, I., Santos, M.A. & Torné, J.M. 1993. Influence of some exogenous amino acids on the production of maize embryogenic callus and on endogenous amino acid content. *Plant Cell, Tissue and Organ Culture* 34: 1-11.
- Côme, D. & Corbineau, F. 1996. Metabolic damage related to desiccation sensitivity. *In*: A.S. Quédrago, K. Poulsen & F. Stubsgaard (eds), *Intermediate/Recalcitrant Tropical Forest Tree Seeds*, pp. 83-97. International Plant Genetic Resources Institute, Rome.
- Corbineau, F., Engelmann, F. & Côme, D. 1990. Ethylene as an indicator of chilling injury in oil palm (*Elaeis guineensis* Jacq) somatic embryos. *Plant Science* 71: 29–34.
- Corbineau, F., Picard, M.A., Fougereux, J.A., Ladonne, F. & Côme, D. 2000. Effects of dehydration conditions on desiccation tolerance of developing pea seeds as related to oligosaccharide content and cell membrane properties. *Seed Science Research* 10: 329-339.
- Corredoira, E., San-José, M.C., Ballester, A. & Vieitez, A.M. 2004. Cryopreservation of zygotic embryo axes and somatic embryos of European chestnut. *CryoLetters* 25: 33-42.
- Crouch, M.L. & Sussex, I.M. 1981. Development and storage-protein synthesis in *Brassica napus* L. embryos *in vivo* and *in vitro*. *Planta* 153: 64-74.
- Crowe, J.H., Carpenter, J.F. & Crowe, L.M. 1998. The role of vitrification in anhydrobiosis. *Annual Review of Physiology* 60: 73-103.

- Crowe, J.H., Carpenter, J.F., Crowe, L.M. & Anchordoguy, T.J. 1990. Are freezing and dehydration similar stress vectors? A comparison of mode of interaction of stabilising solutes with biomolecules. *Cryobiology* 27: 213-219.
- Crowe, J.H., Crowe, L.M., Carpenter, J.F. & Wistrom, C.A. 1987. Stabilisation of dry phospholipid bilayers and proteins by sugars. *Biochemistry Journal* 242: 1–10.
- Dale, P.J. & Deambrogia, E. 1979. A comparison of callus induction and plant regeneration from different explants of *Hordeum vulgare*. *Zeitschrift fur Pflanzenphysiologie* 94: 65-77.
- David, A. 1987. Conifer protoplast. In: J.M. Bonga & D.J. Durzan (eds), *Specific Principles and Methods: Growth and Developments. Cell and Tissue Culture in Forestry*, pp. 2-15. Martinus Nijhoff, Dordrecht.
- Davies Jr., F.T., Geneve, R.L., Hartmann, H.T. & Kester, D.E. 1997. Principles of tissue culture for micropropagation. In: F.T. Davies Jr., H.T. Hartmann & D.E. Kester (eds), *Plant Propagation: Principles and Practices*, pp. 549-589. Prentice Hall, New Jersey, U.S.A.
- Davis-Searles, P.R., Saunders, A.J., Erie, D.A., Winzor, D.J. & Pielak, G.J. 2001. Interpreting the effects of small uncharged solutes on protein-folding equilibria. *Annual Review of Biophysics and Biomolecular Structure* 30: 271-306.
- Daws, M.I. & Pritchard, H.W. 2008. Fruit/seed freezing tolerance is dependent on developmental status in relation to provenance: a study of *Acer pseudoplatanus* across Europe. Agrifood Research Working papers, pp. 153.
- de Boucaud, M.T., Brison, M. & Negrier, P. 1994. Cryopreservation of walnut somatic embryos. *CryoLetters* 15: 151-160.

- de Boucaud, M.T., Brison, M., Ledoux, C., Germain, E. & Lutz, A. 1991. Cryopreservation of embryonic axes of recalcitrant seed *Juglans regia* L. cv Franquette. *CryoLetters* 12: 163-166.
- Demeulemeester, M.A.C., Vandebussche, B. & de Proft, M.P. 1993. Regeneration of chicory plants from cryopreserved *in vitro* shoot tips. *CryoLetters* 14: 57-64.
- Deng, M.L., Wang, Z.Y. & Li, X.Q. 1990. A preliminary study on artificial seeds of wheat. In: X.Q. Li (ed.), *Studies on Artificial Seeds of Plants*, pp. 65. Beijing University, Beijing.
- Dereuddre, J. 1992. Cryopreservation of *in vitro* cultures of plant cells and organs by vitrification and dehydration. In: Y. Dattée, C. Dumas & A. Gallais (eds), *Reproductive Biology and Plant breeding*, pp. 291-300. Springer-Verlag, Berlin.
- Dereuddre, J., Blandin, S. & Hassen, N. 1991. Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen: Effects of pre-culture. *CryoLetters* 12: 125-134.
- Dereuddre, J., Fabre, J. & Bassaglia, C. 1988. Resistance to freezing in liquid nitrogen of carnation (*Dianthus caryophyllus* L. Vareolo) apical and axillary shoot tips excised from different aged *in vitro* plantlets. *Plant Cell Reports* 7: 170-173.
- Dijak, M., Smith, D.L., Wilson, T.J. & Brown, D.C.W. 1986. Stimulation of direct embryogenesis from mesophyll protoplasts of *Medicago sativa*. *Plant Cell Reports* 5: 468-470.
- Dodds, J.H. 1983. Introduction. In: J.H. Dodds (ed.), *Tissue Culture of Trees*, pp. 1-21. Avi Publishing Company, Connecticut, U.S.A.

- Dodds, J.H. 1991. Introduction: conservation of plant genetic resources – the need for tissue culture. *In: J.H. Dodds (ed.), In Vitro Methods for Conservation of Plant Genetic Resources*, pp. 3-8. Chapman and Hall, London.
- Dodds, J. H. & Roberts, L. W. 1985. Experiments in plant tissue culture. *In: J.H. Dodds & L.W. Roberts (eds), Experiments in Plant Tissue Culture*, pp. 195-203. Cambridge University Press, Cambridge, U.K.
- Dodeman, V.L., Ducreux, G. & Kreis, M. 1997. Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany* 48: 1493-1509.
- Dudits, D., Bögre, L. & Györgyey, J. 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. *Journal of Cell Science* 99: 475-484.
- Dudits, D., Nemet, G. & Haydu, Z. 1975. Study of callus growth and organ formation in wheat (*Triticum aestivum*) tissue cultures. *Canadian Journal of Botany* 53: 957-963.
- Dumet, D. & Benson, E.E. 2000. The use of physical and biochemical studies to elucidate and reduce cryopreservation-induced damage in hydrated/desiccated plant germplasm. *In: F. Engelmann & H. Takagi (eds), Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 43-56. International Plant Genetic Resources Institute, Rome.
- Dumet, D., Berjak, P. & Engelmann, F. 1997. Cryopreservation of zygotic and somatic embryos of tropical species producing recalcitrant seeds or intermediate seeds. *In: M.K. Razdan & E.C. Cocking (eds), Conservation of Plant Genetic Resources In Vitro*, pp. 153-172. Science Publishers Inc., Enfield, U.S.A.

- Dumet, D., Engelmann, F., Chabrillange, N. & Duval, Y. 1993a. Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. *Plant Cell Reports* 12: 352-355.
- Dumet, D., Engelmann, F., Chabrillange, N., Duval, Y. & Dereuddre, J. 1993b. Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *CryoLetters* 14: 243-250.
- Dumet, D., Engelmann, F., Chabrillange, N., Dussert, S. & Duval, Y. 1994. Effect of various sugars and polyols on the tolerance to desiccation and freezing of oil palm polyembryonic cultures. *Seed Science Research* 4: 307-313.
- Duncan, D.R. & Widholm, J.M. 1986. Cell selection for crop improvement. *Plant Breeding Reviews* 4: 153-173.
- Duncan, D.R., Williams, M.E., Zehr, B.E. & Widholm, J.M. 1985. The production of callus capable of plant regeneration from immature embryos of numerous *Zea mays* genotypes. *Planta* 165: 322-332.
- Dussert, S., Chabrillange, N., Vasquez, N., Engelmann, F., Anthony, F., Guyot, A. & Hamon, S. 2000. Beneficial effect of post-thawing osmoconditioning on the recovery of cryopreserved coffee (*Coffea Arabica* L.) seeds. *CryoLetters* 21: 47-52.
- Eksomtramage, T., Paulet, F., Guiderdoni, E., Glaszmann, J.C. & Engelmann, F. 1992. Development of a cryopreservation process for embryogenic callus of a commercial hybrid of sugarcane (*Saccharum* sp.) and application to different varieties. *CryoLetters* 13: 239-252.
- Ellis, R.H. 1984. Revised table of seed storage characteristics. *Plant Genetic Resources Newsletter* 58: 16-33.

- Ellis, R.H., Hong, T.D. & Roberts, E.H. 1990. An intermediate category of seed behaviour? I. Coffee. *Journal of Experimental Botany* 41: 1167-1174.
- Ellis, R.H., Hong, T.D., Roberts, E.H. & Soetisna, U. 1991. An intermediate category of seed storage behaviour II. Effects of provenance, immaturity, and imbibition on desiccation tolerance in coffee. *Journal of Experimental Botany* 42: 653-657.
- Emons, A.M.C. 1994. Somatic embryogenesis: cell biological aspects. *Acta Botanica Neerlandica* 43: 1-14.
- Engelmann, F. 1990. Cryopreservation of plant organs: oil palm somatic embryos (*Elaeis guineensis* Jacq.). *International Journal of Refrigeration* 13: 26-30.
- Engelmann, F. 1994. Cryopreservation for the long-term conservation of tropical crops of commercial importance. In: N.A. Shamaam (ed.), *Applications of Plant In Vitro Technology*, pp. 64-77. Proceedings of the International Symposium, University of Pertanian, Selangon, Malaysia.
- Engelmann, F. 1997. *In vitro* conservation methods. In: B.V. Ford-Lloyd, J.H. Newbury & J.A. Callow (eds), *Biotechnology and Plant Genetic Resources: Conservation and Use*, pp. 119-162. CABI Publishing, Wellington.
- Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. In: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Application*, pp. 8-20. International Plant genetic resources Institute, Rome.
- Engelmann, F. 2004. Plant cryopreservation: progress and prospects. *In Vitro Cellular and Developmental Biology – Plant* 40: 427-433.

- Engelmann, F., Chabrillange, N. & Duval, Y. 1995a. Cryopreservation of zygotic embryos and kernels of oil palm (*Elaeis guineensis* Jacq.). *Seed Science Research* 5: 81-86.
- Engelmann, F., Duval, Y. & Dereuddre, J. 1985. Survie et prolifération d'embryons somatiques de palmier à huile (*Elaeis guineensis* Jacq.) après conservation dans l'azote liquide. *Compte Rendus de l'Académie des Sciences* 301: 111-116.
- Engelmann, F., Dumet, D., Chabrillange, N., Abdelnour-Esquivel, A., Assy-Bah, B., Dereuddre, J. & Duval, Y. 1995b. Factors affecting the cryopreservation of coffee, coconut and oil palm embryos. *Plant Genetic Resources Newsletter* 103: 27-31.
- Escobar, R.H., Mafla, G. & Roca, W.M. 1997. A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. *Plant Cell Reports* 16: 474-478.
- Evans, D.A., Sharp, W.R. & Flick, C.E. 1981a. Growth and behaviour of cell cultures: embryogenesis and organogenesis. In: T.A. Thorpe (ed.), *Plant Tissue Culture: Methods and Applications in Agriculture*, pp. 45-113. Academic Press, New York.
- Evans, D.A., Sharp, W.R. & Flick, C.E. 1981b. Plant regeneration from cell cultures. *Horticultural Reviews* 3: 214-314.
- Fabre, J. & Dereuddre, J. 1990. Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot tips. *CryoLetters* 11: 413-426.
- Fahy, G.M. 1986. The relevance of cryoprotectant toxicity to cryobiology. *Cryobiology* 23:1-13.
- Fahy, G.M., MacFarlane, D.R., Angell, C.A. & Meryman, H.T. 1984. Vitrification as an approach to cryopreservation. *Cryobiology* 21: 407-426.

- Farrant, J.M. 1977. Water transport and cell survival in cryobiological procedures. *Philosophical Transactions of the Royal Society of London*, B278: 291-306.
- Farrant, J.M. 1980. General observations on cell preservation. In: M. J. Ashwood-Smith & J.M. Farrant (eds), *Low Temperature Preservation in Medicine and Biology*, pp. 121-138. Pitman Medical. Tunbridge-Wells, U.K.
- Farrant, J.M. 2007. Mechanisms of desiccation tolerance in angiosperm resurrection plants. In: M.A. Jenks & A.J. Wood (eds), *Plant Desiccation Tolerance*, pp. 51-81. Blackwell Publishing, Iowa, U.S.A.
- Fatima, T., Jan, A., Husnain, T. & Riazuddin, S. 2002. Initiation and cryopreservation of cell suspension of rice basmati varieties. *Pakistan Journal of Biological Sciences* 5(6): 679-682.
- Feher, A. 2008. The initiation phase of somatic embryogenesis: what we know and what we don't. *Acta Biologica Szegediensis* 52(1): 53-56.
- Finch, R.P., Lynch, P.T., Jotham, J.P. & Cocking, E.C. 1991. Isolation, culture and fusion of rice protoplast. In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry*, pp. 251-268. Springer-Verlag, Berlin.
- Finch-Savage, W.E., Blake, P.S. & Clay, H.A. 1996. Desiccation stress in *Quercus robur* seeds results in lipid peroxidation and increased synthesis of jasmonates and abscisic acid. *Journal of Experimental Botany* 47: 661-667.
- Finkle, B.J. & Ulrich, J.M. 1979. Effects of cryoprotectants in combination on the survival of frozen sugarcane cells. *Plant Physiology* 63: 598-604.

- Finkle, B.J., Zavala, M.E. & Ulrich, J.M. 1985. Cryoprotective compounds in the viable freezing of plant tissues. *In*: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 75-113. CRC Press, Boca Raton, Florida.
- Fleck, R.A., Day, J.G., Clarke, K.J. & Benson, E.E. 1999. Elucidation of the metabolic and structural basis for the cryopreservation recalcitrance of *Vaucheria sessilis*. *CryoLetters* 20: 271-282.
- Ford-Lloyd, B.V. & Jackson, M.T. 1991. Biotechnology and methods of conservation of plant genetic resources. *Journal of Biotechnology* 17: 247-256
- Fowler, J., Cohen, L. & Jarvis, P. 1999. *Practical Statistics for Field Biology*, pp. 187-195. John Wiley & Sons, New York.
- Frankel, O.H. & Soule, M.E. 1981. *Conservation and Evolution*, pp. 110. Cambridge University Press, Cambridge, U.K.
- Fransz, P.F. & Schel, J.H.N. 1991. Cytodifferentiation during the development of friable embryonic callus of maize (*Zea mays*). *Canadian Journal of Botany* 69: 26-33.
- Fu, J.R., Xia, Q.H. & Tang, L.F. 1993. Effects of desiccation on excised embryonic axes of three recalcitrant seeds and studies on cryopreservation. *Seed Science and Technology* 21: 85-95.
- Fu, J.R., Zhang, B.Z., Wang, X.P., Qiao, Y.Z. & Huang, X.L. 1990. Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three fruit species and their excised embryonic axes. *Seed Science Technology* 18: 743-754.

- Funatsuki, H., Kihara, M., Takahashi, S., Lazzeri, P.A. & Lörz, H. 1996. Regeneration of plants from protoplasts of *Hordeum vulgare* (barley). In: Y.P.S. Bajaj (ed.), *Plant Protoplasts and Genetic Engineering VII: Biotechnology in Agriculture and Forestry*, pp. 64-78. Springer-Verlag, Berlin.
- Gagliardi, R.F., Pacheco, G.P., Valls, J.F.M. & Mansur, E. 2002. Cryopreservation of cultivated and wild *Arachis* species embryonic axes using desiccation and vitrification methods. *CryoLetters* 23: 61-68.
- Galau, G.A. & Hughes, D.W. 1987. Coordinate accumulation of homologous transcripts of 7 cotton *lea* gene families during embryogenesis and germination. *Developmental Biology* 123: 213-221.
- Gamborg, O.L. 1970. The effects of amino acids and ammonium on the growth of plant cells in suspension culture. *Plant Physiology* 45: 372-375.
- Gamborg, O.L. & Shyluk, J.P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. In: T.A. Thorpe (ed.), *Plant Tissue Culture: Methods and Applications in Agriculture*, pp. 21-44. Academic Press, London.
- Gamborg, O. L., Miller, R. A. & Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50: 151-158.
- Gámez-Pastrana, R., Martínez-Ocampo, Y., Beristain, C.I. & González-Arno, M.T. 2004. An improved cryopreservation protocol for pineapple apices using encapsulation-vitrification. *CryoLetters* 25: 405-414.
- Gallo-Meagher M. & Irvine J.E. 1996. Herbicide resistant transgenic sugarcane plants containing the *bar* gene. *Crop Science Society of America* 36: 1367-1374.

- Gazeau, C., Hansz, J., Jondet, M. & Dereuddre, J. 1992. Cryomicroscopic comparison of freezing (to -40°C) and thawing effects on *Catharanthus* cells and their protoplasts. *CryoLetters* 13: 137-148.
- George, E. 1993. *Plant Propagation by Tissue Culture*, pp. 3-93. Exegetics Ltd., England.
- George, E.F., Hall, M.A. & De Klerk, D.J. 2008a. Micropropagation: Uses and methods. *In*: E.F. George, M.A. Hall & D.J. De Klerk (eds), *Plant Propagation by Tissue Culture*, pp. 29-64. Springer-Verlag, Netherlands.
- George, E.F., Hall, M.A. & De Klerk, D.J. 2008b. The components of plant tissue culture II: organic additions, osmotic and pH effects, and support systems. *In*: E.F. George, M.A. Hall & D.J. De Klerk (eds), *Plant Propagation by Tissue Culture*, pp. 115-173. Springer-Verlag, Netherlands.
- George, E.F., Hall, M.A. & De Klerk, D.J. 2008c. Plant growth regulators III: gibberellins, ethylene, abscisic acid, their analogues and inhibitors; miscellaneous compounds. *In*: E.F. George, M.A. Hall & D.J. De Klerk (eds), *Plant Propagation by Tissue Culture*, pp. 227-281. Springer-Verlag, Netherlands.
- Gnanapragasam, S. & Vasil, I.K. 1990. Plant regeneration from a cryopreserved embryogenic cell suspension of a commercial sugarcane hybrid (*Saccharum* sp.). *Plant Cell Reports* 9: 419-423.
- Gnanapragasam, S. & Vasil, I.K. 1992. Cryopreservation of immature embryos, embryogenic callus and cell suspension cultures of *gramineous* species. *Plant Science* 83: 205-215.

- Gonzales, A.E., Schopke, C., Taylor, N.J., Beachy, R.N. & Fauquet, C.M. 1998. Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) through *Agrobacterium* mediated transformation of embryogenic suspension cultures. *Plant Cell Reports* 17: 827-831.
- González-Arno, M.T., Moreira, T. & Urra, C. 1995. Importance of pre-growth with sucrose and vitrification for the cryopreservation of sugarcane apices using encapsulation dehydration. *CryoLetters* 17: 141-148.
- González-Arno, M.T., Engelmann, F., Huet, C. & Urra, C. 1993. Cryopreservation of encapsulated apices of sugarcane: effect of freezing procedure and histology. *CryoLetters* 14: 303-308.
- González-Arno, M.T., Urra, C., Engelmann, F., Ortíz, R. & de la Fe, C. 1999. Cryopreservation of encapsulated sugarcane apices: effect of storage temperature and storage duration. *CryoLetters* 20: 347-352.
- Gonzalez-Benito, M.E. & Perez, C. 1994a. Cryopreservation of embryonic axes of two cultivars of hazelnut (*Corylus avellana* L). *CryoLetters* 15: 41-46.
- Gonzalez-Benito, M.E. & Perez, C. 1994b. Cryopreservation of *Centaureum* shoot tips by vitrification. *Botanical Gardens Micropropagation Newsletter* 1: 82-84.
- Gopal, J., Chamail, A. & Sarkar, D. 2002. Slow-growth *in vitro* conservation of potato germplasm at normal propagation temperature. *Potato Research* 45: 203-213.
- Goveia, M., Kioko, J.I. & Berjak, P. 2004. Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation: A study of *Trichilia dreageana* Sond. *Seed Science Research* 14: 241-248.

- Gray, D.J. 1995. Somatic embryogenesis in grape. *In*: S.M. Jain, P.K. Gupta & R.J. Newton (eds), *Somatic embryogenesis in Woody Plants*, pp. 191–218. Kluwer Academic, Dordrecht, Netherlands.
- Gray, D.J. & Purohit, A. 1991. Somatic embryogenesis and the development of synthetic seed technology. *Critical Reviews in Plant Sciences* 10: 33-61.
- Gray, D.J., Compton, M.E., Harrell, R.C. & Cantliffe, D.J. 1995. Somatic embryogenesis and the technology of synthetic seed. *In*: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry 30. Somatic Embryogenesis and Synthetic Seed I*, pp. 126-151. Springer-Verlag, Berlin.
- Green, C.E. & Rhodes, C.A. 1982. Plant regeneration in tissue cultures of maize. *In*: W.F. Sheridan (ed.), *Maize for Biological Research*, pp. 367-372. Plant Molecular Biology Association, Charlottesville.
- Gresshoff, P.M. 1978. Phytohormones and growth and differentiation of cells and tissues cultured *in vitro*. *In*: D.S. Letham, P.B. Goodwin & T.J.V. Higgins (eds), *Phytohormones and Related Compounds – A Comprehensive Treatise*, pp. 1-29. North-Holland, Amsterdam.
- Grisham, M.P. & Bourg, D. 1989. Efficiency of *in vitro* propagation of sugarcane plants by direct regeneration from leaf tissue and by shoot tip culture. *Journal of the American Society of Sugarcane Technologists* 9: 97-102.
- Grout, B.W.W. 1995. Introduction to the *in vitro* preservation of plant cells, tissues and organs. *In*: B.W.W. Grout, (ed.), *Genetic Preservation of Plant Cells In Vitro*, pp. 1–20. Springer-Verlag, Berlin.

- Grout, B.W.W. & Henshaw, G.G. 1980. Structural observations on the growth of potato shoot-tips after thawing from liquid nitrogen. *Annals of Botany* 46: 243.
- Häggman, H.M., Aronen, T.S. & Rynänen, L.A. 2000. Cryopreservation of embryogenic cultures of conifers. In: S.M. Jain, P.K. Gupta & R.J. Newton (eds.), *Somatic Embryogenesis in Woody Plants*, pp. 707-728. Kluwer Academic Publishers, Dordrecht.
- Haissig, B. E. 1972. Meristematic activity during adventitious root primordium development. Influences of endogenous auxin and applied gibberellic acid. *Plant Physiology* 49: 886-892.
- Halperin, S.J. & Koster, K.L. 2006. Sugar effects on membrane damage during desiccation of pea embryo protoplasts. *Journal of Experimental Biology* 57: 2303.
- Hao, Y.-J., You, C.-X. & Deng, X.-X. 2002. Effects of cryopreservation on developmental competency, cytological and molecular stability of citrus callus. *CryoLetters* 23: 3-22.
- Harding, K. 2004. Genetic integrity of cryopreserved plant cells: A review. *CryoLetters* 25: 3-22.
- Harrington, J.F. 1972. Seed storage and longevity. In: T.T. Kozlowski (ed.), *Seed Biology*, pp. 145-245. Academic Press, London, U.K.
- Hatanaka, T., Yasuda, T., Yamaguchi, T. & Sakai, A. 1994. Direct re-growth of encapsulated somatic embryos of coffee (*Coffea canephora*) after cooling in liquid nitrogen. *CryoLetters* 15: 47-52.

- Hatanaka, T., Arawaka, O., Yasuda, T., Uchida, N. & Yamaguchi, T. 1991. Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Reports* 10: 179-182.
- Hawkes, J.G. 1987. A strategy for seed banking in botanic gardens. In: D. Bramwell, O. Hamann, V. Heywood & H. Synge (eds), *Botanic Gardens and the World Conservation Strategy*, pp. 131-149. Academic Press, London, U.K.
- Heimer, Y.M. & Filner, P. 1970. Regulation of the nitrate assimilation pathway of cultured tobacco cells. II. Properties of a variant cell line. *Biochimica et Biophysica Acta* 215: 152-165.
- Heinz, D.J. & Mee, G.W.P. 1969. Plant differentiation from callus tissue of *Saccharum* spp. *Crop Science* 9: 346-348.
- Heinz, D.J., Krisnamurthi, M., Nickell, L.G. & Maretzke, A. 1977. Cell, tissue and organ culture in sugarcane improvement. In: J.Reinert & Y.P.S. Bajaj (eds), *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture*, pp. 3-17. Springer-Verlag, Berlin.
- Heller, R. 1953. Researches on the mineral nutrition of plant tissues. *Annales des Sciences Naturelles (Botanique) Biologie Vegetale* 14: 1-223.
- Hendre, R.R., Mascarenhas, A.F., Nadgir, A.L., Pathak, M. & Jajannathan, V. 1975. Growth of mosaic virus-free sugarcane plants from apical meristems. *Indian Phytopathology* 28: 175-178.
- Hendre, R.R., Iyer, R.S., Kotwal, M. Khuspe, S.S. & Mascarenhas, A.F. 1983. Rapid multiplication of sugarcane by tissue culture. *Sugar Cane* 1: 5-8.

- Henry, G.A.F. 1993 Oxygen, free radical processes and seed longevity. *Seed Science Research* 3: 141-153.
- Henry, G.A.F., Finch-Savage, W.E., Thorpe, P.C.T., Atherton, N.M., Buckland, S., Nilsson, K.A. & Seel, W.E. 1992. Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. *New Phytologist* 122: 273-279.
- Henshaw, G.G. & Blakesley, D. 1996. The scope of *in vitro* technology in conservation of biological diversity. In: M.N. Normah, M.K. Narimah & M.M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 217-227. Percetakan Watan, Kuala Lumpur.
- Heszky, L.E., Jekkel, Z. & Ali, A-H. 1990. Effect of cooling rate, cryoprotectant and holding time at different transfer temperatures on the survival of cryopreserved cell suspension culture (*Puccinellia distans* L.). *Plant Cell, Tissue and Organ Culture* 21: 217-226.
- Hirai, D., Shira, K., Shira, S. & Sakai, A. 1998. Cryopreservation of *in vitro*-grown meristems of strawberry (*Fragaria × ananassa* Duch.) by encapsulation-vitrification. *Euphytica* 101: 109-115.
- Hirai, D. & Sakai, A. 1999. Cryopreservation of *in vitro*-grown axillary shoot-tip meristems of mint (*Mentha spicata* L.) by encapsulation vitrification. *Plant Cell Reports* 19: 150-155.
- Ho, W. & Vasil, I.K. 1983a. Somatic embryogenesis of sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. *Protoplasma* 118: 169-180.

- Ho, W. & Vasil, I.K. 1983b. Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.). II. Growth and plant regeneration from embryogenic cell suspension cultures. *Annals of Botany* 51: 719-726.
- Hoekstra, F.A., Golovina, E.A., Van Aelst, A.C. & Hemminga, M.A. 1999. Imbibitional leakage from anhydrobiotes revisited. *Plant Cell and Environment* 22: 1121–1131.
- Hong, T.D. & Ellis, R.H. 1996. A protocol to determine seed storage behaviour. In: J.M.M. Engels, & J. Toll (eds), *International Plant Genetic Resources Institute, Technical Bulletin No. 1*, pp. 62. International Plant Genetic Resources Institute, Rome.
- Horbowicz, M. & Obendorf, R.L. 1994. Seed desiccation tolerance and storability; dependence on flatulence-producing oligosaccharides and cyclitols – review and survey. *Seed Science Research* 4: 385-405.
- Hornung, R., Domas, R. & Lynch, P.T. 2001. Cryopreservation of plumular explants of coconut (*Cocos nucifera* L.) to support programmes for mass clonal propagation through somatic embryogenesis. *CryoLetters* 22: 211-220.
- Huang, C.N., Wang, J.H., Yan, Q.S., Zhang, X.Q. & Yan, Q.F. 1995. Plant regeneration from rice (*Oryza sativa* L.) embryogenic suspension cells cryopreserved by vitrification. *Plant Cell Reports* 14: 730-734.
- Hussain, A. 1983. Conservation of genetic resources of medicinal plants in India. In: S.K. Jain & K.L. Mehra, (eds), *Conservation of Medicinal Plant Resources*, pp. 110-117. National Bureau of Plant Genetic Resources, New Delhi.
- Hutchinson, M.J., Senaratna, T., Sahi, S. & Saxena, P.K. 2000. Light mediates endogenous plant growth substances in thiodiazuron induced somatic embryogenesis in geranium hypocotyls cultures. *Journal of Plant Biochemistry and Biotechnology* 9: 1-6.

- Illing, N., Denb, K.J., Collett, H., Shen, A. & Farrant, J.M. 2005. The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. *Integrative and Comparative Biology* 45: 771-787.
- Irvine, J.E. & Benda, G.T.A. 1985. Sugarcane mosaic virus in plantlets regenerated from diseased leaf tissue. *Plant Cell, Tissue and Organ Culture* 5: 101-106.
- Ishikawa, M., Tandon, P., Suzuki, M. & Yamaguishi-Ciampi, A. 1996. Cryopreservation of bromegrass (*Bromus inermis* Leyss) suspension cultured cells using slow pre-freezing and vitrification procedure. *Plant Science* 120: 81–88.
- ISTA. 1999. International Rules for Seed Testing Rules. *Seed Science and Technology* 27: 33-35.
- James, E. 1983. Low temperature preservation of living cells. In: S.H. Mantell & H. Smith (eds), *Plant Biotechnology*, pp. 163-186. Cambridge University Press, London.
- Jana, A.F. 1994. Rapid somatic embryogenesis from the nucelli of monoembryonic mango varieties. *In Vitro Cellular and Developmental Biology – Plant* 30: 55-57.
- Jarret, R.L. & Gawel, N. 1991. Abscisic acid-induced growth inhibition of sweet potato (*Ipomoea batatas* L.) *in vitro*. *Plant Cell, Tissue and Organ Culture* 24: 13-18.
- Jian, L.C., Sun, D.L. & Sun, L.H. 1987. Sugarcane callus cryopreservation. In: P.H. Li (ed.), *Plant Cold Hardiness*, pp. 323-337. Alan R. Liss Inc., New York.
- Jiménez, V.M. 1996. El cultivo de protoplastos en cítricos y su potencial para el mejoramiento genético. *Agronomía Costarricense* 20: 187-204.

- Jiménez, V.M. 2001. Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones. *Revista Brasileira de Fisiologia Vegetal* 13: 196-223.
- Kane, M.E. 2004. Shoot culture procedures. *In: R.N. Gray & D.J. Gray (eds), Plant Development and Biotechnology*, pp. 145-158. CRC Press Inc., Boca Raton, Florida.
- Karp, A. 1994. Origins, causes and uses of variation in plant tissue cultures. *In: I.K. Vasil & T.A. Thorpe (eds), Plant Cell and Tissue Culture*, pp. 139-152. Kluwer Academic Publishers, Dordrecht.
- Kartha, K.K. 1985. Meristem culture and germplasm preservation. *In: K.K. Kartha (ed.), Cryopreservation of Plant Cells and Organs*, pp. 115-134. CRC Press Inc., Boca Raton, Florida.
- Kartha, K.K. & Engelmann, F. 1994. Cryopreservation and germplasm storage. *In: I.K. Vasil & T.A. Thorpe (eds), Plant Cell and Tissue Culture*, pp. 195-230. Kluwer Academic Publishers, Dordrecht.
- Kartha, K.K., Mroginski, L.A., Pahl, K. & Leung, N.L. 1981. Germplasm preservation of coffee (*Coffea arabica* L.) by *in vitro* culture of shoot apical meristem. *Plant Science Letters* 22: 301-307.
- Kasai, M., Komi, J. H., Takakamo, A., Tsudera, H., Sakurai, T. & Machida, T. 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *Journal of Reproduction and Fertility* 89: 91-97.
- Kim, J.-B, Kim, H.-H, Baek, H.-J, Cho, E.-G, Kim, Y.-H. & Engelmann, F. 2005. Changes in sucrose and glycerol content in garlic shoot tips during freezing using PVS3 solution. *CryoLetters* 26: 103-112.

- King, M.W. & Roberts, E.H. 1980. Maintenance of recalcitrant seeds in storage. *In*: H.F. Chin & E.H. Roberts (eds), *Recalcitrant Crop Seeds*, pp. 53-89. Tropical Press, Kuala Lumpur.
- Kioko, J.I. 2003. Aspects of post-harvest seed physiology and cryopreservation of the germplasm of three medicinal plants indigenous to Kenya and South Africa. Ph.D. Thesis, University of Natal, Durban, South Africa.
- Kioko, J.I., Berjak, P., Pritchard, H.W. & Daws, M.I. 2000. Seeds of the African pepperbark (*Warburgia salutaris*) can be cryopreserved after rapid dehydration in silica gel. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm – Current Research Progress and Application*, pp. 371-377. International Plant Genetic Resources Institute, Rome.
- Kioko, J.I., Berjak, P., Pammenter, N.W., Watt, M.P. & Mycock, D.J. 1998. Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana*. *CryoLetters* 19: 5-14.
- Kishor, P.B.K., Hong, Z., Miao, G.H., Hu, C.A.A. & Verma, D.P.S. 1995. Overexpression of Δ^1 -pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* 108: 1387-1394.
- Kobayashi, S., Sakai, A. & Oiyama, I. 1990. Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix eurolepis*) and black spruce (*Picea mariana*). *Journal of Experimental Botany* 43: 73-79.
- Kobayashi, T., Niino, T. & Kobayashi, M. 2005. Simple cryopreservation protocol with an encapsulation technique for tobacco BY-2 suspension cell cultures. *Plant Biotechnology* 22: 105-112.

- Kobayashi, T., Niino, T. & Kobayashi, M. 2006. Cryopreservation of tobacco BY-2 suspension cultures by vitrification with encapsulation. *Plant Biotechnology* 23: 333-337.
- Koga, Y. & Kudo, M. 1977. An efficient method of producing callus and plantlets by tassel culture for sugarcane breeding. *Japan Agricultural Research Quarterly* 11: 193-197.
- Kohlenbach, H. 1978. Comparative somatic embryogenesis. In: T. A. Thorpe (ed.), *Frontiers of Plant Tissue Culture 1978*, pp. 59-66. Proceedings 4th International Congress of Plant Tissue and Cell Culture, University of Calgary, Calgary, Alberta, Canada.
- Komamine, A., Matsumoto, M., Tsukahara, M., Fujiwara, A., Kawahara, R., Ito, M., Smith, J., Nomura, K. & Fujimura, T. 1990. Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry and molecular biology. In: H.H.J. Nijkamp, L.H.W. Van der Plas & J. Van Aartrijk (eds), *Progress in Plant Cellular and Molecular Biology*, pp. 307-313. Kluwer Academic Publishers, Dordrecht.
- Koster, K.L. 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiology* 96: 302-304.
- Koster, K.L. & Leopold, A.C. 1988. Sugars and desiccation tolerance in seeds. *Plant Physiology* 88: 829-832.
- Koster, K.L. & Lynch, V. 1992. Solute accumulation and compartmentalisation during the cold acclimation of Puma Rye. *Plant Physiology* 98: 108-113.
- Krøgstrop, P., Baldursson, S. & Nørgaard, J.V. 1992. *Ex situ* genetic conservation by use of tissue culture. *Opera Botanica* 113: 49-53.
- Kundu, M. & Kachari, J. 2000. Desiccation sensitivity and recalcitrant seed behaviour of seeds of *Aquilaria agallocha* Roxb. *Seed Science and Technology* 28: 755-760.

- Kwon, H.M. & Handler, J.S. 1995. Cell volume regulated transporters of compatible osmolytes. *Current Opinions in Cell Biology* 7: 465-471.
- Lambardi, M., De Carlo, A. & Capuana, M. 2005. Cryopreservation of embryogenic callus of *Aesculus hippocastanum* L. by vitrification/one-step freezing. *CryoLetters* 26: 185-192.
- Langis, R. & Steponkus, P. 1990. Cryopreservation of rye protoplasts by vitrification. *Plant Physiology* 92: 666-671.
- Larkin, P.J. & Scowcroft, W.R. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* 60: 197-214.
- Le Rudulier, D., Strom, A.R., Dandekar, A.M., Smith, L.T. & Valentine, R.C. 1984. Molecular biology of osmoregulation. *Science* 224: 1064-1068.
- Lee, T.S.G. 1986. Multiplication of sugarcane by apex culture. *Turrialba* 36: 231-235.
- Lee, T.S.G. 1987. Micropropagation of sugarcane (*Saccharum* spp.). *Plant Cell, Tissue and Organ Culture* 10: 47-55.
- Leopold, A.C. 1990. Coping with desiccation. In: G.J. Alscher & J.R. Cumming (eds), *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*, pp. 37-56. Wiley-Liss, New York.
- Leprince, O. & Hoekstra, F.A. 1998. The response of cytochrome redox state and energy metabolism to dehydration support a role for cytoplasmic viscosity in desiccation tolerance. *Plant Physiology* 118: 1253-1264.

- Leprince, O. & Walters-Vertucci, C. 1995. A calorimetric study of the glass transition behaviors in axes of *Phaseolus vulgaris* L. seeds with relevance to storage stability. *Plant Physiology* 109: 1471-1481.
- Leprince, O., Buitink, J. & Hoekstra, F.A. 1999. Axes and cotyledons of recalcitrant seeds of *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. *Journal of Experimental Botany* 50: 1515-1524.
- Leprince, O., Hendry, G.A.F. & McKersie, B.D. 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research* 3: 231-246.
- Leprince, O., Harren, F.J.M., Buitnik, J., Alberda, M., & Hoekstra, F.A. 2000. Metabolic dysfunction and unabated respiration precede the loss of membrane integrity during dehydration of germinating radicles. *Plant Physiology* 122: 597-608.
- Leprince, O., Van Aelst, A.C., Pritchard, H.W. & Murphy, D.J. 1998. Oleosins prevent oil-body coalescence during seed imbibition as suggested by a low-temperature scanning electron microscope study of desiccation-tolerant and -sensitive oilseeds. *Planta* 204: 109-119.
- Leunufna, S. & Keller, E.R.J. 2005. Cryopreservation of yams using vitrification modified by including droplet method: effects of cold acclimation and sucrose. *CryoLetters* 26: 93-102.
- Li, B. & Sun, D-W. 2002. Novel methods for rapid freezing and thawing of foods: a review. *Journal of Food Engineering* 54: 175-182.
- Liang, Y.H. & Sun, W.Q. 2000. Desiccation tolerance of recalcitrant *Theobroma cacao* embryonic axes: the optimal drying rate and its physiological basis. *Journal of Experimental Botany* 51: 1911-1919.

- Lindsey, K. & Jones, M.G.K. 1990. Selection of transformed cells. *In*: P.J. Dix (ed.), *Plant Cell Line Selection – Procedures and Applications*, pp. 317-339. New York.
- Litz, R.E. & Gray, D.J. 1992. Organogenesis and somatic embryogenesis. *In*: F.A. Hammerschlag & R.E. Litz (eds), *Biotechnology of Perennial Fruit Crops*, pp. 3-34. CABI International, Wallingford, U.K.
- Litz, R.E. & Gray, D.J. 1995. Somatic embryogenesis for agricultural improvement. *World Journal of Microbiology and Biotechnology* 11: 416-425.
- Liu, M.C. 1984. Sugarcane. *In*: W.H. Sharp, D.A. Evans, P.V. Ammirato & Y. Yamada (eds), *Handbook of Plant Cell Culture*, pp. 572-605. Macmillan, New York.
- Liu, M.C. & Chen, W.H. 1974. Histological studies on the origin and process of plantlet differentiation in sugarcane callus mass. *Proceedings of the International Society for Sugarcane Technology* 15: 188-128.
- Liu, F., Cao, M.Q. & Li, X.Q. 1990. Studies on storage methods and vigour change during storage of carrot artificial seeds under sterile conditions. *In*: X.Q. Li (ed.), *Studies on Artificial Seeds of Plants*, pp. 71. Beijing University, Beijing.
- Liu, M, Yeh, H. & Chan, W. 1984. Tissue and cell culture as aids to sugarcane breeding. *Taiwan Sugar* 31: 77-83.
- Lloyd, D.G. & McCown, B.H. 1981. Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot-tip culture. *Proceedings of the International Plant Propagation Society* 30: 421-427.
- Lorenzo, J.C. & Gonzalez, B.L. 1998. New sugarcane shoot formation in temporary immersion systems. *Plant Cell, Tissue and Organ Culture* 54: 35-36.

- Lovelock, J.E. 1954. The protective action of neutral solutes against haemolysis by freezing and thawing. *Journal of Biochemistry* 56: 265-270.
- Lowe, K., Taylor, D.B., Ryan, P. & Patterson, K.E. 1985. Plant regeneration via organogenesis and embryogenesis in the maize inbred line B73. *Plant Science* 41: 125-132.
- Lulsdorf, M.M., Tautorus, T.E. & Kikcio, S.I. 1993. Germination of encapsulated embryos of interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.). *Plant Cell Reports* 12: 385-389.
- Luyet, B., Tanner, J. & Rapatz, G. 1962. X-Ray diffraction study of the structure of rapidly frozen gelatin solutions. *Biodynamica* 9: 21-46.
- Lyndsey, K. & Jones, M.G.K. 1989. Consequences of tissue culture – variability and instability. In: K. Lyndsey & M.G.K. Jones (eds), *Plant Biotechnology in Agriculture*, pp. 78-93. The Biotechnology Series. Open University Press, Milton Keynes, U.K.
- Lyznik, L.A. & Hodges, T.K. 1994. Transformation of maize protoplasts. In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry, Maize*, pp. 217-240. Springer-Verlag, Berlin.
- Maheswaran, G. & Williams, E.E. 1985. Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens in vitro*. *Annals of Botany* 56: 619-630.
- Malaurie, B., Trouslout, M.F., Engelmann, F. & Chabrillange, N. 1998. Effect of pre-treatment conditions on the cryopreservation of *in vitro*-cultured yam (*Discorea alata* and *Discorea bulbifera*) shoot apices by encapsulation-dehydration. *CryoLetters* 19: 15-26.

- Mandal, B.B. 1997. Application of *in vitro*/cryopreservation techniques in conservation of horticultural crop germplasm. *Acta Horticulturae* 447: 483-489.
- Mantell, S.H., Matthews, J.A. & McKee, R.A. 1985. Cultural tools and techniques. *In: (eds), Principles of Plant Biotechnology: An Introduction to Genetic Engineering in Plants*, pp. 62-88. Blackwell Scientific Publications, Oxford, London.
- Martínez-Montero, M.E., Martínez, J. & Engelmann, F. 2008. Cryopreservation of sugarcane somatic embryos. *CryoLetters* 29: 229-242.
- Martínez-Montero, M.E., González-Arno, M.T., Borroto-Nordelo, C., Puentes-Díaz, C. & Engelmann, F. 1998. Cryopreservation of sugarcane embryogenic callus using a simplified freezing process. *CryoLetters* 19: 171-176.
- Martínez-Montero, M.E., Gonzalez-Arno, M.T., Borroto-Nordelo, C., Puentes-Diaz, C. & Engelmann, F. 2000. Cryopreservation Techniques. Cryopreservation of sugarcane embryogenic callus. *Japan International Research Centre for Agricultural Sciences* 8: 110-114.
- Martínez-Montero, M.E., Ojeda, E., Espinosa, A., Sánchez, M., Castillo, R., González-Arno, M.T., Engelmann, F. & Lorenzo, J.C. 2002. Field performance of sugarcane (*Saccharum* sp.) plants derived from cryopreserved calluses. *CryoLetters* 23: 21-26.
- Masirevec, S.N., Secor, G.A. & Gulya, T.J. 1988. Use of cell culture to screen sunflower germplasm for resistance to *Phomopsis* brown/grey stem spot. *Plant Cell Reports* 7: 528-530.
- Matsumoto, T. & Sakai, A. 1995. An approach to enhance dehydration tolerance of alginate-coated dried meristem cooled to -196°C. *CryoLetters* 16: 299-396.

- Matsumoto, T., Sakai, A. & Nako, Y. 1998. Cryopreservation of *in vitro* cultured axillary shoot tips of grape (*Vitis vinifera*) by vitrification. *Journal of the Japanese Society of Horticultural Science* 67: 27-36.
- Matsumoto, T., Sakai, A., Takahashi, C. & Yamada, K. 1995. Cryopreservation of *in vitro*-grown meristems of wasabi (*Wasabia japonica*) by encapsulation-vitrification method. *CryoLetters* 16: 189-196.
- Mazur, P. 1970. Cryobiology: the freezing of biological systems. *Science* 168: 939-949.
- Mazur, P. 1977. The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology* 14: 251-272.
- Mazur, P. 1984. Freezing of living cells: mechanisms and applications. *American Journal of Physiology, Cell Physiology* 247: C125-C142.
- Mazur, P. 1990. Equilibrium, quasi-equilibrium and non-equilibrium freezing of mammalian embryos. *Cell Biophysics* 17: 53-92.
- Mazur, P., Leibo, S.P., Chu, E.H.Y. 1972. A two-factor hypothesis of freezing injury. *Experimental Cell Research* 71: 345-355.
- McCown, B.H. & Russell, J.A. 1987. Protoplast culture in hardwoods. In: J.M. Bonga & D.J. Durzan (eds), *Specific Principles and Methods: Growth and Developments*, pp. 16-30. Martinus Nijhoff, Dordrecht.
- Meryman, H.T. 1960. The mechanisms of freezing in biological systems. In: A.S. Parkes & I.U. Smith (eds), *Recent Research in Freezing and Drying*, pp. 23-39. Blackwell Scientific Publications, Oxford.

- Meryman, H.T. & Williams, R.J. 1980. Mechanisms of freezing injury and natural tolerance and the principles of artificial cryoprotection. *In*: L.A. Withers & J.T. Williams (eds), *Crop Genetic Resources – The Conservation of Difficult Material*, pp. 5-37. International Union of Biological Sciences, Reading.
- Meryman, H.T. & Williams, R.J. 1985. Basic principles of freezing injury to plant cells: natural tolerance and approaches to cryopreservation. *In*: K.K. Kartha (eds), *Cryopreservation of Plant Cells and Organs*, pp. 13-48. CRC Press, Boca Raton, Florida.
- Miaja, M.L., Gribaudo, I., Vallania, R. & Fernandez, L.F. 2000. Low temperature storage and cryopreservation of a *Vitis vinifera* L. germplasm collection: first results. *Acta Horticulturae* 538: 177-181.
- Morgan, J.M. 1984. Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology* 35: 299-319.
- Murashige, T. 1974. Plant propagation through tissue culture. *Annual Review of Plant Physiology* 25: 135-166.
- Murashige, T. & Skoog, F. 1962. Revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.
- Mycock, D. 1999. Addition of calcium and magnesium to a glycerol and sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. *CryoLetters* 20: 77-82.
- Mycock, D.J., Blakeway, F.C. & Watt, M.P. 2003. General applicability of *in vitro* storage technology to the conservation and maintenance of plant germplasm. *South African Journal of Botany* 70: 31-36.

- Mycock, D.J., Watt, M.P. & Berjak, P. 1991. A simple procedure for the cryopreservation of hydrated embryonic axes of *Pisum sativum*. *Journal of Plant Physiology* 138: 728-733.
- Mycock, D.J., Wesley-Smith, J. & Berjak, P. 1995. Cryopreservation of somatic embryos of four species with and without cryoprotection pre-treatment. *Annals of Botany* 75: 331-336.
- Mycock, D.J., Berjak, P., Pammenter, N.W. Vertucci, C.W. 1997. Cryopreservation of somatic embryoids of *Phoenix dactylifera*. In: R.H. Ellis, M. Black, A.J. Murdoch & T.D. Hong (eds), *Basic Applied Aspects of Biology*, pp. 75-82. Kluwer Academic Publishers, Dordrecht.
- Mycock, D.J. Blakeway, F.C. & Watt, M.P. 2004. General applicability of *in vitro* storage technology to the conservation and maintenance of plant germplasm. *South African Journal of Botany* 70: 31-36.
- Nadar, H.M. & Heinz, D.J. 1977. Root and shoot development from sugarcane callus tissue. *Crop Science* 17: 814-816.
- Nadar, H.M., Soeprapto, S., Heinz, J. & Ladd, S.L. 1978. Fine structure of sugarcane (*Saccharum* sp.) callus and the role of auxin in embryogenesis. *Crop Science* 18: 210-216.
- Naidu, B.P., Aspinall, D. & Paleg, L.G. 1992. Variability in proline-accumulatory ability of barley (*Hordeum vulgare* L.) cultivars induced by vapour pressure deficit. *Plant Physiology* 98: 716-722.
- Ng, S.Y.C. & Ng, N.Q. 1991. Reduced-growth storage of germplasm. In: J.H. Dodds (ed.), *In Vitro Methods for Conservation of Plant Genetic Resources*, pp.11-35. Chapman and Hall, London.

- Nieves, N., Martínez, M.E., Castillo, R., Blanco, M.A., and González-Olmedo, J.L. 2001. Effect of abscisic acid and jasmonic acid on partial desiccation of encapsulated somatic embryos of sugarcane. *Plant Cell, Tissue and Organ Culture* 65: 15-21.
- Nieves, N., Lorenzo, J.C., Blanco, M.A., González, J., Peralta, H., Hernández, M., Santos, R., Concepción, O., Borroto, C.G., Borroto, E., Tapia, R., Martínez, M.E., Fundora, Z. & González, A. 1998. Artificial endosperm of *Cleopatra tangerine* zygotic embryos: a model for somatic embryo encapsulation. *Plant Cell, Tissue and Organ culture* 54: 77-83.
- Niino, T. & Sakai, A. 1992. Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear and mulberry. *Plant Science* 8: 199-206
- Niino, T., Sakai, A. & Nojiri, K. 1992. Cryopreservation of *in vitro* grown shoot tips of apple and pear by vitrification. *Plant Cell, Tissue and Organ Culture* 28: 261-266.
- Nishizawa, S., Sakai, A., Amano, Y. & Matsuzawa, T. 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. *Plant Science* 91: 67-73.
- Normah, M.N. & Vengadasalam, M. 1992. Effects of moisture content on cryopreservation of *Coffea* and *Vigna* seeds and embryos. *CryoLetters* 13: 199-208.
- Normah, M.N., Chin, H.F. & Hor, Y.L. 1986. Desiccation and cryopreservation of embryonic axes of *Hevea brasiliensis* Muell-Arg. *Pertanika* 9: 299-303.
- Nuti, V.R., Caligo, M.A., Nozolini, M. & Luccarini, G. 1984. Stimulation of carrot somatic embryogenesis by proline and serine. *Plant Cell Reports* 3: 210-214.

- O'Brien, S. 2001. Studies towards the cryopreservation of sugarcane somatic embryos. Honours Thesis, University of Natal, Durban, South Africa.
- Obendorf, R.L. 1997. Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. *Seed Science Research* 7: 61-74.
- Özkavukcu, S. & Erdemli, E. 2002. Cryopreservation: basic knowledge and biophysical effects. *Journal of Ankara Medical School* 24: 187-196.
- Pammenter, N.W. & Berjak, P. 1999. A review of recalcitrant seed physiology in relation to desiccation-tolerant mechanisms. *Seed Science Research* 9: 13-37.
- Pammenter, N.W., Vertucci, C.W. & Berjak, P. 1991. Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii*. *Plant Physiology* 96: 1093-1098.
- Pammenter, N.W., Berjak, P., Wesley-Smith, J. & Vander Willigen, C. 2002. Experimental aspects of drying and recovery. In: M. Black & H.W. Pritchard (eds), *Drying without Dying: Desiccation and Survival in Plants*, pp. 93-110. CABI Publishing, New York, U.S.A.
- Pammenter, N.W., Gregains, V., Kioko, J.I., Wesley-Smith, J., Berjak, P. & Finch-Savage, W.E. 1998. Effects of differential drying rates on viability retention of recalcitrant seeds of *Ekerbergia capensis*. *Seed Science Research* 8: 363-461.
- Partenen, C. R. 1963. Plant tissue culture in relation to developmental cytology. *International Review of Cytology* 15: 215-243.
- Pence, V.C. 1990. Cryostorage of embryo axes of several large-seeded temperate tree species. *Cryobiology* 27: 212-218.

- Pence, V.C. 1992. Desiccation and the survival of *Aesculus*, *Castanea* and *Quercus* embryo axes through cryopreservation. *Cryobiology* 29: 391-399.
- Pence, V.C. 1995. Cryopreservation of recalcitrant seeds. In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry 32: Cryopreservation of Plant Germplasm 1*, pp. 29-50. Springer-Verlag, Berlin.
- Perán, R., Pammenter, N.W., Naicker J. & Berjak, P. 2004. The influence of rehydration techniques on the response of recalcitrant seed embryos to desiccation. *Seed Science Research* 14: 179-184.
- Phunchindawan, M., Hirata, K., Sakai, A. & Miyamoto, K. 1997. Cryopreservation of encapsulated shoot primordia induced in horse radish (*Amoracia rusticana*) hairy root cultures. *Plant Cell Reports* 16: 469-473.
- Piccioni, E. 1997. Plantlets from encapsulated micropropagated buds of M.26 apple rootstock. *Plant Cell, Tissue and Organ Culture* 47: 255-260.
- Pitt, R. E. 1992. Thermodynamics and intracellular ice formation. In: P. Steponkus (ed.), *Advances in Low Temperature Biology*, pp. 63-99. JAI Press, London.
- Plessis, P., Leddet, C. & Dereuddre, J. 1991. Cryopreservation of alginate-coated shoot-tips of grape after progressive increase of sucrose concentration in culture medium. In: *Proceedings of the 28th Annual Meeting of the Society for Cryobiology*, Leuven, Belgium.
- Pliegoalfaro, F., Litz, R.E., Moon, P.A. & Gray, D.J. 1996. Effect of abscisic acid, osmolarity and temperature on *in vitro* development of recalcitrant mango nucellar embryos. *Plant Cell, Tissue and Organ Culture* 44: 53-61.

- Popov, A.S. 1993. Mechanisms of *in vitro* cryoinjury in plant cell cryopreservation. *Russian Journal of Plant Physiology* 40: 421-430.
- Potts, S.E. & Lumpkin, T.A. 1997. Cryopreservation of *Wasabia* spp. seeds. *CryoLetters* 18: 185-190.
- Poulsen, K.M. 1992. Sensitivity to desiccation and low temperatures (-196°C) of embryo axes from acorns of the pedunculate oak (*Quercus robur* L.). *CryoLetters* 13: 75-82.
- Pritchard, H.W. & Prendergast, F.G. 1986. Effects of desiccation and cryopreservation on the *in vitro* viability of the recalcitrant seed species *Araucaria hunsteinii* K. Shum. *Journal of Experimental Botany* 37: 1388-1397.
- Raemakers, C.J.J.M., Jacobsen, E. & Visser, R.G.F. 1995. Secondary somatic embryogenesis and applications in plant breeding. *Euphytica* 81: 93-107.
- Rao, N.K. 2004. Plant genetic resources: advancing conservation and use through biotechnology. *African Journal of Biotechnology* 3: 136-145.
- Rao, P.V.L. & Singh, B. 1991. Plantlet regeneration from encapsulated somatic embryos of hybrid *Solanum melongena* L. *Plant Cell Reports* 10: 7-11.
- Razdan, M.K. 1993. Somatic embryogenesis. In: M.K. Razdan (ed.), *An Introduction to Plant Tissue Culture*, pp. 398. Intercept, Andover, Oxford U.K. & IBH, New Delhi.
- Razdan, M.K. & Cocking, E.C. 1997. Biotechnology in conservation of genetic resources. In: M.K. Razdan & E.C. Cocking (eds), *Conservation of Plant Genetic Resources In Vitro, Volume 1: General Aspects*, pp. 1-19. Science Publishers Inc., U.S.A.

- Redenbergh, K. 1990. Application of artificial seed to tropical crops. *Horticultural Science* 25: 225-251.
- Redenbaugh, K. 1993. Introduction. In: K. Redenbaugh (ed.), *Synseeds: An Application of Synthetic Seeds to Crop Improvement*, pp. 1-3. CRC Press Inc., Boca Raton, Florida.
- Redenbaugh, K., Fujii, J.A.A. & Slade, D. 1993. Hydrated coatings for synthetic seeds. In: K. Redenbaugh (ed.), *Synseeds: Applications of Synthetic Seeds to Crop Improvement*, pp. 35-46. CRC Press Inc., Boca Raton, Florida.
- Reed, B.M. 1996. Pretreatment strategies for cryopreservation of plant tissues. In: M.N. Normah, M.K. Narimah & M.M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 73-87. Percetakan Watan, Kuala Lumpur.
- Reed, B.M. & Chang, Y. 1997. Medium and long-term storage of *in vitro* cultures of temperate fruit and nut crops. In: M.K. Razdan & E.C. Cocking (eds), *Conservation of Plant Genetic Resources In Vitro*, pp. 67-105. Science Publishers Inc., New Hampshire.
- Reed, B. M., Denoma, J., Luo, J., Chang, Y.J. & Towill, L.E. 1998. Cryopreservation and long-term storage of pear germplasm. *In Vitro Cellular & Developmental Biology - Plant* 34: 256-260.
- Reinert, J. 1973. Aspects of organisation – organogenesis and embryogenesis. In: H.E. Street (ed.), *Plant Tissue and Cell Culture*, pp. 338-355. Blackwell Scientific Publications, London.
- Reinert, J., Bajaj, Y.P.S. & Zbell, B. 1977. Aspects of organisation – organogenesis, embryogenesis, cytodifferentiation. In: H.E. Street (ed.), *Plant Tissue and Cell Culture*, pp. 398-427. Blackwell Scientific Publications, Oxford.

- Reinoud, P.J., Van Iren, F. & Kijne, W. 2000a. Cryopreservation of undifferentiated plant cells. *In: F. Engelmann & H. Takagi (eds.), Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 91-102. International Plant Genetic Resources Institute, Japan International Research Centre of Agricultural Sciences, Tsukubu, Japan.
- Reinoud, P.J., Schrijnemakers, W.M., Van Iren, F. & Kijne, J.W. 1995. Vitrification and a heat shock treatment improve cryopreservation of tobacco cell suspensions compared to two-step freezing. *Plant Cell, Tissue and Organ Culture* 42: 261-267.
- Reinoud, P.J., Versteeg, I., Kars, I., Van Iren, F. & Kijne, J.W. 2000b. Physiological and molecular changes in tobacco suspension cells during development of tolerance to cryopreservation by vitrification. *In: F. Engelmann & H. Takagi (eds.), Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 57-66. International Plant Genetic Resources Institute, Japan International Research Centre of Agricultural Sciences, Tsukubu, Japan.
- Ring, R.A. & Danks, H.V. 1994. Desiccation and cryoprotection: overlapping adaptations. *CryoLetters* 15: 181-190.
- Roberts, E.H. 1973. Predicting the viability of seeds. *Seed Science Technology* 1: 499-514.
- Roberts, E.H., King, M.W., & Ellis, R.H. 1984. Recalcitrant seeds: their recognition and storage. *In: J.H.W. Holden & J.T. Williams (eds), Crop Genetic Resources: Conservation and Evaluation*, pp. 38-52. George Allen and Unwin, London.
- Roca, W.M., Rodriguez, J.A., Mafla, G. & Roa, J.C. 1984. Procedures for recovering cassava clones, pp. 8. International Center for Tropical Agriculture, Colombia.

- Rudolph, A.S. & Crowe, J.H. 1985. Membrane stabilisation during freezing: the role of two natural cryoprotectants, trehalose and proline. *Cryobiology* 2: 367-377.
- Runthala, R., Jana, M.K. & Mohanan, K. 1993. Cryopreservation of groundnut (*Arachis hypogaea* L.) embryonic axes for germplasm conservation. *CryoLetters* 14: 323-334.
- Sakai, A. 1960. Survival of the twig of woody plants at -196°C. *Nature* 185: 393-394.
- Sakai, A. 1965. Determining the degree of frost-hardiness in highly hardy plants. *Nature* 206: 1064-1065.
- Sakai, A. 1986. Cryopreservation of germplasm of woody plants. In: Y.P.S. Bajaj (ed.), *Biotechnology in Agriculture and Forestry, Trees 1*, pp. 113-129. Springer-Verlag, Berlin.
- Sakai, A. 1995. Cryopreservation of germplasm of woody plants. In: Y.P.S. Bajaj (eds), *Biotechnology in Agriculture and Forestry*, pp. 53-69. Springer-Verlag, Heidelberg.
- Sakai, A. 1997. Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In: M.K. Razdan & E.C. Cocking (eds), *Conservation of Plant Genetic Resources In Vitro Volume 1: General Aspects*, pp. 53-66. Science Publishers Inc., Enfield, U.S.A.
- Sakai, A. 2000. Development of cryopreservation techniques. In: F. Engelmann & H. Takagi (eds.), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 1-7. International Plant Genetic Resources Institute, Japan International Research Centre of Agricultural Sciences, Tsububu, Japan.
- Sakai, A. 2004. Plant Cryopreservation. In: B.J. Fuller, N. Lane & E.E. Benson (eds), *Life in the Frozen State*, pp. 329-345. CRC Press, Boca Raton, Florida.

- Sakai, A., Kobayashi, S. & Oiyama, I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9: 30-33
- Sakai, A., Kobayashi, S. & Oiyama, I. 1991. Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var. *brasiliensis* Tanaka) cooled to -196°C. *Journal of Plant Physiology* 137: 465-470.
- Sakai, A., Niino, T., Yamada, T. & Kohmura, H. 1992. Cryopreservation by vitrification of mulberry meristems, meristematic callus of white clover and multiple bud cultures of asparagus. *Cryobiology* 29: 747-748.
- Santarius, K.A. & Franks, F. 1998. Cryopreservation of lactate dehydrogenase – interactions between various cryoprotectants. *CryoLetters* 19: 37-48.
- Sauvaire, D. & Galzy, R. 1978. Micro-propagation de la canne a sucre par bouturage *in vitro* action d'une auxine et d'une cytokinine. *Agronomia Tropical* 36: 63-69.
- Schemske, D.W., Husband, B.C., Ruckelshans, M.H., Goodwillie, C., Parker, I.M. & Bishop, J.G. 1994. Evaluating approaches to the conservation of rare and endangered plants. *Ecology* 75: 584-606.
- Schobert, B. & Tschesche, G. 1978. Unusual solution properties of proline and its interaction with proteins. *Biochimica et Biophysica Acta* 541: 270-277.
- Scocchi, A., Faloci, M., Medina, R., Olmos, S. & Mroginski, L. 2004. Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation-dehydration and assessment of their genetic stability. *Euphytica* 135: 29-38.

- Scowcroft, W.R. 1984. *Genetic Variability in Tissue Culture. Impact on Germplasm Conservation and Utilisation*. International Board for Plant Genetic Resources Report, pp. 184-152.
- Sershen, Pammenter, N.W., Berjak, P., Wesley-Smith, J. 2007. Cryopreservation of embryonic axes of selected amaryllid species. *CryoLetters* 28: 387-399.
- Shands, H.L. 1993. The U.S. national plant germplasm system. *Canadian Journal of Plant Science* 75: 9-15.
- Shatnawi, M.A., Engelmann, F., Frattarelli, A. & Damiani, C. 1999. Cryopreservation of apices of *in vitro* plantlets of almond (*Prunus dulcis* Mill.). *CryoLetters* 20: 13-20.
- Shaw, P.W., Bernard, A.G., Fuller, B.J., Hunter, J.H. & Shaw, R.W. 1992. Vitrification of mouse oocytes using short cryoprotectant exposure: effects of varying exposure times on survival. *Molecular Reproduction and Development* 33: 21-214.
- Shaw, J.M., Kola, I., MacFarlane, D.R. & Trounson, A.O. 1991. An association between chromosomal abnormalities in rapidly frozen 2-cell mouse embryos and the ice-forming properties of the cryoprotective solution. *Journal of Reproduction and Fertility* 91: 9-18.
- Shibli, R.A. & Al-Juboory, K.H. 2000. Cryopreservation of 'Nabali' olive (*Olea europea* L.) somatic embryos by encapsulation-vitrification. *CryoLetters* 21: 357-366.
- Shikama, K. & Yamazaki, I. 1961. Denaturation of catalase by freezing and thawing. *Nature* 190: 83-84.

- Shimonishi, K., Fujita, E., Kouzuma, M., Ichi, K., Ikeda, K. & Torigoe, H. 2000. Problems in sweet potato production related to 'cultivar decline' in Kagoshima Prefecture. *In: Y. Nakatani & K. Ishiguro (eds), Proceedings of International Workshop on Sweet Potato Cultivar Decline*, pp. 33-41. Miyakonojo, Miyazaki, Japan.
- Skoog, F. & Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. *Symposium of the Society for Experimental Biology* 11: 118-131.
- Smith, M.T. & Berjak, P. 1995. Deteriorative changes associated with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. *In: J. Kigel & G. Galili (eds), Seed Development and Germination*, pp. 701-746. Marcel Dekker Inc., New York.
- Smith, D.L. & Krikorian, A.D. 1990. Somatic pro-embryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. *Plant Cell Reports* 9: 468-470.
- Snyman, S.J., Meyer, G.M., Carlson, D.L. & Botha, F.C. 1996. Establishment of embryogenic callus and transient gene expression in selected sugarcane varieties. *South African Journal of Botany* 62: 151-154.
- Snyman, S.J., Watt, M.P., Hockett, B.I. & Botha, F.C. 2000a. A comparison of direct and indirect somatic morphogenesis for the production of transgenic sugarcane (*Saccharum* spp. Hybrids). Presented at The 4th International Symposium on *in vitro* Culture and Horticultural Breeding, in Tempere, Finland 2-7 July 2000.
- Snyman, S.J., Watt, M.P., Hockett, B.I. & Botha, F.C. 2000b. Direct somatic embryogenesis for rapid, cost effective production of transgenic sugarcane (*Saccharum* spp. Hybrids). *Proceedings of the South African Sugar Technologists' Association* 74: 186-187.

- Sousa, R., 1995. Use of glycerol, polyols and other protein structure stabilising agents in protein crystallisation. *Acta Crystallographica* 51: 271–277.
- Stanwood, P.C. 1985. Cryopreservation of seed germplasm for genetic conservation. In: KK Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 199-226. CRC Press Inc., Boca Raton, Florida.
- Steponkus, P.L. 1984. The role of the plasma membrane in freezing injury and cold acclimation. *Annual Review of Plant Physiology* 35: 543-584.
- Steponkus, P.L. & Lynch, D.V. 1989. Freeze/thaw-induced destabilisation of the plasma membrane and the effects of cold acclimation. *Journal of Bioenergetics and Biomembranes* 21: 21-41.
- Steponkus, P.L., Langis, R. & Fugikawa, S. 1992. Cryopreservation of plant tissues by vitrification. In: P.L. Steponkus (ed.), *Advances in Low-Temperature Biology*, pp. 1-61. JAI Press, Greenwich, Connecticut.
- Steward, F.C., Mapes, M.O. & Mears, K. 1958. Growth and organised development of cultured cells. Organisation in cultures grown from freely suspended cells. *American Journal of Botany* 45: 705-708.
- Storey, K.B. & Storey, J.M. 1996. Natural freezing survival in animals. *Annual Review of Ecology and Systematics* 27: 365-386.
- Strömquist, L. & Hansen, J. 1980. Effects of auxin and irradiance on the rooting of cuttings of *Pinus sylvestris*. *Physiologia Plantarum* 49: 346-350.

- Sudha, C.G. & Seeni, S. 2006. Spontaneous somatic embryogenesis of *in vitro* root segment cultures of *Rauvolfia micrantha* Hook. F. – a rare medicinal plant. *In Vitro Cellular Developmental Biology – Plant* 42: 119-123.
- Sugawara, Y. & Sakai, A. 1974. Survival of suspension-cultured sycamore cells cooled to temperature of liquid nitrogen. *Plant Physiology* 54: 722-724.
- Swan, T.W., Ohare, D., Gill, R.A. & Lynch, P.T. 1999. Influence of pre-culture conditions on the post thaw recovery of suspension cultures of Jerusalem Artichoke (*Helianthus tuberosus* L.). *CryoLetters* 20: 325-336.
- Sweby, D.L., Hockett, B.I. & Botha, F.C. 1994. Minimising somaclonal variation in tissue cultures of sugarcane. *Proceedings of the South African Sugar Technologists' Association* 1: 46-50.
- Taylor, W.R. 1986. The classification of amino acid conservation. *Journal of Theoretical Biology* 119: 205- 218.
- Taylor, P.W.J & Dukic, S. 1993. Development of an *in vitro* culture technique for conservation of *Saccharum* spp. Hybrid germplasm. *Plant Cell, Tissue and Organ Culture* 34: 217-222.
- Taylor, M., Pone, S. & Palupe, A. 1996. Slow growth strategies. In: M.N. Normah, M.K. Narimah & M.M. Clyde (eds), *In Vitro Conservation of Plant Genetic Resources*, pp. 119-134. Percetakan Watan, Kuala Lumpur.
- Teixeira da Silva, J.A. 2005. Simple multiplication and effective genetic transformation (four methods) of *in vitro* grown tobacco by stem thin cell layers. *Plant Science* 16: 1046-1058.

- Terzi, M. & Lo Schiavo, F. 1990. Somatic embryogenesis. *In*: S.S. Bhojwani (ed.), *Plant Tissue Culture: Applications and Limitations*, pp. 54-66. Elsevier, Amsterdam.
- Tessereau, H. 1993. Développement d'une méthode simplifiée de cryoconservation de tissus et d'embryons somatiques végétaux et étude de l'acquisition de la tolérance à la congélation. Thèse de Doctorat d' Université, Paris, France.
- Tessereau, H., Florin, B., Meschine, M. C., Thierry, C. & Pétiard, V. 1994. Cryopreservation of somatic embryos: a tool for germplasm storage and commercial delivery of selected plants. *Annals of Botany* 74: 547-555.
- Tetteroo, F.A.A., Hoekstra, F.A. & Karssen, C.M. 1995. Induction of complete desiccation tolerance in carrot (*Daucus carota*) embryoids. *Journal of Plant Physiology* 145: 349-356.
- Thierry, C., Tessereau, H., Florin, B., Meschine, M-C., Petiard, V. 1997. Role of sucrose for the acquisition of tolerance to cryopreservation of carrot somatic embryos. *CryoLetters* 18: 283-292.
- Thorpe, T.A. 1983. Morphogenesis and regeneration in tissue culture. *In*: L.D. Owens (ed.), *Genetic engineering: Applications to Agriculture*, pp. 285-303. Rowman and Allanheld Publishers, London.
- Tisserat, B. 1984. Clonal propagation: palms. *In*: I.K. Vasil & F.L. Orlando (eds), *Cell Culture and Somatic Cell Genetics of Plants. Vol. 1: Laboratory Procedures and Their Applications*, pp. 74-81. Academic Press, London.
- Tomes, D.T & Smith, O.S. 1985. The effect of parental genotype on initiation of embryogenic callus from the elite maize (*Zea mays* L.) germplasm. *Theoretical and Applied Genetics* 70: 505-509.

- Tompsett, P.B. 1987. Desiccation and storage studies on *Dipterocarpus* seeds. *Annals of Applied Biology* 110: 371-379.
- Touchell, D.H. & Walters, C. 2000. Recovery of embryos of *Zizania palustris* following exposure to liquid nitrogen. *CryoLetters* 21: 261-270.
- Towill, L.E. 1990. Cryopreservation of isolated mint shoot tips by vitrification. *Plant Cell Reports* 9: 178-180.
- Towill, L.E. 1996. Vitrification as a method to cryopreserve shoot tips. In: D.J. Gray & R.N. Trigiano (eds), *Plant Tissue Culture Concepts and Laboratory Exercises*, pp. 297-304. CRC Press, Inc., Boca Raton, Florida.
- Valdez, C.A., Abas Mazni, O., Takahashi, Y., Fujikawa, S. & Kanagawa, H. 1992. Successful cryopreservation of mouse blastocysts using a new vitrification solution. *Journal of Reproduction and Fertility* 96: 793-802.
- Vandenbussche, B. & De Proft, M.P. 1996. Cryopreservation of *in vitro* sugar beet shoot-tips using the encapsulation-dehydration technique: development of a basic protocol. *CryoLetters* 17: 137-140.
- Vardjan, M. & Nitsch, J. P. 1961. La regeneration chez *Cichorium endivia* L. *Bulletin de la Société Botanique de France* 108: 363-374.
- Vasil, I.K. 1982a. Plant cell culture and somatic cell genetics of cereals and grasses. In: I.K. Vasil, W.R. Scowcroft & K.J. Frey (eds.), *Plant Improvement and Somatic Cell Genetics*, pp. 179-203. Academic Press, New York.
- Vasil, I.K. 1982b. Somatic embryogenesis and plant regeneration in cereals and grasses. In: A. Fujiwara (ed.), *Plant Tissue Culture*, pp. 101-104. Maruzen, Tokyo.

- Vasil, I.K. 1983. Regeneration of plants from single cells of cereals and grasses. *In*: P. Le Rouin & A. Kleinhofs (eds), *Genetic Engineering of Eukaryotes*, pp. 233-252. Plenum Press, New York.
- Vasil, I.K. 1987. Developing cell and tissue culture systems for the improvement of cereal and grass crops. *Journal of Plant Physiology* 128: 193-218.
- Vasil, I.K. & Vasil, V. 1994. *In vitro* cultures of cereals and grasses. *In*: I.K. Vasil & T.A. Thorpe (eds), *Plant Cell and Tissue Culture*, pp. 293-312. Academic Publishers, Dordrecht.
- Vasil, V., Lu, C. & Vasil, I.K. 1985. Histology of somatic embryogenesis in immature embryos of maize (*Zea Mays* L.). *Protoplasma* 127: 1-8.
- Vasil, I.K., Lu, C., Ozias-Akins, P., Haydi, Z., Wang, D.Y. 1982. Somatic embryogenesis in cereals and grasses. *In*: E. Earle & Y. Demarly (eds), *Variability in Plants Regenerated from Tissue Culture*, pp. 3-21. Praeger Press, New York.
- Vertucci, C.W. 1989. The effects of low water contents on physiological activities of seeds. *Physiologia Plantarum* 77: 172-176.
- Vertucci, C.W. 1990. Calorimetric studies of the state of water in seed tissues. *Biophysical Journal* 58: 1463-1471.
- Vertucci, C.W. & Farrant, J.M. 1995. Acquisition and loss of desiccation tolerance. *In*: J. Kigel & G. Galili (eds), *Seed Development and Germination*, pp. 237-271. Marcel Dekker, New York.

- Vertucci, C.W., Berjak, P., Pammenter, N.W. & Crane, J. 1991. Cryopreservation of embryonic axes of an homeohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water. *CryoLetters* 12: 339-350.
- Vos, O. & Kaalen, M.C.A. 1965. Prevention of freezing damage to proliferating cells in tissue culture. *Cryobiology* 1: 249-260.
- Wagih, M.R.E. 1989. The establishment and use of *in vitro* tissue culture techniques for the production of pathogen and Fiji disease virus-free clones of sugarcane, pp. 191. PhD Thesis. Department of Agriculture, University of Queensland, Australia (pdf download).
- Walters, C., Wheeler, L.M. & Grotenhuis, J.M. 2005. Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* 15: 1-20.
- Walters, C., Farrant, J.M., Pammenter, N.W. & Berjak, P. 2002. Desiccation stress and damage. In: M. Black & H.W. Pritchard (eds), *Desiccation and Survival in Plants: Drying Without Dying*, pp. 263-291. CABI Publishing, Wallingford, U.K.
- Walters, C., Pammenter, N.W., Berjak, P. & Crane, J. 2001. Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research* 11: 135-148.
- Watanabe, K., Yamada, Y., Ueno, S. & Mitsuda, H. 1985. Change of freezing resistance and retention of metabolic and differentiation potentials in cultured green *Lavandula vera* cells which survived repeated freeze-thaw procedures. *Agricultural and Biological Chemistry* 49: 1727-1731.

- Waterworth, P. & Kahn, R.P. 1978. Thermotherapy and aseptic bud culture of sugarcane to facilitate the exchange of germplasm and passage through quarantine. *Plant Disease Reports* 62: 772-776.
- Weigel Jr, R.C. & Hughes, K.W. 1985. Long-term regeneration by somatic embryogenesis in barley (*Hordeum vulgare* L.) tissue cultures derived from apical meristem explants. *Plant Cell, Tissue and Organ Culture* 5: 151-162.
- Werner, O., Ros Espin, T.M., Bopp, M. & Atzorn, R. 1991. Abscisic-acid-induced drought tolerance in *Funaria hygrometrica* Hedw. *Planta* 186: 99-103.
- Wesley-Smith, J. 2002. Investigations into the Responses of axes of recalcitrant seeds to dehydration and cryopreservation. Unpublished Ph.D. Thesis, University of Natal, Durban, South Africa.
- Wesley-Smith, J., Pammenter, N.W. & Vertucci, C.W. 1995. Ultrastructural evidence for the effects of freezing in embryonic axes of *Pisum sativum* L. at various water contents. *Annals of Botany* 76: 59-64.
- Wesley-Smith, J., Pammenter, N.W., Berjak, P. & Walters, C. 2001b. The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. *Annals of Botany* 88: 653-664.
- Wesley-Smith, J., Walters, C., Berjak, P. & Pammenter, N.W. 1999. A method for the cryopreservation of embryonic axes at ultra-rapid cooling rates *In*: M. Marzalina, K.C. Khoo, N. Jayanthi, F.Y. Isan & B. Krishnapillay (eds), *IUFRO Seed Symposium 1998 "Recalcitrant seeds"*. *Proceedings of the Conference*. Kuala Lumpur, Malaysia.
- Wesley-Smith, J., Walters, C., Berjak, P. & Pammenter, N.W. 2004a. Non-equilibrium cooling of *Poncirus trifoliata* (L.) embryonic axes at various water contents. *CryoLetters* 25: 121-128.

- Wesley-Smith, J., Walters, C., Berjak, P. & Pammenter, N.W. 2004b. The influence of water content, cooling and warming rate upon survival of embryonic axes of *Poncirus trifoliata* (L.) *CryoLetters* 25(2): 129-138.
- Wesley-Smith, J., Walters, C., Pammenter, N.W. & Berjak, P. 2001a. Interactions among water content, rapid (non-equilibrium) cooling to -196°C and survival of embryonic axes of *Aesculus hippocastanum* L. seeds. *Cryobiology* 42: 196-206.
- Wesley-Smith, J., Vertucci, C.W., Berjak, P., Pammenter, N.W. & Crane, J. 1992. Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology* 140: 596-604.
- Wetherell, D.F. & Dougall, D.K. 1976. Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. *Physiologia Plantarum* 37: 97-103.
- White, P.R. 1954. *A Handbook of Plant Tissue Culture*, pp. 6-187. Ronald Press, New York.
- Whittam, J.H., & Rosano, H.L. 1973. Effects of the freeze-thaw process on alpha-amylase. *Cryobiology* 10: 240-243.
- Widholm, J.M. 1974. Control of aromatic amino acid biosynthesis in cultured plant tissues: effect of intermediates and aromatic amino acids on free levels. *Physiologia Plantarum* 30: 13-18.
- Wiest, S.C. & Steponkus, P.L. 1978. Freeze-thaw injury to isolated spinach protoplasts and its simulation at above-freezing temperatures. *Plant Physiology* 62: 699-705.

- Wilkins, C.P. & Dodds, J.H. 1983. The application of tissue culture techniques to plant genetic conservation. In: L.A. Withers & P.G. Anderson (eds), *Plant Tissue Culture and its Agricultural Applications*, pp. 259-284. Butterworths, London.
- Williams, E.G. & Maheshwaran, G. 1986. Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. *Annals of Botany* 57: 443-462.
- Withers, L.A. 1980. Low temperature storage of plant tissue cultures. In: A. Fiechter (ed.), *Advances in Biochemical Engineering/Biotechnology*, pp. 101-150. Springer-Verlag, Berlin.
- Withers, L.A. 1982. *Institutes Working on Tissue Culture for Genetic Conservation, A Revised List*, pp. 104. International Board for Plant Genetic Resources, Secretariat, Rome.
- Withers, L.A. 1985. Cryopreservation of cultured plant cells and protoplasts. In: K.K. Kartha (ed.), *Cryopreservation of Plant Cells and Organs*, pp. 243-267. CRC Press, Boca Raton, Florida.
- Withers, L.A. 1988. Germplasm preservation. In: G. Bocl & J. Marsh (eds), *Application of Plant Cell and Tissue Culture*, pp. 163-177. John Wiley and sons, Chichester, U.K.
- Withers, L.A. & Davey, M.R. 1978. A fine-structural study of the freeze-preservation of plant tissue cultures. I. The frozen state. *Protoplasma* 94: 207-219.
- Withers, L.A. & Engelmann, F. 1998. *In vitro* conservation of plant genetic resources. In: A. Altman (ed.), *Biotechnology in Agriculture*, pp. 57-79. Marcel Dekker Inc., New York.
- Withers, L.A. & King, P.J. 1979. Proline: a novel cryoprotectant for freeze-preservation of cultured cells of *Zea mays* L. *Plant Physiology* 64: 675-678.

- Witjaksono, T. & Litz, R.E. 1999. Induction and growth characteristics of embryogenic avocado (*Persea americana* Mill.) cultures. *Plant Cell, Tissue and Organ Culture* 58: 19-29.
- Wolfe, J. & Steponkus, P.L. 1983. Mechanical properties of the plasma membrane of isolated plant protoplasts. *Plant Physiology* 71: 276-285.
- Wolkers, W.F., Oldenhof, H., Alberda, M. & Hoekstra, F.A. 1998. A Fourier transform infrared microspectroscopy study of sugar glasses: application to anhydrobiotic higher plant cells. *Biochimica et Biophysica Acta* 1379: 83-96.
- Wolkers, W.F., Tetteroo, F.A.A., Alberda, M. & Hoekstra, F.A. 1999. Changed properties of the cytoplasmic matrix associated with desiccation tolerance of dried carrot somatic embryos. An *in situ* Fourier transform infrared spectroscopic study. *Plant Physiology* 120: 153-163.
- Wu, Y-J, Huan, X-L.; Xiao, J-N., Li, X-J., Zhou, M-D. & Engelmann, F. 2003. Cryopreservation of mango (*Mangifera indica* L.) embryogenic cultures. *CryoLetters* 24: 303-314.
- Yamada, T., Kuroda, K., Jitsuyama, Y., Takezawa, D., Arakawa, K. & Fujikawa, S. 2002. Roles of the plasma membrane and the cell wall in the responses of plant cells to freezing. *Planta* 215: 770-778.
- Yoshida, S., Hattanda, Y. & Suyama, T. 1993. Variations in chilling sensitivity of suspension cultured cells of mung bean (*Vigna radiata* [L.] Wilczek) during the growth cycle. *Plant Cell Physiology* 34: 673-679.

- Yoshimatsu, K., Touno, K. & Shimomura, K. 2000. Cryopreservation of medicinal plant resources: retention of biosynthetic capabilities in transformed cultures. *In*: F. Engelmann & H. Takagi (eds), *Cryopreservation of Tropical Plant Germplasm: Current Research Progress and Application*, pp. 77-88. Japan International Research Center for Agricultural Sciences, International Plant Genetic Resources Institute, Rome.
- Zimmerman, J.L. 1993. Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* 5: 1411-1423.
- Zhong, H. & Wang, F.X. 1989. Somatic embryogenesis in cell suspension culture of alfalfa roots and encapsulation of embryoids. *Chinese Journal of Botany* 1: 35.