School of Biomedical Sciences

The Role of Oxidative Stress in Successful Cryopreservation of South-west Western Australian Plant Species

Bryn Funnekotter

This thesis is presented for the Degree of

Doctor of Philosophy

of

Curtin University

February 2015

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Bryn Funnekotter

Date: 16/06/2015

Acknowledgements

First of all, I would like to express my sincerest gratitude to my supervisors Prof Ricardo Mancera, Dr Eric Bunn, Dr Anja Kaczmarczyk, Dr Shane Turner and Dr Louise Colville for their support, guidance and encouragement, and knowledge they gave me throughout my PhD. I cannot thank you enough for all your time, patience and constructive feedback, which has been invaluable to me when writing this thesis. I would also like to thank Dr Kylie Munyard for chairing my Thesis Committee.

I would like to convey my gratitude to the staff at Kings Park and Botanic Garden, the School of Biomedical Sciences and the School of Pharmacy, Curtin University, and the Royal Botanic Gardens, Kew, for all their support and help during my PhD. During the course of this thesis I was supported by an Australian Postgraduate Award, and additional funding and scholarships were received from the Minerals & Energy Research Institute of Western Australia and from Curtin University.

To the many friends I made during my PhD, it has been an honour getting to work with you all and I would like to thank everyone for all the help and advice given to me along the way.

To my family, in particular my mother and father, I would like to thank you for all the love and support you have given me, not just during my PhD, but throughout my life.

And last but not least, I would like to thank Anna Williams. Your encouragement and reassurances got me through all the difficult times in my PhD and made the journey so much more enjoyable.

Table of Contents

Declaration ..... **II**

Acknowledgements ..... **III**

Abbreviations ..... **VII**

List of Figures ..... **VIII**

List of Tables ..... **XIV**

Abstract ..... **XV**

Chapter 1 – Introduction **1**

Introduction 1

Cryopreservation 2

Cryopreservation techniques 3

Factors affecting successful cryopreservation 5

Objectives 7

Structure of the thesis 8

References 9

Chapter 2 – The Role of Oxidative Stress in the Successful Cryopreservation of Plant Species **15**

Abstract 15

Introduction 15

Oxidative stress 16

Antioxidant defence 22

Analysis of oxidative stress during cryopreservation 24

Alleviating oxidative stress during cryopreservation 25

Conclusions 26

References 27

Chapter 3 – Influence of abiotic stress factors during preconditioning on post-cryogenic survival, antioxidant enzymes and electrolyte leakage in the south-west Australian Mediterranean climate species *Lomandra sonderi* 37

Abstract.....37

Introduction.....38

Materials and methods.....41

Results.....45

Discussion.....53

References.....59

Chapter 4 – Antioxidant analysis of low-molecular weight thiols and ascorbic acid during cryopreservation of native Australian plant shoot tips.....64

Abstract.....64

Introduction.....64

Materials and methods.....68

Results.....73

Discussion.....84

References.....88

Chapter 5 – Evaluation of the new vacuum infiltration vitrification (VIV) cryopreservation technique for native Australian plant shoot tips.....94

Preface.....94

Abstract.....94

Introduction.....94

Materials and methods.....96

Results.....98

Discussion.....102

References.....109

Chapter 6 – Application of exogenous ascorbic acid and lipid peroxidation analysis in the improved protocol for the successful cryopreservation of Australian plant species *Loxocarya cinerea*.....113

Abstract.....113

Introduction.....113

Materials and methods.....115

Results.....118

Discussion.....122

References.....127

Chapter 7 – Conclusions and Future Directions.....132

Introduction.....132

Developing a successful cryopreservation protocol for *Loxocarya cinerea*.....132

Oxidative stress during cryopreservation.....133

Future work.....135

Conclusions.....137

References.....138

References.....140

Appendix.....164

Additional Publications.....164

Chapter 5 – Response to Reviewers.....165

Permissions.....170

Abbreviations

AsA,	ascorbic acid
BA,	6-benzylaminopurine
CAT,	catalase
CPAs,	cryoprotective agents
CV,	conventional vitrification
Cys,	cysteine
DW,	dry weight
FDA,	fluorescein diacetate
FW,	fresh weight
γ-Glu-Cys,	*gamma*-glutamylcysteine
GPx,	glutathione peroxidase
GR,	glutathione reductase
GSH,	glutathione
GSSG,	glutathione disulphide
H_2O_2,	hydrogen peroxide
LMW,	low molecular weight
LN,	liquid nitrogen
LPO,	lipid peroxidation
MDA,	malondialdehyde
MS,	Murashige and Skoog salts
PUFA,	polyunsaturated fatty acid
ROS,	reactive oxygen species
SOD,	superoxide dismutase
VIV,	vacuum infiltration vitrification

List of Figures

Figure 2.1. Chemical pathways of production of ROS (reproduced from Ďuračková (2014), with permission from Springer). 17

Figure 2.2. Fenton's Reaction. 18

Figure 2.3. Lipid peroxidation reaction sequence (this research was originally published in Biochemical Society Transactions. Young, I., and J. McEneny. (2001). "Lipoprotein Oxidation and Atherosclerosis." *Biochemical Society Transactions* 29: 358-362. © copyright holder). 20

Figure 2.4. Formation of DNA adducts with MDA (reproduced from Dedon et al. (1998), with copyright permission from PNAS).....21

Figure 3.1. Synergistic effect of antioxidant enzymes in the removal of reactive oxygen species. $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; H_2O_2, hydrogen peroxide. 40

Figure 3.2. Representative preconditioned *in vitro* shoots. *In vitro* shoots of *Lomandra sonderi* exposed to room temperature (A), high sucrose (B), low light (C), high light (D), alternating temperature (E), high temperature (F) preconditioning regimes for 3 weeks. 46

Figure 3.3. Survival of *L. sonderi* shoot tips. Effect of various preconditioning regimes under control (-LN) conditions. (B) Effect of droplet-vitrification protocol (+LN). Preconditioning regimes: RT, room temperature; AT, alternating temperature; HT, high temperature; LL, low light; HL, high light; HS, high sucrose. The bars represent the standard error of the means. Bars labelled with different letters have significant differences across the preconditioning regimes ($P < 0.05$).....47

Figure 3.4. Effects of various preconditioning regimes on the antioxidant enzyme activity of *L. sonderi*. The bars represent the standard error of the means Preconditioning regimes: RT, room temperature; AT, alternating temperature; HT, high temperature; LL, low light; HL, high light; HS, high sucrose.....49

Figure 3.5. Scatter plot comparing control survival, electrolyte leakage and antioxidant activity to post-cryogenic survival. Preconditioning regimes: RT, room temperature; AT, alternating temperature; HT, high temperature; LL, low light; HL, high light; HS, high sucrose. 50

Figure 3.6. Antioxidant activity of CAT, GPx and GR through the cryopreservation protocol comparing between the 3 week room temperature (RT, black bars) or alternating temperature (AT, grey bars) preconditioning regime.....52

Figure 4.1. Preconditioned *in vitro* shoots. Preconditioned *in vitro* shoots of *Anigozanthos viridis* (A), *Loxocarya cinerea* (B) and *Lomandra sonderi* (C) exposed to 25°C (I), 25/5°C day/night cycle (II) or 5°C (III) preconditioning temperatures (with a 12 h photoperiod, PFFD of ~30 µmol $m^{-2}s^{-1}$) after 3 weeks. 69

Figure 4.2. Cryopreservation protocol. Dotted arrows signify where shoot tips were analysed for antioxidant content. Adapted from Kaczmarczyk et al. (2012); originally published under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/32860 71

Figure 4.3. Survival and regeneration of *Anigozanthos viridis, Loxocarya cinerea* and *Lomandra sonderi* following cryopreservation. Survival (black bars) and regeneration (grey bars) of shoot tips before (-LN) and after (+LN) cryopreservation in *A. viridis*, *L. cinerea* and *L. sonderi*. Data shows mean ± SE. Different letters donate significant differences (One-way ANOVA Tukey's Test $P < 0.05$). 74

Figure 4.4. Analysis of total concentration of LMW thiols cysteine, *gamma*-glutamyl cysteine and glutathione for *Anigozanthos viridis*. White bars show reduced thiol concentration and black bars show corresponding disulphide thiol concentration ± SE of total thiol concentration. Red circles show the respective half-cell reduction potential of each redox couple ($E_{cystine/2Cys}$, $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$ and $E_{GSSG/2GSH}$) ± SE (right hand y-axis). Different letters indicate significant differences between treatments: black lower case letters for total thiol concentration and red upper case letters for half-cell reduction potentials (One-way ANOVA Tukey's Test $P < 0.05$). 76

Figure 4.5. Analysis of total concentration of LMW thiols cysteine, *gamma*-glutamyl cysteine and glutathione for *Loxocarya cinerea*. White bars show reduced thiol concentration and black bars show corresponding disulphide thiol concentration ± SE of total thiol concentration. Red circles show the respective half-cell reduction potential of each redox couple ($E_{cystine/2Cys}$, $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$ and $E_{GSSG/2GSH}$) ± SE (right hand y-axis). Different letters indicate significant differences between treatments: black lower case letters for total thiol concentration and red upper case letters for half-cell reduction potentials (One-way ANOVA Tukey's Test P < 0.05). 77

Figure 4.6. Analysis of total concentration of LMW thiols cysteine, *gamma*-glutamyl cysteine and glutathione for *Lomandra sonderi*. White bars show reduced thiol concentration and black bars show corresponding disulphide thiol concentration ± SE of total thiol concentration. Red circles show the respective half-cell reduction potential of each redox couple ($E_{cystine/2Cys}$, $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$ and $E_{GSSG/2GSH}$) ± SE (right hand y-axis). Different letters indicate significant differences between treatments: black lower case letters for total thiol concentration and red upper case letters for half-cell reduction potentials (One-way ANOVA Tukey's Test P < 0.05). 78

Figure 4.7. Scatterplots comparing half-cell reduction potential of the thiols $E_{cystine/2Cys}$ (A, B), $E_{bis\text{-}\gamma\text{-}Glu\text{-}Cys/2\gamma\text{-}Glu\text{-}Cys}$ (C, D), $E_{GSSG/2GSH}$ (E, F) and the overall redox environment (G, H) in the preconditioned shoot tips (A, C, E, G) and the recovered shoot tips (B, D, F, H) vs post-cryogenic survival in *Anigozanthos viridis* (black circles), *Loxocarya cinerea* (white squares) and *Lomandra sonderi* (grey triangles). Data shows mean ± SE. 80

Figure 4.8. Analysis of total ascorbic acid concentration after different steps of the cryopreservation protocol in *Anigozanthos viridis* (A), *Loxocarya cinerea* (B) and *Lomandra sonderi* (C). 25°C (black bars), 25/5°C (grey bars) and 5°C (white bars) preconditioned shoot tips at the respective cryopreservation stages shown in each graph. Data represents mean ± SE, different letters denote significant differences (One-way ANOVA, Tukey's Test P < 0.05). 82

Figure 4.9. Scatterplot comparing ascorbic acid (AsA) concentration to post-cryogenic survival and regeneration. Graph (A) shows post-cryogenic survival vs AsA concentration in the preconditioned shoot tips (closed symbols) or post-cryogenic survival vs AsA concentration in the recovered shoot tips (open symbols). Graph (B) shows the percentage change in AsA concentration between the preconditioning and recovery stages vs either post-cryogenic survival (closed symbols) or post- cryogenic regeneration (open symbols). Results show *Anigozanthos viridis* (circles)*, Loxocarya cinerea* (squares) and *Lomandra sonderi* (triangles) ± SE, dotted lines show regression line with regression values indicated. 83

Figure 5.1. Post-cryogenic regeneration. Representative shoot tip showing post-cryogenic regeneration of *Anigozanthos viridis* (A), *Lomandra sonderi* (B), *Loxocarya cinerea* (C) and *Androcalva perlaria* (D).....98

Figure 5.2. Survival and regeneration of *Anigozanthos viridis* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A and C) and after (+LN, B and D) cryopreservation comparing conventional (A and B) and vacuum infiltration (C and D) vitrification techniques. A 20 min loading solution (LS) was applied to CV treatments, while a 5 min LS +vacuum infiltration step was applied to VIV treatments. Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE. 101

Figure 5.3. Survival and regeneration of *Lomandra sonderi* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A and C) and after (+LN, B and D) cryopreservation comparing conventional (A and B) and vacuum infiltration (C and D) vitrification techniques. A 20 min loading solution (LS) was applied to CV treatments, while a 5 min LS +vacuum infiltration step was applied to VIV treatments. Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE. 102

Figure 5.4. Survival and regeneration of *Loxocarya cinerea* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A and C) and after (+LN, B and D) cryopreservation comparing conventional (A and B) and vacuum infiltration (C and D) vitrification techniques. A 20 min loading solution (LS) was applied to CV treatments, while a 5 min LS +vacuum infiltration step was applied to VIV treatments. Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE. 103

Figure 5.5. Survival and regeneration of *Androcalva perlaria* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A) and after (+LN, B) cryopreservation utilising the vacuum infiltration vitrification technique (+5 min LS step) Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE. 104

Figure 6.1. Regeneration of *Loxocarya cinerea* shoot tips 4 weeks post-cryopreservation. Comparison of regeneration of non-cryopreserved (-LN, A) and cryopreserved (+LN, B) shoot tips precultured for 2, 3, 5 and 7 days. Black bars show regeneration of control shoot tips with no ascorbic acid exposure while grey bars show regeneration of shoot tips exposed to 0.5 mM ascorbic acid during the preculture period. Different letters denote a statistically significant difference between all treatments ($P < 0.05$) in -LN or +LN. 118

Figure 6.2. MDA concentration in shoot tips 4 weeks post-cryopreservation for various preculture times. Black bars show MDA concentration of control shoot tips with no ascorbic acid exposure while grey bars show MDA concentration of shoot tips exposed to 0.5 mM ascorbic acid during the preculture period. Different letters denote a statistically significant difference between all treatments ($P < 0.05$). 119

Figure 6.3. Representative shoot tips stained with C11-BODIPY$^{581/591}$. (A) Freshly isolated shoot tip, 2 (B), 3 (C), 5 (D) and 7 (E) day precultured shoot tips 4 weeks post-cryopreservation. (I) red, (II) green and (III) merged red and green fluorescence images. 120

Figure 6.4. Analysis of C11-BODIPY$^{581/591}$ relative proportion of green fluorescence (green intensity/red+green intensity) in freshly isolated shoot tips or shoot tips 4 weeks post-cryopreservation. Comparison of 2, 3, 5 & 7 d precultured shoot tips with AsA (0.5 mM) and without AsA (control). Different letters denote a statistically significant difference between the average green fluorescence conditions ($P < 0.05$). 121

Figure 6.5. Representative shoot tips stained with FDA and DAPI. (A) Freshly isolated shoot tip, (B) dead shoot tip, (C) surviving shoot tip, (D) regenerating shoot tip 4 weeks post-cryopreservation. Scale bar represents 200 µm. 123

List of Tables

Table 2.1. Common reactive oxygen species (ROS) (modified from Halliwell and Gutteridge (2007) with permission from Oxford University Press). 16

Table 3.1. Preconditioning treatments of *L. sonderi in vitro* shoots applied 3 weeks prior to the cryopreservation experiment, determination of electrolyte leakage or antioxidant analysis 42

Table 3.2. Effect of 3 weeks preconditioning regimes. Comparison of formation of new shoots across the various preconditioning regimes. Asterisks indicate the frequency of dead leaves occurring (No asterisk = none, * = low, ** = medium, *** = high). Different letters denote significant differences across the preconditioning regimes ($P < 0.05$). 45

Table 3.3. Effects of preconditioning regimes on LT_{50} temperature (°C ± SE).....48

Table 5.1. Comparison of ice formation in DSC themographs. CV – conventional vitrification technique, VIV – vacuum infiltration vitrification technique, LS – loading solution (2 M glycerol + 0.4 M sucrose), PVS2 – plant vitrification solution 2 (30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO + 0.4 M sucrose), ND – not detected. VIV incubation applied at room temperature with 80 kPa vacuum tested on four plant species: *Anigozanthos viridis, Lomandra sonderi, Loxocarya cinerea* and *Androcalva perlaria* (n = 3).....99

Abstract

This thesis reports findings of investigations into cryopreservation of three endemic Western Australian species: *Anigozanthos viridis* ssp. *terrapectans* (Haemodoraceae), *Loxocarya cinerea* (Restionaceae) and *Lomandra sonderi* (Asparagaceae). *Anigozanthos viridis* ssp. *terraspectans* is an endangered rhizomatous perennial herb, while *L. cinerea* and *L. sonderi* are dioecious rhizomatous perennial herbs, which are important in post-mining rehabilitation programs. These species are considered recalcitrant for the rehabilitation process due to their low seed production and dormancy issues. Consequently, these species are micropropagated in large numbers and planted back into restoration sites to maintain as much species diversity as possible, thus they represent a substantial investment in the rehabilitation process.

The application of a variety of preconditioning stresses (low and high temperatures, low and high light levels, and high osmotic stress), and characterising the antioxidant activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), in shoot tips after preconditioning and throughout the cryopreservation protocol were examined with *L. sonderi*. The application of the preconditioning stress regimes reduced post-cryogenic survival in this species contrary to expectations, motivating a further detailed analysis of antioxidant levels. Antioxidant activity of SOD in the preconditioned shoot tips of *L. sonderi* showed a positive correlation to post-cryogenic survival whilst GR and CAT activity showed little correlation. Analysis of the antioxidants throughout the cryopreservation protocol showed that GR activity decreased significantly after cryopreservation, whilst GPx and CAT did not change. The high light preconditioned shoot tips showed increased antioxidant activity, with the greatest GR and GPx activity of all the preconditioning regimes assessed, but, nonetheless, shoot tips exposed to these conditions had the lowest post-cryogenic survival.

Low molecular weight (LMW) thiols (cysteine, *gamma*-glutamylcysteine and glutathione) and ascorbic acid, representing the major water-soluble antioxidants in plants, were analysed through the cryopreservation protocol in all three species. Post-cryogenic regeneration was greatest in *A. viridis* followed by *L. sonderi*, whilst *L. cinerea* did not show any post-cryogenic regeneration in this part of the study. The application of a 3 week, 5°C preconditioning regime resulted in significantly lower post-cryogenic regeneration for *A. viridis* but had little effect on the other two species.

Total antioxidant concentration decreased significantly with each step through the cryopreservation protocol, particularly in the cryoprotected and washed shoot tips. Antioxidant levels increased during the 7 day post-cryopreservation recovery period, with the greatest increase seen in *A. viridis* shoot tips. LMW thiols and their corresponding disulphides were used to calculate the half-cell reduction potentials, revealing the ability of the plants to maintain a strong reducing environment throughout the cryopreservation protocol correlating to post-cryogenic survival.

Loxocarya cinerea was analysed for lipid peroxidation damage during the recovery phase by comparing different preculture incubation times and the addition of exogenous ascorbic acid to the preculture medium, to further our understanding of the recalcitrant nature of this species during the cryopreservation process. Individual shoot tips (4 weeks post-cryopreservation) were stained using a C11-BODIPY$^{581/591}$ fluorescent probe to provide a ratiometric indication of oxidative stress. MDA formation as a by-product of lipid peroxidation indicated that lipid peroxidation was occurring, possibly as a result of oxidative stress incurred during one or more phases of the cryopreservation process.

The application of vacuum during shoot tip incubation in cryoprotective agents was also investigated (vacuum infiltration vitrification), resulting in a significantly reduced PVS2 incubation time for cryogenic survival and regeneration for four species: *A. viridis, L. cinerea*, *L. sonderi* and *Androcalva perlaria*. This method substantially reduced the time needed to adequately protect shoot tips compared to a conventional droplet-vitrification technique.

This study confirms that oxidative stress is a fundamental cause of poor post-cryopreservation performance, but the exact details of antioxidant response to preconditioning treatments in order to mitigate this oxidative stress appears to be complex and generally species specific. Vacuum assistance during the cryoprotectant treatment phase shows promise in reducing exposure times to phytotoxic cryprotectants and potentially reducing oxidative stress. As such, this thesis will greatly improve the knowledge base for the improvement of cryopreservation for conservation of indigenous flora.

Chapter 1 – Introduction

Introduction

Plants are important sources of food and shelter for terrestrial animal and human communities, and many species have cultural, horticultural or pharmaceutical significance. The South-west Western Australian Floristic Region (SWAFR) represents one of the 34 recognised global biodiversity hotspots (Myers et al. 2000). Conservation efforts in this area, which contains over 7,300 species (Hopper and Gioia 2004; Lambers 2014), are of great importance to preserve this unique terrestrial ecosystem.

Plants can be conserved through two basic methods, i.e. *in situ* conservation or *ex situ* conservation (Offord and Meagher 2009). *In situ* conservation involves maintaining plants in their natural environment through creation of reserves and parks, and protecting these against development or destructive processes with legislation, monitoring and maintenance. This is, however, not always possible due to already large losses of the natural environment caused by urban, agricultural and mining developments, as well as other threatening processes that may be largely beyond the control of land managers, such as wild fires, climate change, and exotic pests and diseases. *Ex situ* conservation approaches to mitigate against range contraction and extinction include seed banking, the establishment of container collections and arboretum, *in vitro* culture/propagation and, finally, cryopreservation.

Seed banks are the most traditional means of conserving plants and provide a cost effective method that can be applied to a wide variety of plant species (Hong et al. 1998). Over 286 endangered plant species from Western Australia have been successfully stored using seed banks, where the seeds are dried to a low moisture content and stored at -18^{o}C (Offord et al. 2009; Touchell et al. 2002). Long-term seed viability varies between species and the cost of storage increases with time, as more detailed information about the seed and the effect of the storage environment on seed viability is needed to effectively manage and store these seed resources (Hong and Ellis 1996). Seeds are classified into various categories (orthodox, intermediate and recalcitrant) dependent on their ability to remain viable during storage under different conditions and their tolerance to low seed moisture environments (Pritchard 2004). Thus, seed banks cannot be used for seeds that have low viability, unusual storage requirements (i.e. desiccation sensitive), are innately short lived or have complex dormancy mechanisms.

In vitro cultures (tissue culture) provide an alternative to seed banks and allow for a larger number of plants to be produced from a very small amount of starting material (i.e. a single seed or plant cutting) due to axillary meristem production. The cultures are also advantageous as they maintain the key genotypic features of elite genotypes, which may be lost when storing genetically variable seed accessions. While a variety of native taxa have been micropropagated at Kings Park and Botanic Garden (Touchell et al. 2002; Kaczmarczyk et al. 2011b; Bunn et al. 2011) and other botanical gardens worldwide (Reed et al. 2011), this conservation method requires constant maintenance of culture media and large amounts of space to store plant cultures, even when using growth-retardant media and cold storage approaches (Touchell et al. 2002; Kaczmarczyk et al. 2012). The cultures are also prone to pathogenic and non-pathogenic microbial contamination (Leifert et al. 1994; Leifert et al. 1991) somaclonal variation or actual alteration of the genetic code (i.e. DNA sequence changes) and epigenetic aberrations (long-lasting changes in genomic expression), all of which lead to unwanted changes and deterioration of the integrity and biosecurity of tissue culture collections over time (George and Sherrington 1993; Smulders and De Klerk 2011). Cryopreservation provides the best long-term storage solution for endangered or valuable plant species which cannot be preserved as seeds, as it allows for storage of metabolically active vegetative material without loss of viability and potentially harmful genetic changes (Kaczmarczyk et al. 2012).

Cryopreservation

Cryopreservation, or storage of material in liquid nitrogen (LN) at ultra-low temperature (-196°C), is a very efficient way of achieving long-term storage of biological samples. This is due to the arrest of all cellular metabolic processes at cryogenic temperatures. This eliminates the time and propagation cycle-induced somaclonal variation, and culture viability decline inherent in long-term tissue culture collections, as well as virtually eliminating the risk of operator introduced contamination (Bajaj 1995; Benson 2008; Day et al. 2008; Kaczmarczyk et al. 2011b; Sakai 2004). A wide variety of different plant materials can be cryopreserved, including seeds, zygotic embryos, somatic embryos, shoot tips and root tips (all containing meristems), as well as pollen, callus and cell cultures (Bajaj 1995). Since the first plant species was cryopreserved by Sakai in the 1960's (Sakai 1960), hundreds of plant species have been successfully cryopreserved, including many Australian taxa (Sakai and Engelmann 2007; Reed 2008; Ashmore et al. 2011; Reed et al. 2011).

Cells are composed of 60 – 85% water (Mazur 2004) and the main limiting factor affecting successful cryopreservation is, therefore, the formation of ice at low temperatures as this water content freezes at sub-zero temperatures. Intracellular ice formation during cryopreservation results in ice crystal damage from physical rupturing of cell membranes while increasing the concentration of excluded solutes to toxic levels (Sakai and Yoshida 1967).

Damage from ice can be minimised through vitrification, which is the phase transition of water from the liquid to an amorphous, metastable glassy state, avoiding the formation of ice crystals. The development of the 'vitrification' procedure has had a notable effect on increasing cryopreservation survival. Vitrification was proposed by Luyet (1937) to avoid the cellular and tissue damage caused by ice crystals through the use of highly concentrated viscous solutions. The formation of a high viscosity, glassy state during cryopreservation stops normal cytoplasm diffusion processes and halts chemical reactions, thereby allowing indefinite sample storage (Benson 2008).

Cryopreservation techniques

There are three main techniques used for cryopreserving samples: two-step cryopreservation, encapsulation-dehydration and vitrification (Hamilton et al. 2009). The two-step protocol involves a freeze-induced dehydration step prior to plunging the sample into LN. The samples are slowly cooled at a controlled rate as extracellular ice nucleation is induced. The extracellular ice crystals cause an osmotic imbalance resulting from a reduction in the free (liquid) water outside the cells as the crystals grow (Day et al. 2008). This desiccates the cells and causes an increase in the intracellular solute concentration, increasing the chances of glass formation when the sample is plunged into LN (Offord et al. 2009). However, it requires the cells to desiccate at a much higher level (between 10-20% final moisture content) to stop intracellular ice nucleation (Hamilton et al. 2009). This high level of desiccation can lead to an excessive solute concentration within the cell causing damage or a lethal reduction in cell volume and damage to the plasmodesmata connections during cell shrinkage (Day et al. 2008),. The two-step cryopreservation technique is not commonly used anymore with plants as better results are achieved using the more recently developed encapsulation-dehydration and vitrification technique. For example, Reinhound et al. (1995) found an increase in survival from 36% to 55% when using a vitrification technique rather than the two-step technique when cryopreserving tobacco cell cultures. Nonetheless, the two-step cryopreservation

technique remains advantageous when cryopreserving large sample numbers, as large numbers of samples can be simultaneously cryopreserved.

Fabre and Dereuddre (1990) developed the encapsulation-dehydration cryopreservation technique, which involves encapsulation of the tissue to be cryopreserved in a calcium alginate bead, incubation on a high sucrose preculture media and then desiccation of the bead (*via* constant air movement or silica gel) before plunging it into LN. The desiccation step saturates the sucrose in the beads so that a glass is formed during cooling in LN (Sakai 2004). This technique has been used to successfully cryopreserve a wide range of plant species including carrots, coffee, eucalypt, mint, sugarcane and tea (Dereuddre et al. 1988; Gonzalez-Arnao et al. 1999; Hatanaka et al. 1994; Hirai and Sakai 1999; Kuranuki and Sakai 1995; Paques et al. 2002).

The vitrification protocol is currently the most common technique used for cryopreservation as it has been used to cryopreserve >200 species (Reed 2008; Sakai and Engelmann 2007). This technique is different from both the two-step and encapsulation-dehydration techniques in that it aims to stop ice nucleation both intra- and extra-cellularly through the use of cryoprotective agents (CPAs) in a highly concentrated solution (Hamilton et al. 2009; Day et al. 2008). Fahy et al. (1984) were the first to demonstrate complete vitrification of tissues using CPAs on kidney slices. The use of CPAs started with the discovery that glycerol can protect spermatozoa from freezing injury (Polge et al. 1949), and that DMSO can be an effective CPA for preserving red blood cells (Lovelock 1953). A large variety of different molecules that are beneficial for avoiding freezing damage have been discovered since and their effects documented. Common CPAs used in plant cryopreservation include glycerol, DMSO, ethylene glycol, propylene glycol and methanol, as well as a large variety of sugars, particularly sucrose, trehalose and raffinose (Benson 2008; Kim et al. 2009b; Sakai and Engelmann 2007; Sakai et al. 1990). These agents can be penetrating or non-penetrating. Penetrating cryoprotectants cross the cell membranes and increase the solute concentration within the cell, which reduces the likelihood of intracellular ice formation (Kim et al. 2009b). Non-penetrating components do not cross the cell membranes and desiccate the cell through the creation of an osmotic imbalance, further increasing the solute concentrations within the cells (Kim et al. 2009b). The most common vitrification solution used in cryopreservation of plant species is Plant Vitrification Solution 2 (PVS2), developed by Sakai et al. (1990), which comprises of 30% glycerol (w/v), 15% DMSO (w/v), 15% ethylene glycol (w/v) and 0.4 M sucrose. Alternative vitrification solutions have been developed that may have lower toxicity

and provide greater survival depending on how the plant species reacts to the vitrification solution (Kim et al. 2009b). Pretreatment of the explants in solutions that are comprised of moderately concentrated cryoprotectants (30-40% PVS2 or PVS3), called loading solutions, before exposure to the highly concentrated vitrification solutions can improve survival through an additional step in the cryogenic process (Kim et al. 2009a). The protection mechanism imparted by the incorporation of a loading solution is not fully understood, but it is thought that the loading solution may reduce the osmotic stress when the explants are placed in the vitrification solution as cells are 'loaded' with CPAs and partially dehydrated before, thus the cells will require less desiccation to achieve vitrification (Kim et al. 2009a).

Factors affecting successful cryopreservation

There are many factors that can affect survival following cryopreservation, such as:

• Initial health of *in vitro* material

• Sensitivity of plant material to excision damage during extraction of shoot tips

• Osmotic stress incurred during desiccation phase

• Phytotoxic effects of CPA's

• Ice crystallization damage

• Potential for cell membrane damage

• Oxidative damage from ROS

Plant tissues undergoing cryogenic storage will experience all these stresses during the cryopreservation process, and the ability of the sample to withstand these stresses will ultimately determine its post-cryogenic regrowth potential (Volk and Walters 2006; Kaczmarczyk et al. 2012; Benson 2008; Hughes et al. 2012; Roach et al. 2008).

This study will investigate the health of the *in vitro* shoots and additional stressing of the shoots using abiotic preconditioning regimes prior to cryopreservation, as preconditioning has routinely been shown to improve post-cryogenic survival (Funnekotter et al. 2013; Menon et al. 2012; Gupta and Reed 2006; Chang et al. 2000; Leunufna and Keller 2005; Kaczmarczyk et al. 2010; Uchendu et al. 2013). The application of abiotic stress conditions have been shown to increase antioxidant concentrations, alter the composition of membranes and the concentration of solutes within cells, which may aid plants during the cryopreservation process (Sasaki et al.

1996; Fujikawa et al. 1999; Kamata and Uemura 2004; Funnekotter et al. 2013; Hincha and Zuther 2014; Menon et al. 2014).

It is also important to seek a balance between protection from ice damage/dehydration and toxicity. The application of CPAs is vital if successful cryopreservation protocols using vitrification approaches are to be developed; however, a major ongoing issue with CPAs is their toxicity. The concentrations of CPAs needed to fully protect cells is often toxic to plant tissues, and sensitivity to CPAs is generally species specific (Volk et al. 2006). The use of differential scanning calorimetry (DSC) in cryopreservation can help to develop cryogenic approaches for new species as it allows the observation of ice formation and glass transitions to determine the least amount of time and concentration required to provide maximum cryoprotection, thereby avoiding overexposure to potentially toxic CPAs (Dumet et al. 2000; Benson 2008; Kaczmarczyk et al. 2013).

The stresses involved in the cryopreservation process can lead to the formation of reactive oxygen species (ROS) that can disrupt the delicate redox balance of cells, leading to oxidative stress and reduced regeneration of plant samples after cryopreservation (Benson and Bremner 2004). ROS include damaging free radicals such as superoxide and hydroxyl radical, as well as non-radicals such as hydrogen peroxide and singlet oxygen, all of which can interact with and damage a wide range of cellular components such as lipids, proteins and nucleic acids (Halliwell and Gutteridge 2007). Little is known about how antioxidants are affected throughout the cryopreservation protocol or indeed whether they can be utilised to inform or improve current cryogenic approaches.

Investigating the many stages of cryopreservation for excessive stress that may contribute to a reduction in post-cryogenic survival is vital for improving our understanding. With this knowledge, improved cryopreservation protocols may be developed that may alleviate these stresses resulting in improved post-cryogenic regrowth.

Objectives

The objectives of this PhD research program were as follows:

- Develop an effective cryopreservation protocol for the plant species *Loxocarya cinerea* through the incremental assessment of various cryopreservation techniques.
- Use differential scanning calorimetry (DSC) in conjunction with the new vacuum infiltration vitrification technique to determine the optimum incubation time in CPAs to promote glass formation and reduce CPA toxicity.
- Determine the effectiveness of stimulating; through temperature, light or osmotic stress, the natural ability of plant cells to resist the effects of oxidative stress experienced during cryopreservation in conjunction with analysing the tissue's antioxidant status. It is hypothesised that increased concentration of reduced antioxidants within the cells will correlate to increased post-cryogenic survival.
- Determine the effectiveness of adding exogenous antioxidants to CPAs to increase post-cryogenic survival. The addition of exogenous enzymes is expected to reduce the formation of reactive oxygen species and boost levels of antioxidants in the cells, thereby increasing overall survival.
- Evaluate the formation of malondialdehyde (MDA) as an end product indicator of lipid peroxidation during cryopreservation. It is predicted that increased levels of MDA will correlate to reduced survival after cryopreservation as it indicates increased lipid peroxidation damage.
- Correlate outcomes to provide tangible improvements to existing cryopreservation protocols by applying antioxidants or modified cryogenic techniques that have shown to reduce oxidative stresses and improve cryogenic survival.

Structure of the thesis

This thesis is composed of six chapters written in the format of one review paper, four research papers, and finally, a general discussion, which synthesises and distils the previous five chapters:

Chapter 2 – This chapter provides a critical review on the role of oxidative stress in the cryopreservation of plants.

Chapter 3 – This chapter investigates the effect of a range of abiotic stresses (temperature, light and osmotic) during the preconditioning regime on post-cryogenic survival in *Lomandra sonderi*. Abiotic stresses were evaluated for their effects on both post-cryogenic survival and the antioxidant enzyme activity, which was measured after the preconditioning phase.

Chapter 4 – This chapter investigates the water-soluble antioxidants glutathione and ascorbic acid in three plant species (*Anigozanthos viridis, Loxocarya cinerea* and *Lomandra sonderi*) throughout different stages in the cryopreservation protocol.

Chapter 5 – This chapter examines the effect of applying a vacuum to shoot tips during their incubation in CPAs to increase the rate of infiltration and effectiveness.

Chapter 6 – This chapter focuses on developing a successful cryopreservation protocol for *L. cinerea*, and investigates an extended preculture time and the addition of ascorbic acid to the preculture medium. Recovering shoot tips were analysed for MDA formation and the C11-BODIPY$^{581/591}$ probe was used as an indicator of oxidative stress.

A bibliography of cited works is provided at the conclusion of each chapter with an overall reference list included at the end of the thesis.

References

Ashmore, S.E., K.N. Hamilton, and C.A. Offord. 2011. "Conservation Technologies for Safeguarding and Restoring Threatened Flora: Case Studies from Eastern Australia." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 99-109

Bajaj, Y.P.S. 1995. "Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity." In *Biotechnology in Agriculture and Forestry 32*, ed. Y.P.S. Bajaj, 1-18. New York: Springer.

Benson, E.E. 2008. "Cryopreservation Theory." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 15-32. New York: Springer.

Benson, E.E., and D. Bremner. 2004. "Oxidative Stress in the Frozen Plant: A Free Radical Point of View." In *Life in the Frozen State*, eds B.J. Fuller, N. Lane and E.E. Benson, 206–241. Boca Raton, Florida: CRC Press.

Bunn, E., S.R. Turner, and K.W. Dixon. 2011. "Biotechnology for Saving Rare and Threatened Flora in a Biodiversity Hotspot." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 188-200

Chang, Y., R.E. Barker, and B.M. Reed. 2000. "Cold Acclimation Improves Recovery of Cryopreserved Grass *(Zoysia* and *Lolium* Sp.)." *CryoLetters* 21 (2): 107-116

Day, J.G., K.C. Harding, J. Nadarajan, and E.E. Benson. 2008. "Cryopreservation: Conservation of Bioresources at Ultra Low Tempratures." In *Molecular Biomethods Handbook*, eds J Walker and R Rapley, 917-947. Hertfordshire UK: Humana Press.

Dereuddre, J., J. Fabre, and Basaglia. 1988. "Resistance of Alginate-Coated Somatic Embryos of Carrot (*Daucus Carota* L.) to Desiccation and Freezing in Liquid Nitrogen." *Plant Cell Reports* 7: 170-173

Dumet, D., W. Block, R. Worland, B.M. Reed, and E.E. Benson. 2000. "Profiling Cryopreservation Protocols for *Ribes Ciliatum* Using Differential Scanning Calorimetry." *CryoLetters* 21 (6): 367-378

Fabre, J., and J. Dereuddre. 1990. "Encapsulation Dehydration: A New Approach to Cryopreservation of *Solanum* Shoot-Tips." *CryoLetters* 11: 413-426

Fahy, G.M., D.R. Mac Farlane, C.A. Angell, and H.T. Meryman. 1984. "Vitrification as an Approach to Cryopreservation." *Cryobiology* 21 (4): 407-426

Fujikawa, S., Y. Jitsuyama, and K. Kuroda. 1999. "Determination of the Role of Cold Acclimation-Induced Diverse Changes in Plant Cells from the Viewpoint of Avoidance of Freezing Injury." *Journal of Plant Research* 112 (2): 237-244

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

George, E.F., and P.D. Sherrington. 1993. *Plant Propagation by Tissue Culture, Part 1. The Technology*. Edington UK: Exegentics.

Gonzalez-Arnao, M.T., C. Urra, F. Engelmann, R. Ortiz, and C. de la Fe. 1999. "Cryopreservation of Encapsulated Sugarcane Apices: Effect of Storage Temperature and Storage Duration." *CryoLetters* 20 (6): 347-352

Gupta, S., and B.M. Reed. 2006. "Cryopreservation of Shoot Tips of Blackberry and Raspberry by Encapsulation-Dehydration and Vitrification." *CryoLetters* 27 (1): 29-42

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Hamilton, K.N., S.R. Turner, and S.E. Ashmore. 2009. "Cryopreservation." In *Plant Germplasm Conservation in Australia*, eds C.A. Offord and P.F. Meagher, 129-148. Sydney: Australian Network for Plant Conservation Inc.

Hatanaka, T., T. Yasuda, T. Yamaguchi, and A. Sakai. 1994. "Direct Regrowth of Encapsulated Somatic Embryos of Coffee (*Coffea Canephora*) after Cooling in Liquid Nitrogen." *CryoLetters* 15: 47-52

Hincha, D., and E. Zuther. 2014. "Introduction: Plant Cold Acclimation and Freezing Tolerance." In *Plant Cold Acclimation*, eds Dirk K. Hincha and Ellen Zuther, 1-6. Springer New York.

Hirai, D., and A. Sakai. 1999. "Cryopreservation of in Vitro-Grown Axillary Shoot-Tip Meristems of Mint *(Mentha Spicata* L.) by Encapsulation Vitrification." *Plant Cell Reports* 19 (2): 150-155

Hong, T.D., and R.H. Ellis. 1996. *A Protocol to Determine Seed Storage Behaviour*. Edited by IPGRI Technical Bulletin No. 1. Rome, Italy: International Plant Genetic Resources Institute.

Hong, T.D., S. Linington, and R.H. Ellis. 1998. *Compendium of Information on Seed Storage Behaviour* UK: Kew Publishing.

Hopper, S.D., and P. Gioia. 2004. "The Southwest Australian Floristic Region: Evolution and Conservation of a Global Hot Spot of Biodiversity." *Annual Review of Ecology, Evolution, and Systematics*: 623-650

Hughes, Z.E., A.E. Mark, and R.L. Mancera. 2012. "Molecular Dynamics Simulations of the Interactions of Dmso with Dppc and Dopc Phospholipid Membranes." *The Journal of Physical Chemistry B* 116 (39): 11911-11923

Kaczmarczyk, A., B. Funnekotter, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., B. Funnekotter, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for *Loxocarya Cinerea*-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519

Kaczmarczyk, A., A. Houben, E. Keller, and M.F. Mette. 2010. "Influence of Cryopreservation on the Cytosine Methylation State of Potato Genomic DNA." *CryoLetters* 31 (5): 380-391

Kaczmarczyk, A., S.R. Turner, E. Bunn, R.L. Mancera, and K.W. Dixon. 2011b. "Cryopreservation of Threatened Native Australian Species—What Have We Learned and Where to from Here?" *In Vitro Cellular & Developmental Biology - Plant* 47 (1): 17-25

Kamata, T., and M. Uemura. 2004. "Solute Accumulation in Wheat Seedlings During Cold Acclimation: Contribution to Increased Freezing Tolerance." *CryoLetters* 25 (5): 311-322

Kim, H.H., Y.G. Lee, S.U. Park, S.C. Lee, H.J. Baek, E.G. Cho, and F. Engelmann. 2009a. "Development of Alternative Loading Solutions in Droplet-Vitrification Procedures." *CryoLetters* 30 (4): 291-299

Kim, H.H., Y.G. Lee, D.J. Shin, H.C. Ko, J.G. Gwag, E.G. Cho, and F. Engelmann. 2009b. "Development of Alternative Plant Vitrification Solutions in Droplet-Vitrification Procedures." *CryoLetters* 30 (5): 320-34

Kuranuki, Y., and A. Sakai. 1995. "Cryopreservation of in Vitro-Grown Shoot Tips of Tea (*Camellia Sinensis*) by Vitrification." *CryoLetters* 16 (6): 345-352

Lambers, H. 2014. *Plant Life on the Sandplains in Southwest Australia: A Global Biodiversity Hotspot*: University of Western Australia Press.

Leifert, C., C.E. Morris, and W.M. Waites. 1994. "Ecology of Microbial Saprophytes and Pathogens in Tissue-Culture and Field-Grown Plants - Reasons for Contamination Problems in Vitro." *Critical Reviews in Plant Sciences* 13 (2): 139-183

Leifert, C., J. Ritchie, and W. Waites. 1991. "Contaminants of Plant-Tissue and Cell Cultures." *World Journal of Microbiology and Biotechnology* 7 (4): 452-469

Leunufna, S., and E.R. Keller. 2005. "Cryopreservation of Yams Using Vitrification Modified by Including Droplet Method: Effects of Cold Acclimation and Sucrose." *CryoLetters* 26 (2): 93-102

Lovelock, J.E. 1953. "Het Mechanism of the Protective Action of Glycerol against Haemolysis by Freezing and Thawing." *Biochimica et Biophysica Acta* 11: 28-36

Luyet, B.J. 1937. "The Vitrification of Organic Colloids and of Protoplasm." *Biodynamica* 1 (29): 1-14

Mazur, P. 2004. "Priciples of Cryobiology." In *Life in the Frozen State*, eds B.J. Fuller, N. Lane and E. E. Benson, 3-65. Boca Raton, Florida: CRC Press.

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. da Fonseca, and J. Kent. 2000. "Biodiversity Hotspots for Conservation Priorities." *Nature* 403 (6772): 853-858

Offord, C.A., E. Bunn, S.R. Turner, K.D. Sommerville, J. Seimon, and S.E. Ashmore. 2009. "Tissue Culture." In *Plant Germplasm Conservation in Australia*, eds

C.A. Offord and P.F. Meagher, 109-128. Sydney: Australian Network for Plant Conservation Inc.

Offord, C.A., and P.F. Meagher. 2009. *Plant Germplasm Conservation in Australia*. Edited by C.A. Offord and P.F. Meagher. Sydney: Australian Network for Plant Conservation Inc.

Paques, M., V. Monod, M. Poissonnier, and J. Dereuddre. 2002. "Cryopreservation of *Eucalyptus* Sp Shoot Tips by the Encapsulation-Dehydration Procedure." In *Cryopreservation of Plant Germplasm Ii*, eds L. E. Towill and YPS Bajaj, 234-245. Berlin: Springer-Verlag Berlin.

Polge, C., A.U. Smith, and A.S. Parkes. 1949. "Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures." *Nature* 164 (4172): 666-666

Pritchard, H.W. 2004. "Classification of Seed Storage 'Types' for Ex Situ Conservation in Relation to Temperature and Moisture." In *Ex Situ Plant Conservation: Supporting Species Survival in the Wild*, eds E. O. Guerrant, K. Havens and M. Maunder, 139-161. Washington, DC: Island Press.

Reed, B.M. 2008. "Cryopreservation - Practical Considerations." In *Plant Cryopreservation. A Practical Guide*, ed. B.M. Reed, 3-13. New York: Springer.

Reed, B.M., V. Sarasan, M. Kane, E. Bunn, and V.C. Pence. 2011. "Biodiversity Conservation and Conservation Biotechnology Tools." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 1-4

Reinhoud, P.J., E.W.M. Schrijnemakers, F. Iren, and J.W. Kijne. 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 (3): 261-267

Roach, T., M. Ivanova, R.P. Beckett, F.V. Minibayeva, I. Green, H.W. Pritchard, and I. Kranner. 2008. "An Oxidative Burst of Superoxide in Embryonic Axes of Recalcitrant Sweet Chestnut Seeds as Induced by Excision and Desiccation." *Physiologia Plantarum* 133 (2): 131-139

Sakai, A. 1960. "Survival of the Twig of Woody Plants." *Nature, London* 185 (4710): 393-4

Sakai, A. 2004. "Plant Cryopreservation." In *Life in the Frozen State*, eds B.J. Fuller, N Lane and E. E. Benson, 329-345. Boca Raton, Florida: CRC Press.

Sakai, A., and F. Engelmann. 2007. "Vitrification, Encapsulation-Vitrification and Droplet-Vitrification: A Review." *CryoLetters* 28 (3): 151-72

Sakai, A., S. Kobayashi, and I. Oiyama. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb. Var. Brasiliensis Tanaka) by Vitrification." *Plant Cell Reports* 9 (1): 30-33

Sakai, A., and S. Yoshida. 1967. "Survival of Plant Tissue at Super-Low Temperature Vi. Effects of Cooling and Rewarming Rates on Survival." *Plant Physiology* 42 (12): 1695-1701

Sasaki, H., K. Ichimura, and M. Oda. 1996. "Changes in Sugar Content During Cold Acclimation and Deacclimation of Cabbage Seedlings." *Annals of Botany* 78 (3): 365-369

Smulders, M., and G. De Klerk. 2011. "Epigenetics in Plant Tissue Culture." *Plant Growth Regulation* 63 (2): 137-146

Touchell, D.H., S.R. Turner, E. Bunn, and K.W. Dixon. 2002. "Cryostorage of Stomatic Tissues of Endangered Australian Species." In *Biotechnology in Agriculture and Forestry 50: Cryopreservation of Plant Germplasm Ii*, eds LE Towill and YPS Bajaj. New York: Springer.

Uchendu, E.E., M.R. Shukla, B.M. Reed, and P.K. Saxena. 2013. "Melatonin Enhances the Recovery of Cryopreserved Shoot Tips of American Elm (*Ulmus Americana* L.)." *Journal of Pineal Research* 55 (4): 435-442

Volk, G.M., J.L. Harris, and K.E. Rotindo. 2006. "Survival of Mint Shoot Tips after Exposure to Cryoprotectant Solution Components." *Cryobiology* 52 (2): 305-308

Volk, G.M., and C. Walters. 2006. "Plant Vitrification Solution 2 Lowers Water Content and Alters Freezing Behavior in Shoot Tips During Cryoprotection." *Cryobiology* 52 (1): 48-61

Chapter 2 – The Role of Oxidative Stress in the Successful Cryopreservation of Plant Species

Abstract

Cryopreservation is an important conservation technique for the long-term storage of valuable plant material. However, cells and tissues experience a wide range of stresses during cryopreservation that will affect their capacity to survive and recover following cryogenic storage. Oxidative stress is a complex phenomenon whereby excessive production of reactive oxygen species (ROS) or reduced antioxidant activity can overwhelm the antioxidant defences of the cell leading to tissue damage and cell death. Understanding where and when oxidative stress occurs, as well as the ability of plants to neutralise this stress during cryopreservation is vital for addressing the current constraints limiting the uptake of this technology, and for developing new and insightful approaches for the successful cryogenic storage of new and diverse plant species.

Introduction

Advances in cryopreservation biotechnology have resulted in the development of efficient long-term storage methods for the preservation of genetically important plant material. Cryopreservation techniques have been developed for a wide range of species and plant tissues, such as dormant buds, seeds, embryos, somatic embryos, shoot tips and callus cultures (Bajaj 1995). While this large range of plant material is cryo-capable, regrowth after cryopreservation can be highly variable not only between plant species, but also within clonal lines of the same plant species, and is significantly affected by the choice of plant material and the health of the tissue prior to cryopreservation (Kaczmarczyk et al. 2012). Samples experience a wide range of stresses during the cryopreservation process, and the ability to neutralise these stresses will determine the rate of post-cryogenic survival.

All aerobic cells exist in a delicate balance between the formation of reactive oxygen species (ROS) and the scavenging of excessive ROS with antioxidants, referred to as the redox environment. Previous work has shown that the cryopreservation process affects this delicate balance (Uchendu et al. 2010a; Uchendu et al. 2010b; Roach et al. 2008; Benson and Bremner 2004). If excessive ROS are produced or the antioxidant defences are damaged, then plant tissues are unlikely to survive.

Increasing our understanding of the processes behind the formation of ROS, the ability of a species to neutralise ROS production, and procedures that can be used to neutralise excessive oxidative damage that may occur during cryopreservation, is vital for improving current cryopreservation methods.

Oxidative stress

ROS are important in respiration, photosynthesis, biotic defence and act as signalling molecules, and without them aerobic life could not exist (Halliwell and Gutteridge 2007). A diverse group of molecules are classified as ROS, including free radicals and non-radical reactive oxygen derivatives (Table 2.1).

ROS production

ROS are frequently produced as by-products during cellular metabolism and photosynthesis in plants. The electron transport chain used in respiration and photosynthesis are the major producers of ROS, caused by the leakage of free electrons onto O_2, resulting in the formation of the superoxide radical ($O_2^{\bullet-}$) (Halliwell and Gutteridge 2007; Benson and Bremner 2004; Benson 1990). Many of the other ROS can then be produced as subsequent reactions of superoxide (Fig 2.1) (Ďuračková 2014).

Table 2.1. Common reactive oxygen species (ROS) (modified from Halliwell and Gutteridge (2007) with permission from Oxford University Press).

Radicals	**Non-Radicals**
Superoxide ($O_2^{\bullet-}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl (OH•)	Peroxynitrite ($ONOO^-$)
Hydroperoxyl (OOH•)	Peroxynitrous acid (ONOOH)
Peroxyl (ROO•)	Hypochlorous acid (HOCl)
Alkoxyl (RO•)	Hypobromous acid (HOBr)
Carbonate ($CO_3^{\bullet-}$)	Ozone (O_3)
Carbon dioxide ($CO_2^{\bullet-}$)	Singlet oxygen ($^1\Delta g$)
Singlet oxygen ($^1\Sigma g^+$)	

The most reactive ROS commonly found in plants are the superoxide ($O_2^{\bullet-}$), hydroxyl radical (OH•), hydroperoxyl (OOH•) radicals as well as singlet oxygen ($1\Sigma g^+$). The formation of superoxide and singlet oxygen often occurs as by-products of the electron transport chain from both metabolism and photosynthesis, while the highly reactive hydroperoxyl and hydroxyl radicals are commonly formed in a process called Fenton's reaction (Fig 2.2), whereby hydrogen peroxide is converted into the hydroxyl or hydroperoxyl radical.

As photosynthetic organisms, plants have an additional source of oxidative stress, as not only will the metabolic pathways expose these organisms to ROS, but the photosynthetic pathways also utilise ROS for the production of carbohydrates from CO_2. The reaction centres (PSI & PSII) are the major generation sites of ROS within the chloroplasts as the formation of ROS is critical for the transfer of energy and electrons during photosynthesis (Fryer et al. 2002; Halliwell and Gutteridge 2007; Foyer et al. 1994). The overproduction of ROS can have detrimental effects on

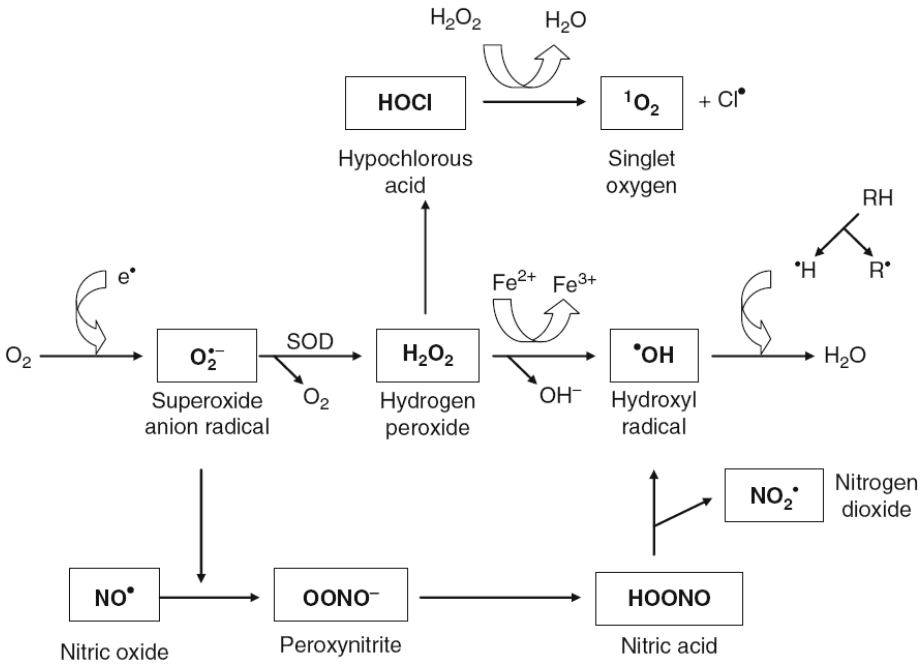

Figure 2.1. Chemical pathways of production of ROS (reproduced from Ďuračková (2014), with permission from Springer).

photosynthetic rate, enzymes and membranes of the chloroplast. As a consequence, high levels of antioxidants and ROS scavengers are found within the chloroplast to control the levels of ROS and reduce their capacity to cause significant biochemical damage (Foyer and Noctor 2009; Foyer et al. 1994).

ROS damage

Under normal conditions, cells are very efficient in preventing ROS damage. However, as plants are sessile and cannot move to escape from stressful environmental conditions such as high temperature, intense light, drought and xenobiotic contamination, which may increase ROS production, these stressful conditions may overwhelm the antioxidant defences leading to cellular damage and plant death (Farrugia and Balzan 2012). Damage caused by ROS is difficult to quantify as these molecules are non-specific in their interactions, reacting freely with lipids, proteins and DNA. ROS are highly reactive (and, therefore, by their very nature are short-lived), and thus, direct measurement of ROS present in cells is difficult and does not accurately reflect the damage that may be done prior to the ROS being quenched by antioxidants. Consequently, it is easier to measure the formation of the by-products of oxidative damage or the antioxidant state of the cells. The ratio of oxidised-to-reduced antioxidants is a good indicator of ROS formation and the ability of cells to regulate oxidative stress. The identification of oxidation end products is an indication of the damage caused, and is a sign that cells have been unable to satisfactorily quench ROS activity, but does not necessarily imply irreversible oxidative damage.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\bullet + H^+$$

Figure 2.2. Fenton's Reaction.

Oxidative damage of DNA

ROS can affect both purine and pyrimidine bases of DNA as well as the deoxyribose sugar backbone (Halliwell and Gutteridge 2007). While not all ROS can damage DNA, it is nevertheless highly susceptible to the hydroxyl radical. This radical indiscriminately oxidises the sugars, pyrimidines and purines, resulting in reduced genetic stability. DNA damage by ROS is commonly found where carcinogenesis, ageing and mutagenesis damage has occurred. While ROS damage to DNA is commonly detected, it is not clear if this type of damage is a major cause of ROS-induced cell death, as it is repairable with base excisions (Halliwell and Gutteridge 2007; Avery 2014). However, DNA repair mechanisms may become overwhelmed resulting in damage beyond repair (Avery 2014; Lowe 2014). As the main aim of cryopreservation is to preserve genetic integrity of the samples for long term storage, even single base mutations may be detrimental and should be avoided.

Little variation in genetic integrity after cryopreservation has been shown in a range of plant species including apple, grape, kiwi and potato (Zarghami et al. 2008; Hao et al. 2002; Zhai et al. 2003; Harding 2004). However, methylation changes have been linked to reduced post-cryogenic regrowth rates (Johnston et al. 2007; Kaity et al. 2013; Kaczmarczyk et al. 2010). Analysis of genetic integrity generally utilises recovering shoot tips that show post-cryogenic growth, and consequently this will preferentially exclude any shoot tips that may have experienced excessive DNA oxidation or damage as they are likely to have undergone apoptosis. While plants naturally eradicate significant genetic modifications through apoptosis, it may nevertheless still account for a reduction in post-cryogenic regeneration of plant material where DNA damage is relatively minor (Harding et al. 2009).

Oxidative damage of lipids

It has long been known that the cell membrane represents one of the major places where cryogenic injury can occur. Any damage to cellular membranes can alter the delicate balance between intra- and extracellular solutes, leading to cell death (Anchordoguy et al. 1987; Dowgert and Steponkus 1984; Gordon-Kamm and Steponkus 1984; Lynch and Steponkus 1987). Lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFA) in phospholipids can cause extensive damage to membranes if the chain reaction is not controlled, leading to large areas where the semi-permeability of the membrane is altered and can thus no longer function normally, possibly leading to apoptosis (Halliwell and Gutteridge 2007; Benson et al. 1992; Møller et al. 2007; Avery 2014). LPO is caused when specific ROS (hydroxyl

and peroxyl radicals and singlet oxygen) interact with a PUFA (Figure 2.3) (Young and McEneny 2001).

LPO can result in the formation of unstable lipid hydroperoxides and aldehydes, such as malondialdehyde (MDA) and 4-hydroxylnonenal (HNE), which can also cause damage to cells (Halliwell and Gutteridge 2007; Lowe 2014). The toxicity of MDA is debatable (Halliwell and Gutteridge 2007); however, there is evidence that MDA can interact with proteins and DNA (Fig 2.4), causing loss of function in proteins and mutations in DNA (Hipkiss et al. 1997; Marnett 1999a; Voulgaridou et al. 2011).

Increased electrolyte leakage after cryopreservation in sugarcane callus tissue was taken as an indicator of membrane damage, and these samples also showed increased MDA formation during the first two days post-cryopreservation indicating the presence of LPO stress (Martinez-Montero et al. 2002). MDA has also been linked to reduced post-cryogenic survival in rice and blackberry (Benson et al. 1992; Uchendu et al. 2010a), while the application of melatonin during cryopreservation

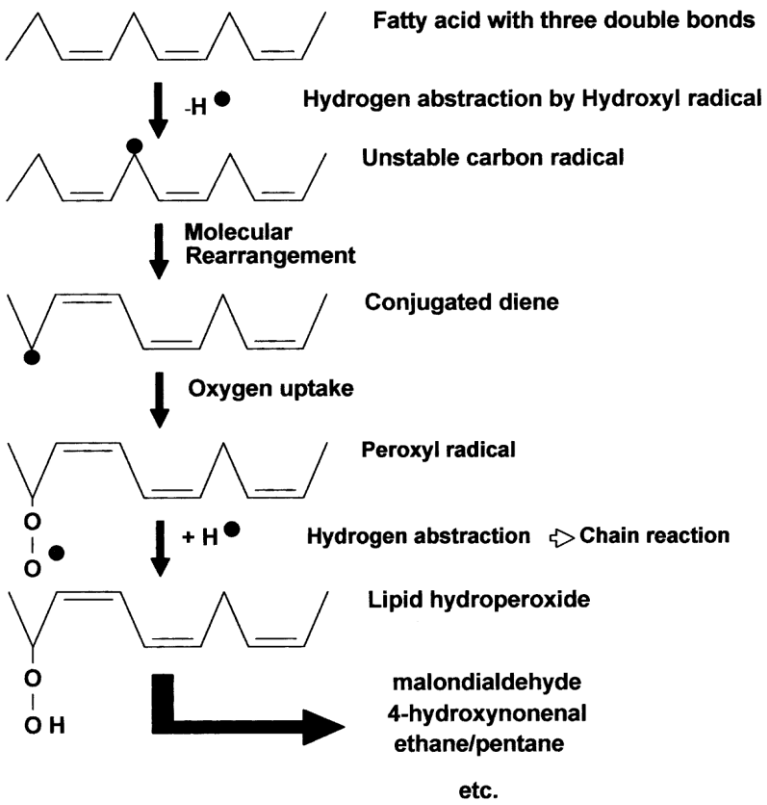

Figure 2.3. Lipid peroxidation reaction sequence (this research was originally published in Biochemical Society Transactions. Young, I., and J. McEneny. (2001). "Lipoprotein Oxidation and Atherosclerosis." *Biochemical Society Transactions* 29: 358-362. © copyright holder).

of *Rhodiola crenulata* reduced the formation of MDA in all stages of the cryogenic process (Zhao et al. 2011). Similar findings were obtained with the addition of ascorbic acid during cryopreservation of wheat and blackberry tissues (Fretz and Lörz 1995; Uchendu et al. 2010a).

Oxidative damage of proteins

Proteins are susceptible to a wide range of oxidative damage. They can be damaged through peroxiredoxin inactivation, formation of methionine sulphoxide, oxidation of side-chain carbonyls, destruction of Fe-S clusters, glutathionylation and glycation, as well as 'secondary damage' from the end-products of lipid peroxidation such as MDA (Halliwell and Gutteridge 2007). Newly synthesised and metabolic proteins are reported to be particularly sensitive to oxidative damage (Avery 2014), thus, during the recovery phase in cryopreservation, oxidative damage may be a significant cause of low survival and reduced regeneration. Indeed Cao et al. (2003) showed a decrease in the heat-shock protein 90 in spermatozoa due to the stresses of cryopreservation, while analysis of carrot somatic embryos linked the formation of polypeptides that stabilise proteins to better survival after freezing stress (Thierry et al. 1999).

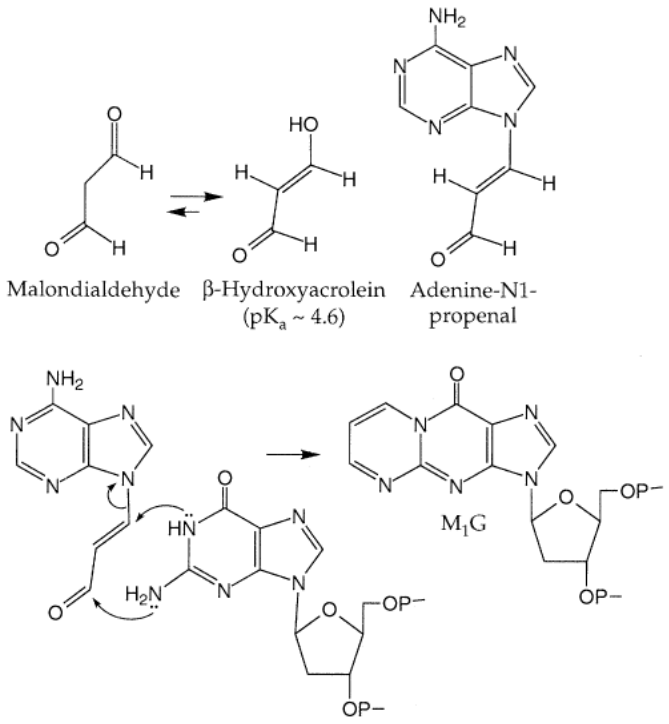

Figure 2.4. Formation of DNA adducts with MDA (reproduced from Dedon et al. (1998), with copyright permission from PNAS)

Antioxidant defence

Antioxidants are defined as molecules that “delay, prevent or removes oxidative damage to target molecules” (Halliwell and Gutteridge 2007), and are vital cellular molecules. Without antioxidants, ROS production would not be controlled and regulated, and complete oxidation of sensitive tissues and molecules would therefore occur. Antioxidants can be classified into two major groups: enzymes that catalytically remove ROS and sacrificial antioxidant molecules that are preferentially oxidised to protect more important molecules by intercepting and quenching ROS (Halliwell and Gutteridge 2007b).

Antioxidant enzymes

Superoxide dismutase, catalase and peroxidases are the primary antioxidant enzymes involved in neutralising ROS before they interact with and damage DNA, lipids and proteins. These enzymes are essential in the removal of superoxide, hydrogen peroxide, aldehydes and lipid peroxides. Indeed it was the discovery of copper zinc superoxide dismutase (CuZnSOD) by McCord and Fridovich (1968) that stimulated interest in free radicals, ROS and neutralisation of these damaging molecules by antioxidants.

The enzyme superoxide dismutase (SOD) catalyses the dismutation of superoxide to oxygen and hydrogen peroxide. Catalase (CAT) or peroxidases will then further break down hydrogen peroxide into water, acting in synergy with SOD for the removal of ROS. Extensive analysis of these antioxidant enzymes have shown increased activity when plants are exposed to environmental stresses, such as cold temperatures, intense light conditions, and drought and high salinity conditions (Tsang et al. 1991; McKersie et al. 1993; Cakmak and Marschner 1992; Gupta et al. 1993; van Camp et al. 1996; Meloni et al. 2003; Zhang and Kirkham 1994; Kim et al. 2014; Xu et al. 2014). However, it is not as simple as suggesting that increased antioxidant activity will result in better stress tolerance, or alternatively, that the application of an abiotic stress (temperature, light, osmotic) to a particular plant species will result in increased antioxidant activity. Extreme abiotic stress in sensitive plants may damage their endogenous antioxidants resulting in decreased activity (Airaki et al. 2012; Shu et al. 2011; Mengutay et al. 2013), or the stress applied may show an initial increase in antioxidant activity that decreases over time when analysed over an extended period (Zhang and Kirkham 1994; Naraikina et al. 2014). Indeed, Malan et al. (1990) showed that the synergistic effect between increased SOD and glutathione reductase (GR)

activity was required for increased tolerance to drought and photooxidative stress in maize.

Sacrificial antioxidants

Sacrificial antioxidants are preferentially oxidised and form stable radicals that will no longer interact with DNA, proteins or lipids. The three major sacrificial antioxidants in plant cells are ascorbic acid (AsA, ascorbate, Vitamin C) and glutathione, which are water soluble, and tocopherol (Vitamin E), which is hydrophobic and protects membranes from LPO. These three antioxidants have been shown to scavenge a wide range of ROS like $O_2^{\bullet-}$, $HO_2^{\bullet}$, $OH^{\bullet}$, $^1O_2$, HOCl, and H_2O_2 (Halliwell and Gutteridge 2007; Foyer et al. 2008; Noctor et al. 2012). Plants contain additional molecules that protect against ROS, such as carotenoids and flavonoids. Caroteniod pigments are primarily found in chloroplasts and absorb excessive energy from chlorophylls, preventing the formation of superoxide radicals (Halliwell and Gutteridge 2007). Flavanoids are phenolic compounds found throughout the plant kingdom and are thought to exert a significant antioxidant effect, and they also possess chelating properties that may reduce the formation of the hydroxyl radical (Ďuračková 2014; Gill and Tuteja 2010).

Similar to the antioxidant enzymes, the concentration of sacrificial antioxidants within plants can show large variations upon exposure to abiotic stress (Munne-Bosch 2005; Foyer and Noctor 2009; Gill and Tuteja 2010). Increased antioxidant content has been detected in response to light, temperature, drought, UV-B and salt stress (Agarwal 2007; Yang et al. 2008; Srivastava et al. 2005; Shao et al. 2007). However, increased antioxidant content is not always beneficial. For example, Creissen et al. (1999) observed increased necrosis in transgenic tobacco despite having a three-fold increase in glutathione content. The ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) is commonly used as an indicator of oxidative stress within the cell, and is important in redox signalling (Foyer and Noctor 2009). If the production of these oxidised antioxidants exceeds the ability of plants to reduce them, then the reduction potential will increase, indicating excessive ROS formation. The half-cell reduction potential is commonly used in seed storage experiments, linking the reduction of germination to a change in the half-cell reduction potential because the seeds become more oxidised over time as they age and accumulate cellular damage during storage (Colville and Kranner 2010; Birtic et al. 2011; Chen et al. 2013; Nagel et al. 2014).

Analysis of oxidative stress during cryopreservation

Excision

The excision of sample material, such as shoot tips from *in vitro* shoots or embryonic axes from seeds, can result in oxidative burst formation due to wounding of sample tissue. Superoxide formation after excision has been detected in *Castanea sativa, Strychnos gerrardii* and *Trichilia dregeana* embryogenic axes (Roach et al. 2008; Whitaker et al. 2010; Berjak et al. 2011). While Skyba et al. (2012) observed the greatest increased H_2O_2 formation after excision of *Hypericum perforatum* shoot tips compared to any other stage in the cryopreservation process. The formation of H_2O_2 in these excised shoot tips was great enough to damage the chloroplasts, to the point where ruptured thylakoid grana were observed (Skyba et al. 2012).

Preculture

The preculture period is defined as the period after excision of the shoot tips (containing actively growing meristematic tissues) from the *in vitro* shoots, but before the shoot tips are exposed to CPAs. This period is typically used to slightly desiccate the shoot tips prior to cryopreservation and allow for shoot tips to recover from the excision damage. Increased ROS and by-products of oxidative stress have been detected due to desiccation stress, such as increased superoxide, H_2O_2 and MDA (Fang et al. 2008; Whitaker et al. 2010; Berjak et al. 2011; Lynch et al. 2011). However, while ROS production is increased, antioxidants, such as GR and ascorbate peroxidase, have also shown to increase during the preculture period (Wen et al. 2010; Lynch et al. 2011).

Incubation in cryoprotectants

Excessive exposure to CPAs can be deleterious as many CPAs are cytotoxic, and can also lead to excessive dehydration causing significant tissue damage. Exposure to CPAs has been shown to increase methane production in *Ribes*, as a marker for the hydroxyl radical (Johnston et al. 2007). The formation of H_2O_2 and increased MDA have also been associated with the cytotoxicity of the CPAs (Uchendu et al. 2010a; Skyba et al. 2010).

Rewarming and recovery

The rewarming and recovery period after cryopreservation is a critical stage where increased oxidative stress has been linked to decreased post-cryogenic regrowth. Skyba et al. (2012) observed increased oxidative stress in *Hypericum perforatum* for

2 months after cryopreservation using 2,7-dichlorofluorescein diacetate (DCF-DA) to detect intracellular ROS. A burst of superoxide, formation of the hydroxyl radical, and increased MDA and 4-hydroxynonenal (4-HNE) have all been observed after the initial stages of recovery (Dussert et al. 2003; Whitaker et al. 2010; Fang et al. 2008). Along with increased ROS, a reduction in total antioxidants and decreased activity of GR and SOD has been observed after cryopreservation (Harding et al. 2009; Johnston et al. 2007; Wen et al. 2010). These results suggest the plants are highly susceptible to oxidative stress throughout the many stages of cryopreservation including the recovery period post-cryopreservation.

Alleviating oxidative stress during cryopreservation

The application of a preconditioning regime prior to cryopreservation has routinely been shown to improve post-cryogenic survival. The utilisation of a low, non-freezing temperature is the most common preconditioning regime as it has shown benefits for a wide range of species (Funnekotter et al. 2013; Menon et al. 2012; Gupta and Reed 2006; Chang et al. 2000; Leunufna and Keller 2005; Kaczmarczyk et al. 2010; Uchendu et al. 2013). As stated in the previous sections, these abiotic stress conditions have been shown to increase antioxidant concentrations, and preconditioning has also been shown to alter the composition of membranes and the concentration of solutes within cells, which may aid plants during the cryopreservation process (Sasaki et al. 1996; Fujikawa et al. 1999; Kamata and Uemura 2004; Funnekotter et al. 2013; Hincha and Zuther 2014; Menon et al. 2014).

The addition of exogenous antioxidants to cryoprotectant solutions or media has been shown to increase post-cryogenic survival in some cases. For example, the application of melatonin in *Rhodiola crenulata* and American Elm (Zhao et al. 2011; Uchendu et al. 2013), ascorbic acid in *Nepheluim ramboutan-ake*, wheat and blackberry cryopreservation (Fretz and Lörz 1995; Uchendu et al. 2010a; Chua and Normah 2011), lipoic acid, glycine betaine and glutathione in *Rubus* (Uchendu et al. 2010b) and glutathione in citrus and potato (Wang and Deng 2004; Canepa et al. 2011) all increased post-cryogenic survival. However, the application of an exogenous antioxidant is not always beneficial, for instance, Uchendu et al. (2010a) and Canepa et al. (2011) both reported decreased post-cryogenic survival with the addition of ascorbic acid when iron was present, likely due to the fact that AsA and iron can participate in Fenton's reaction to produce highly destructive hydroxyl radicals (Halliwell and Gutteridge 2007).

The addition of specific chelating agents (such as desferrioxamine) has been shown to reduce the levels of iron in cryopreserved tissues, which subsequently decreased the levels of the hydroxyl radical (Benson 1995; Fleck et al. 2000; Obert et al. 2005). Avoiding light exposure after cryopreservation has also been shown to increase post-cryogenic survival because of the reduction in photo-oxidative stress on the recovering shoot tips (Touchell et al. 2002).

Conclusions

This review focuses on the large variation of damage that can be induced by oxidative stresses on the various cellular components susceptible to the non-specific interactions of ROS. Any damage to DNA, lipids and proteins will reduce the chances of post-cryogenic survival and regeneration. Indeed, plant material undergoing cryostorage is likely to experience a wide range of stresses during the cryopreservation process which have the capacity to cause significant oxidative damage under some circumstances. Cryopreservation relies on the ability of plants to overcome these stresses in order to regenerate after cryogenic storage. Alleviating these stresses, even slightly, may help to improve post-cryogenic regeneration regardless of the species, clonal line or plant material used. There are multiple examples of the advantages of using preconditioning regimes and exogenous antioxidants to improve post-cryogenic survival; however, there is still further scope for additional experimentation to analyse the natural antioxidant status of plants and their ability to survive and regenerate following cryopreservation. Increasing our understanding of when and where oxidative damage occurs, such that it disrupts the sensitive redox balance within cells is vital for the development of new cryopreservation protocols and forms the underlying basis for this dissertation.

References

Agarwal, S. 2007. "Increased Antioxidant Activity in *Cassia* Seedlings under Uv-B Radiation." *Biologia Plantarum* 51 (1): 157-160

Airaki, M., M. Leterrier, R.M. Mateos, R. Valderrama, M. Chaki, J.B. Barroso, L.A. Del Rio, J.M. Palma, and F.J. Corpas. 2012. "Metabolism of Reactive Oxygen Species and Reactive Nitrogen Species in Pepper (*Capsicum Annuum* L.) Plants under Low Temperature Stress." *Plant, Cell & Environment* 35 (2): 281-295

Anchordoguy, T.J., A.S. Rudolph, J.F. Carpenter, and J.H. Crowe. 1987. "Modes of Interaction of Cryoprotectants with Membrane Phospholipids During Freezing." *Cryobiology* 24 (4): 324-331

Avery, S. 2014. "Oxidative Stress and Cell Function." In *Systems Biology of Free Radicals and Antioxidants*, ed. Ismail Laher, 89-112. Springer Berlin Heidelberg.

Bajaj, Y.P.S. 1995. "Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity." In *Biotechnology in Agriculture and Forestry 32*, ed. Y.P.S. Bajaj, 1-18. New York: Springer.

Benson, E.E. 1990. *Free Radical Damage in Stored Plant Germplasm*. Rome: International Board for Plant Genetic Resources.

Benson, E.E. 1995. "Cryopreservation of Shoot-Tips and Meristems." In *Methods in Molecular Biology Volume 38: Cryopreservation and Freeze-Drying Protocols*, eds J.G. Day and M.R. McLellan, 121-132. Totwa, NJ: Humana Press Inc.

Benson, E.E., and D. Bremner. 2004. "Oxidative Stress in the Frozen Plant: A Free Radical Point of View." In *Life in the Frozen State*, eds B.J. Fuller, N. Lane and E.E. Benson, 206–241. Boca Raton, Florida: CRC Press.

Benson, E.E., P.T. Lynch, and J. Jones. 1992. "The Detection of Lipid Peroxidation Products in Cryoprotected and Frozen Rice Cells: Consequences for Post-Thaw Survival." *Plant Science* 85 (1): 107-114

Berjak, P., B. Varghese, and N. Pammenter. 2011. "Cathodic Amelioration of the Adverse Effects of Oxidative Stress Accompanying Procedures Necessary for Cryopreservation of Embryonic Axes of Recalcitrant-Seeded Species." *Seed Science Research* 21 (03): 187-203

Birtic, S., L. Colville, H.W. Pritchard, S.R. Pearce, and I. Kranner. 2011. "Mathematically Combined Half-Cell Reduction Potentials of Low-Molecular-Weight Thiols as Markers of Seed Ageing." *Free Radical Research* 45 (9): 1093-1102

Cakmak, I., and H. Marschner. 1992. "Magnesium Deficiency and High Light Intensity Enhance Activities of Superoxide Dismutase, Ascorbate Peroxidase, and Glutathione Reductase in Bean Leaves." *Plant Physiology* 98 (4): 1222-1227

Canepa, P., A. Panta, and D. Tay. 2011. "The Effect of Antioxidants on the Cryopreservation Recovery of Two Potato Cultivars Following Post-Thawing Culture." In *International Symposium on Cryopreservation in Horticultural Species*, 101-105. Acta Hort. (ISHS).

Cao, W.-L., Y.-X. Wang, Z.-Q. Xiang, and Z. Li. 2003. "Cryopreservation-Induced Decrease in Heat-Shock Protein 90 in Human Spermatozoa and Its Mechanism." *Asian journal of andrology* 5 (1): 43-46

Chang, Y., R.E. Barker, and B.M. Reed. 2000. "Cold Acclimation Improves Recovery of Cryopreserved Grass *(Zoysia* and *Lolium* Sp.)." *CryoLetters* 21 (2): 107-116

Chen, H., D. Osuna, L. Colville, O. Lorenzo, K. Graeber, H. Küster, G. Leubner-Metzger, and I. Kranner. 2013. "Transcriptome-Wide Mapping of Pea Seed Ageing Reveals a Pivotal Role for Genes Related to Oxidative Stress and Programmed Cell Death." *PloS One* 8 (10): e78471

Chua, S., and M. Normah. 2011. "Effect of Preculture, Pvs2 and Vitamin C on Survival of Recalcitrant *Nephelium Ramboutan*-Ake Shoot Tips after Cryopreservation by Vitrification." *CryoLetters* 32 (6): 506-515

Colville, L., and I. Kranner. 2010. "Desiccation Tolerant Plants as Model Systems to Study Redox Regulation of Protein Thiols." *Plant Growth Regulation* 62 (3): 241-255

Creissen, G., J. Firmin, M. Fryer, B. Kular, N. Leyland, H. Reynolds, G. Pastori, F. Wellburn, N. Baker, and A. Wellburn. 1999. "Elevated Glutathione Biosynthetic Capacity in the Chloroplasts of Transgenic Tobacco Plants Paradoxically Causes Increased Oxidative Stress." *The Plant Cell Online* 11 (7): 1277-1291

Dedon, P.C., J.P. Plastaras, C.A. Rouzer, and L.J. Marnett. 1998. "Indirect Mutagenesis by Oxidative DNA Damage: Formation of the Pyrimidopurinone

Adduct of Deoxyguanosine by Base Propenal." *Proceedings of the National Academy of Sciences* 95 (19): 11113-11116

Dowgert, M.F., and P.L. Steponkus. 1984. "Behavior of the Plasma Membrane of Isolated Protoplasts During a Freeze-Thaw Cycle." *Plant Physiology* 75 (4): 1139-1151

Ďuračková, Z. 2014. "Free Radicals and Antioxidants for Non-Experts." In *Systems Biology of Free Radicals and Antioxidants*, ed. Ismail Laher, 3-38.

Dussert, S., N. Chabrillange, J.L. Montillet, J.P. Agnel, F. Engelmann, and M. Noirot. 2003. "Basis of Coffee Seed Sensitivity to Liquid Nitrogen Exposure: Oxidative Stress or Imbibitional Damage?" *Physiologia Plantarum* 119 (4): 534-543

Fang, J.Y., A. Wetten, and J. Johnston. 2008. "Headspace Volatile Markers for Sensitivity of Cocoa (*Theobroma Cacao* L.) Somatic Embryos to Cryopreservation." *Plant Cell Reports* 27 (3): 453-461

Farrugia, G., and R. Balzan. 2012. "Oxidative Stress and Programmed Cell Death in Yeast." *Molecular and Cellular Oncology* 2 (64): 1-21

Fleck, R.A., E.E. Benson, D.H. Bremner, and J.G. Day. 2000. "Studies of Free Radical-Mediated Cryoinjury in the Unicellular Green Alga *Euglena Gracilis* Using a Non-Destructive Hydroxyl Radical Assay: A Novel Approach for Developing Protistan Cryopreservation Strategies." *Free Radical Research* 32 (2): 157-70

Foyer, C.H., L.D. Gomez, and D.R. van Heerden. 2008. "Glutathione." In *Antioxidants and Reactive Oxygen Species in Plants*, ed. N. Smirnoff, 1-24. Wiley.

Foyer, C.H., M. Lelandais, and K.J. Kunert. 1994. "Photooxidative Stress in Plants." *Physiologia Plantarum* 92 (4): 696-717

Foyer, C.H., and G. Noctor. 2009. "Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications." *Antioxidants & Redox Signaling* 11 (4): 861-905

Fretz, A., and H. Lörz. 1995. "Cryopreservation of *in Vitro* Cultures of Barley *(Hordeum Vulgare L*. And *H. Murinum L*.) and Transgenic Cells of Wheat (*Triticum Aestivum L*.)." *Journal of Plant Physiology* 146 (4): 489-496

Fryer, M.J., K. Oxborough, P.M. Mullineaux, and N.R. Baker. 2002. "Imaging of Photo‐Oxidative Stress Responses in Leaves." *Journal of Experimental Botany* 53 (372): 1249-1254

Fujikawa, S., Y. Jitsuyama, and K. Kuroda. 1999. "Determination of the Role of Cold Acclimation-Induced Diverse Changes in Plant Cells from the Viewpoint of Avoidance of Freezing Injury." *Journal of Plant Research* 112 (2): 237-244

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Gill, S.S., and N. Tuteja. 2010. "Reactive Oxygen Species and Antioxidant Machinery in Abiotic Stress Tolerance in Crop Plants." *Plant Physiology and Biochemistry* 48 (12): 909-930

Gordon-Kamm, W.J., and P.L. Steponkus. 1984. "Lamellar-to-Hexagonalii Phase Transitions in the Plasma Membrane of Isolated Protoplasts after Freeze-Induced Dehydration." *Proceedings of the National Academy of Sciences of the United States of America* 81 (20): 6373-7

Gupta, A.S., J.L. Heinen, A.S. Holaday, J.J. Burke, and R.D. Allen. 1993. "Increased Resistance to Oxidative Stress in Transgenic Plants That Overexpress Chloroplastic Cu/Zn Superoxide Dismutase." *Proceedings of the National Academy of Sciences* 90 (4): 1629-1633

Gupta, S., and B.M. Reed. 2006. "Cryopreservation of Shoot Tips of Blackberry and Raspberry by Encapsulation-Dehydration and Vitrification." *CryoLetters* 27 (1): 29-42

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Hao, Y.J., C.X. You, and X.X. Deng. 2002. "Analysis of Ploidy and the Patterns of Amplified Fragment Length Polymorphism and Methylation Sensitive Amplified Polymorphism in Strawberry Plants Recovered from Cryopreservation." *CryoLetters* 23 (1): 37-46

Harding, K. 2004. "Genetic Integrity of Cryopreserved Plant Cells: A Review." *CryoLetters* 25 (1): 3-22

Harding, K., J.W. Johnston, and E.E. Benson. 2009. "Exploring the Physiological Basis of Cryopreservation Success and Failure in Clonally Propagated in Vitro Crop Plant Germplasm." *Agricultural and Food Science* 18 (2): 103-116

Hincha, D., and E. Zuther. 2014. "Introduction: Plant Cold Acclimation and Freezing Tolerance." In *Plant Cold Acclimation*, eds Dirk K. Hincha and Ellen Zuther, 1-6. Springer New York.

Hipkiss, A.R., J.E. Preston, D. Himswoth, V.C. Worthington, and N.J. Abbot. 1997. "Protective Effects of Carnosine against Malondialdehyde-Induced Toxicity Towards Cultured Rat Brain Endothelial Cells." *Neuroscience Letters* 238 (3): 135-138

Johnston, J.W., K. Harding, and E.E. Benson. 2007. "Antioxidant Status and Genotypic Tolerance of *Ribes in Vitro* Cultures to Cryopreservation." *Plant Science* 172 (3): 524-534

Kaczmarczyk, A., B. Funnekotter, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., A. Houben, E. Keller, and M.F. Mette. 2010. "Influence of Cryopreservation on the Cytosine Methylation State of Potato Genomic DNA." *CryoLetters* 31 (5): 380-391

Kaity, A., R.A. Drew, and S. Ashmore. 2013. "Genetic and Epigenetic Integrity Assessment of Acclimatised Papaya Plants Regenerated Directly from Shoot-Tips Following Short-and Long-Term Cryopreservation." *Plant Cell, Tissue and Organ Culture* 112 (1): 75-86

Kamata, T., and M. Uemura. 2004. "Solute Accumulation in Wheat Seedlings During Cold Acclimation: Contribution to Increased Freezing Tolerance." *CryoLetters* 25 (5): 311-322

Kim, Y.J., M.G. Jang, H.Y. Noh, H.J. Lee, J. Sukweenadhi, J.H. Kim, S.Y. Kim, W.S. Kwon, and D.C. Yang. 2014. "Molecular Characterization of Two Glutathione Peroxidase Genes of *Panax Ginseng* and Their Expression Analysis against Environmental Stresses." *Gene* 535 (1): 33-41

Leunufna, S., and E.R. Keller. 2005. "Cryopreservation of Yams Using Vitrification Modified by Including Droplet Method: Effects of Cold Acclimation and Sucrose." *CryoLetters* 26 (2): 93-102

Lowe, F. 2014. "Biomarkers of Oxidative Stress." In *Systems Biology of Free Radicals and Antioxidants*, ed. Ismail Laher, 65-87. Springer Berlin Heidelberg.

Lynch, D.V., and P.L. Steponkus. 1987. "Plasma Membrane Lipid Alterations Associated with Cold Acclimation of Winter Rye Seedlings (*Secale Cereale* L. Cv Puma)." *Plant Physiology* 83 (4): 761-767

Lynch, P.T., A. Siddika, J.W. Johnston, S.M. Trigwell, A. Mehra, C. Benelli, M. Lambardi, and E.E. Benson. 2011. "Effects of Osmotic Pretreatments on Oxidative Stress, Antioxidant Profiles and Cryopreservation of Olive Somatic Embryos." *Plant Science* 181: 47-56

Malan, C., M.M. Greyling, and J. Gressel. 1990. "Correlation between Cuzn Superoxide Dismutase and Glutathione Reductase, and Environmental and Xenobiotic Stress Tolerance in Maize Inbreds." *Plant Science* 69 (2): 157-166

Marnett, L.J. 1999a. "Chemistry and Biology of DNA Damage by Malondialdehyde." *IARC Scientific Publications* (150): 17-27

Martinez-Montero, M., N. Mora, J. Quinones, M. Gonzalez-Arnao, F. Engelmann, and J. Lorenzo. 2002. "Effect of Cryopreservation on the Structural and Functional Integrity of Cell Membranes of Sugarcane (*Saccharum* Sp.) Embryogenic Calluses." *CryoLetters* 23 (4): 237-244

McCord, J.M., and I. Fridovich. 1968. "The Reduction of Cytochrome C by Milk Xanthine Oxidase." *Journal of Biological Chemistry* 243 (21): 5753-5760

McKersie, B.D., Y. Chen, M. de Beus, S.R. Bowley, C. Bowler, D. Inzé, K. D'Halluin, and J. Botterman. 1993. "Superoxide Dismutase Enhances Tolerance of Freezing Stress in Transgenic Alfalfa (*Medicago Sativa* L.)." *Plant Physiology* 103 (4): 1155-1163

Meloni, D.A., M.A. Oliva, C.A. Martinez, and J. Cambraia. 2003. "Photosynthesis and Activity of Superoxide Dismutase, Peroxidase and Glutathione Reductase in Cotton under Salt Stress." *Environmental and Experimental Botany* 49 (1): 69-76

Mengutay, M., Y. Ceylan, U.B. Kutman, and I. Cakmak. 2013. "Adequate Magnesium Nutrition Mitigates Adverse Effects of Heat Stress on Maize and Wheat." *Plant and Soil* 368 (1-2): 57-72

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Møller, I.M., P.E. Jensen, and A. Hansson. 2007. "Oxidative Modifications to Cellular Components in Plants." *Annual Review of Plant Biology* 58: 459-481

Munne-Bosch, S. 2005. "The Role of *A*-Tocopherol in Plant Stress Tolerance." *Journal of Plant Physiology* 162 (7): 743-748

Nagel, M., I. Kranner, K. Neumann, H. Rolletschek, C.E. Seal, L. Colville, B. Fernández-Marín, and A. Börner. 2014. "Genome-Wide Association Mapping and Biochemical Markers Reveal That Seed Ageing and Longevity Are Intricately Affected by Genetic Background and Developmental and Environmental Conditions in Barley." *Plant, Cell & Environment*: doi: 10.1111/pce.12474

Naraikina, N., M. Sin'kevich, I. Demin, A. Selivanov, I. Moshkov, and T. Trunova. 2014. "Changes in the Activity of Superoxide Dismutase Isoforms in the Course of Low-Temperature Adaptation in Potato Plants of Wild Type and Transformed with Δ12-Acyl-Lipid Desaturase Gene." *Russian Journal of Plant Physiology* 61 (3): 332-338

Noctor, G., A. Mhamdi, S. Chaouch, Y. Han, J. Neukermans, B. Marquez‐Garcia, G. Queval, and C.H. Foyer. 2012. "Glutathione in Plants: An Integrated Overview." *Plant, Cell & Environment* 35 (2): 454-484

Obert, B., E.E. Benson, S. Millam, A. Pretová, and D.H. Bremner. 2005. "Moderation of Morphogenetic and Oxidative Stress Responses in Flax in Vitro Cultures by Hydroxynonenal and Desferrioxamine." *Journal of Plant Physiology* 162 (5): 537-547

Roach, T., M. Ivanova, R.P. Beckett, F.V. Minibayeva, I. Green, H.W. Pritchard, and I. Kranner. 2008. "An Oxidative Burst of Superoxide in Embryonic Axes of Recalcitrant Sweet Chestnut Seeds as Induced by Excision and Desiccation." *Physiologia Plantarum* 133 (2): 131-139

Sasaki, H., K. Ichimura, and M. Oda. 1996. "Changes in Sugar Content During Cold Acclimation and Deacclimation of Cabbage Seedlings." *Annals of Botany* 78 (3): 365-369

Shao, H.-B., L.-Y. Chu, G. Wu, J.-H. Zhang, Z.-H. Lu, and Y.-C. Hu. 2007. "Changes of Some Anti-Oxidative Physiological Indices under Soil Water Deficits among 10 Wheat (*Triticum Aestivum* L.) Genotypes at Tillering Stage." *Colloids and Surfaces B: Biointerfaces* 54 (2): 143-149

Shu, D.-F., L.-Y. Wang, M. Duan, Y.-S. Deng, and Q.-W. Meng. 2011. "Antisense-Mediated Depletion of Tomato Chloroplast Glutathione Reductase Enhances Susceptibility to Chilling Stress." *Plant Physiology and Biochemistry* 49 (10): 1228-1237

Skyba, M., L. Petijová, J. Košuth, D.P. Koleva, T.G. Ganeva, V.M. Kapchina-Toteva, and E. Čellárová. 2012. "Oxidative Stress and Antioxidant Response in *Hypericum Perforatum* L. Plants Subjected to Low Temperature Treatment." *Journal of Plant Physiology* 169 (10): 955-964

Skyba, M., M. Urbanová, V. Kapchina-Toteva, J. Košuth, K. Harding, and E. Čellárová. 2010. "Physiological, Biochemical and Molecular Characteristics of Cryopreserved *Hypericum Perforatum* L. Shoot Tips." *CryoLetters* 31 (3): 249-260

Srivastava, A.K., P. Bhargava, and L.C. Rai. 2005. "Salinity and Copper-Induced Oxidative Damage and Changes in the Antioxidative Defence Systems of Anabaena Doliolum." *World Journal of Microbiology and Biotechnology* 21 (6-7): 1291-1298

Thierry, C., B. Florin, and V. Pétiard. 1999. "Changes in Protein Metabolism During the Acquisition of Tolerance to Cryopreservation of Carrot Somatic Embryos." *Plant Physiology and Biochemistry* 37 (2): 145-154

Touchell, D.H., S.R. Turner, E. Bunn, and K.W. Dixon. 2002. "Cryostorage of Stomatic Tissues of Endangered Australian Species." In *Biotechnology in Agriculture and Forestry 50: Cryopreservation of Plant Germplasm Ii*, eds LE Towill and YPS Bajaj. New York: Springer.

Tsang, E., C. Bowler, D. Hérouart, W. Van Camp, R. Villarroel, C. Genetello, M. Van Montagu, and D. Inzé. 1991. "Differential Regulation of Superoxide Dismutases in Plants Exposed to Environmental Stress." *The Plant Cell Online* 3 (8): 783-792

Uchendu, E.E., S.W. Leonard, M.G. Traber, and B.M. Reed. 2010a. "Vitamins C and E Improve Regrowth and Reduce Lipid Peroxidation of Blackberry Shoot Tips Following Cryopreservation." *Plant Cell Reports* 29 (1): 25-35

Uchendu, E.E., M. Muminova, S. Gupta, and B.M. Reed. 2010b. "Antioxidant and Anti-Stress Compounds Improve Regrowth of Cryopreserved *Rubus* Shoot Tips." *In Vitro Cellular & Developmental Biology-Plant*: 1-8

Uchendu, E.E., M.R. Shukla, B.M. Reed, and P.K. Saxena. 2013. "Melatonin Enhances the Recovery of Cryopreserved Shoot Tips of American Elm (*Ulmus Americana* L.)." *Journal of Pineal Research* 55 (4): 435-442

van Camp, W., K. Capiau, M. Van Montagu, D. Inze, and L. Slooten. 1996. "Enhancement of Oxidative Stress Tolerance in Transgenic Tobacco Plants Overproducing Fe-Superoxide Dismutase in Chloroplasts." *Plant Physiology* 112 (4): 1703-1714

Voulgaridou, G.-P., I. Anestopoulos, R. Franco, M.I. Panayiotidis, and A. Pappa. 2011. "DNA Damage Induced by Endogenous Aldehydes: Current State of Knowledge." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 711 (1): 13-27

Wang, Z.C., and X.X. Deng. 2004. "Cryopreservation of Shoot-Tips of Citrus Using Vitrification: Effect of Reduced Form of Glutathione." *CryoLetters* 25 (1): 43-50

Wen, B., R. Wang, H. Cheng, and S. Song. 2010. "Cytological and Physiological Changes in Orthodox Maize Embryos During Cryopreservation." *Protoplasma* 239 (1-4): 57-67

Whitaker, C., R.P. Beckett, F.V. Minibayeva, and I. Kranner. 2010. "Production of Reactive Oxygen Species in Excised, Desiccated and Cryopreserved Explants of *Trichilia Dregeana* Sond." *South African Journal of Botany* 76 (1): 112-118

Xu, J., J. Yang, X. Duan, Y. Jiang, and P. Zhang. 2014. "Increased Expression of Native Cytosolic Cu/Zn Superoxide Dismutase and Ascorbate Peroxidase Improves Tolerance to Oxidative and Chilling Stresses in Cassava (*Manihot Esculenta* Crantz)." *BMC Plant Biology* 14 (1): 208

Yang, Y., C. Han, Q. Liu, B. Lin, and J. Wang. 2008. "Effect of Drought and Low Light on Growth and Enzymatic Antioxidant System of *Picea Asperata* Seedlings." *Acta Physiologiae Plantarum* 30 (4): 433-440

Young, I., and J. McEneny. 2001. "Lipoprotein Oxidation and Atherosclerosis." *Biochemical Society Transactions* 29: 358-362

Zarghami, R., M. Pirseyedi, S. Hasrak, and B.P. Sardrood. 2008. "Evaluation of Genetic Stability in Cryopreserved *Solanum Tuberosum*." *African Journal of Biotechnology* 7 (16): 2798-2802

Zhai, Z., Y. Wu, F. Engelmann, R. Chen, and Y. Zhao. 2003. "Genetic Stability Assessments of Plantlets Regenerated from Cryopreserved in Vitro Cultured Grape and Kiwi Shoot-Tips Using Rapd." *CryoLetters* 24 (5): 315-322

Zhang, J., and M. Kirkham. 1994. "Drought-Stress-Induced Changes in Activities of Superoxide Dismutase, Catalase, and Peroxidase in Wheat Species." *Plant and Cell Physiology* 35 (5): 785-791

Zhao, Y., L.W. Qi, W.M. Wang, P.K. Saxena, and C.Z. Liu. 2011. "Melatonin Improves the Survival of Cryopreserved Callus of *Rhodiola Crenulata*." *Journal of Pineal Research* 50 (1): 83-88

Chapter 3 – Influence of abiotic stress factors during preconditioning on post-cryogenic survival, antioxidant enzymes and electrolyte leakage in the south-west Australian Mediterranean climate species *Lomandra sonderi*

Abstract

Lomandra sonderi (Asparagaceae) is endemic to south-west Western Australia and is found in the region's Jarrah (*Eucalyptus marginata*) forest. It is a plant species widely used in post-mining restoration, which is aided by a recently developed cryopreservation protocol. In order to investigate the effect of abiotic stress preconditioning on improving post-cryogenic survival, plants were exposed to a wide range of light, temperature and osmotic induced stresses under tissue culture conditions. Room temperature preconditioning showed the greatest post-cryogenic survival, followed by low light preconditioning. Alternating temperature, high temperature, high sucrose and high light preconditioning all showed significantly and progressively lower post-cryogenic shoot survival than the room temperature regime. Analysis of freezing tolerance showed little correlation to post-cryogenic survival; however, *L. sonderi* showed limited increase in the LT_{50} freezing tolerance. The resistance of plants to the deleterious effects of cryopreservation may be enhanced by increasing the availability of antioxidants in the tissue, before exposure to the stresses of cryopreservation. Antioxidant activity of superoxide dismutase in preconditioned shoot tips showed a positive correlation to post-cryogenic survival, whilst glutathione reductase, glutathione peroxidase and catalase activity showed little correlation. Analysis of the antioxidant enzymes throughout the cryopreservation protocol showed that the activity of glutathione reductase decreased significantly after cryopreservation, whilst the activity of glutathione peroxidase and catalase did not change. The high light preconditioned shoot tips showed increased antioxidant activity, exhibiting the greatest levels of activity of glutathione reductase and glutathione peroxidase of all the preconditioning regimes, yet exhibited the lowest post-cryogenic survival. There are many other factors that can influence post-cryogenic survival, and analysis of the antioxidant enzyme showed no clear trend for increasing the antioxidant enzyme activity with a specific preconditioning regime or if this increased activity will result in greater post-cryogenic survival.

Introduction

Lomandra sonderi (Asparagaceae) is an endemic plant species of south-west Western Australia found in the region's Jarrah (*Eucalyptus marginata*) forest. This region is subject to bauxite mining that requires restoration of native plant species following ore extraction. *Lomandra sonderi* is a "dioecious rhizomatous, perennial herb" (Paczkowska 1998) that does not easily re-establish following mining (Plummer et al. 1995). It has complex seed germination properties, making seed-based propagation approaches unreliable and problematic. Therefore, this species is usually propagated by tissue culture for the production of large numbers of plants for mine site restoration (Koch and Samsa 2007; Willyams 2005). However, tissue culture is labour-intensive and expensive (compared with conventional propagation) and involves significant risk of contamination, human error and somaclonal variation, which increase with repeated subculturing (Panis and Lambardi 2006). To address these limitations, cryopreservation has been developed as a more reliable tool for the long-term storage of this species (Menon et al. 2012).

Plant cryopreservation is the use of liquid nitrogen (-196C) for ultra-low temperature, long-term storage of plant tissue, whilst retaining its viability after rewarming (Benson 2008). During cryopreservation, all cell division, metabolic and biochemical processes are suspended, which in principle allows the indefinite storage of plant material without degeneration or alteration (Bajaj 1995). Cryogenic survival of different species, even cultivars of the same species, may often be highly variable for reasons not yet fully understood (Kaczmarczyk et al. 2011b). As species or cultivars can be very specific in their response to cryogenic conditions, cryopreservation protocols need to be adjusted at critical points to optimise survival for each plant species (Reed 2011). Preconditioning of explant material with temperature, light or osmotic stresses prior to cryopreservation has been shown to be beneficial to improving cryogenic survival (Pritchard et al. 1986; Huner et al. 1998; Chang et al. 2000; Kaczmarczyk et al. 2008; Funnekotter et al. 2013; Menon et al. 2012). A positive correlation has been observed between shoot tip regeneration and several biochemical changes (such as increased sugars, antioxidant activity and altered fatty acid content) which occur during preconditioning (e.g. cold acclimation) (Vandenbussche et al. 1999; Crowe et al. 1988; Kaczmarczyk et al. 2008; Funnekotter et al. 2013; Menon et al. 2014). Increased sugar content may help to promote the formation of intracellular glassy water, which minimises the deleterious formation of ice crystals, and may substitute for water in maintaining hydrophilic structures in their hydrated state (Smallwood and Bowles 2002). An increase in the level of unsaturated fatty acids in cell membranes

can improve cell membrane stability by reducing the likelihood of the liquid crystalline-to-gel transition at lower temperatures, allowing plants to better withstand freezing stresses (Uemura et al. 2006; Uemura et al. 1995), whilst increased antioxidants may aid in reducing reactive oxygen species (ROS) damage following warming from cryogenic temperatures and recovery.

The metabolism of plants under stress is generally characterised by increased formation of ROS (Tausz et al. 2004). ROS like superoxide and hydrogen peroxide possess the ability to initiate cascade chemical reactions resulting in the production of hydroxyl and peroxide radicals, as well as other destructive species that can interact with and cause damage to cell membranes, proteins and DNA, resulting in lipid peroxidation, protein oxidation, enzyme inhibition and, in extreme cases, breakages in nucleic acids leading to cell decline and death (Halliwell and Gutteridge 2007). Formation of ROS in chloroplasts or mitochondria causes changes in the transcriptome, affecting the transmission of information from these organelles to the nucleus, ultimately affecting gene expression (Apel and Hirt 2004). Formation of ROS has been detected at multiple stages in the cryopreservation protocol and has been suggested as a likely reason for the reduction in post-cryogenic survival (Whitaker et al. 2010).

Plants can protect themselves against ROS through antioxidant mechanisms. In general, the antioxidant defence system can be classified into enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) are all enzymatic anti-oxidants (Kranner and Birtić 2005; Gill and Tuteja 2010). These antioxidant enzymes either catalyse reactions that remove ROS or directly process them to interrupt the cascades of uncontrolled oxidation (Noctor and Foyer 1998). Non-enzymatic antioxidants such as glutathione (GSH), ascorbic acid, tocopherols and carotenoids are sacrificial molecules that are preferentially oxidised by ROS, protecting important cellular components (Kranner and Birtić 2005; Noctor and Foyer 1998; Gill and Tuteja 2010).

When environmental stresses cause increase in the production of ROS, SODs play an important role in plant stress tolerance and constitute the first line of defence against the toxic effects of increased levels of the ROS superoxide (Halliwell and Gutteridge 2007). SODs remove the superoxide radical, but form hydrogen peroxide (H_2O_2) in the process (Fig 3.1). This H_2O_2 has the capacity to form the hydroxyl free radical and thus has the potential to be highly cytotoxic if not neutralised in cells (Gill & Tuteja, 2010). CAT and peroxidase enzymes, such as GPx, are the major defence against H_2O_2. CAT decomposes H_2O_2 into water and oxygen, whilst GPx can reduce

H_2O_2 into water and also neutralise organic peroxides, however, this requires the sacrificial antioxidant glutathione (GSH) or thioredoxins (Navrot et al. 2006). Oxygen-sensitive enzymes and proteins are protected by GSH from oxidative degradation of their sulfhydryl thiol group. In normal cells, the ratio of GSH to glutathione disulphide (GSSG) is high. GR catalyses the reaction that converts GSSG back into GSH utilising NADPH (Noctor et al. 2011; Halliwell and Gutteridge 2007). The interplay between GR and GSH has an important role in regulating plant stress tolerance, and without GR the GSH present in the cells will quickly become oxidised and non-functional (Rao and Reddy 2008; Gill and Tuteja 2010).

The action of the various antioxidant enzymes maintains the equilibrium between the production and scavenging of ROS, having a synergistic effect in neutralising ROS damage (Fig 3.1). This equilibrium can be disturbed by various biotic and abiotic stress factors, leading to an abrupt increase in intracellular ROS that may cause significant damage to cell structures (Gill & Tuteja, 2010). The subtle spatial and time balance between ROS formation and scavenging determines whether ROS will lead to damage, protection (e.g. pathogen defence) or systematic signalling (Gratão et al. 2005). Plant stress tolerance may thus be enhanced by the increase in activity of antioxidant enzymes (Mishra et al. 1993).

Although a cryopreservation protocol for *L. sonderi* has been developed, shoot tip survival remains relatively modest (~30%) (Menon et al. 2012). Exposure to sub-lethal levels of stress resulting in the protection from lethal levels of the same stress at a

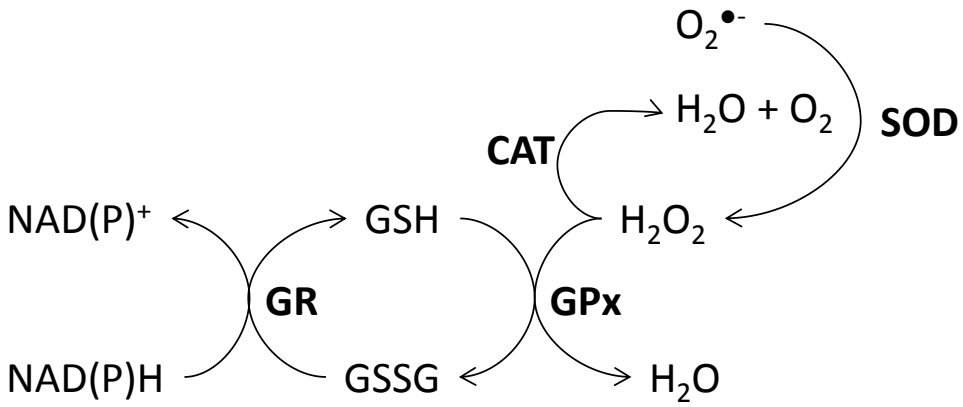

Figure 3.1. Synergistic effect of antioxidant enzymes in the removal of reactive oxygen species. $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulphide; H_2O_2, hydrogen peroxide.

later stage is termed stress acclimation (Apel and Hirt 2004). The study of different preconditioning stress factors may thus provide increased understanding of how these stresses impact the post cryogenic survival of *L. sonderi*. This study examined specific environmental stresses that could lead to changes in antioxidant responses and electrolyte leakage (as a measure of cell membrane stability), and which may improve post cryogenic survival, providing the first characterisation of the role of anti-oxidants in stress tolerance in Australian plant species. Three-week alternating cold temperature, room temperature (control), high temperature, low light, high light and high sucrose preconditioning regimes were applied prior to cryopreservation. Increasing understanding of these various stresses and ways to mitigate them is important because the development and optimisation of novel biotechnology approaches is a crucial step towards the successful conservation of the mega-diverse Australian flora.

Materials and methods

Plant material

In vitro shoots from *Lomandra sonderi* were obtained from Kings Park and Botanic Garden, Western Australia. A basal medium (BM) was prepared, consisting of half strength Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962), 500 µM myo-inositol, 500 µM 4-morpholineethanesulfonic acid (MES), 100 µM NaFeEDTA, 4 µM niacin, 3 µM thiamine-HCl, 2.5 µM pyridoxine-HCl, 60 mM sucrose, and 0.8% (w/v) agar, with the pH adjusted to 6 before autoclaving (121°C for 20 minutes). This medium was used for all tissue culture propagation unless otherwise specified.

Preconditioning

Lomandra sonderi in vitro shoots were incubated for three weeks under six different stress conditions (as described in Table 3.1). Room temperature (control) preconditioning was propagated on BM at 23±1°C with a 12 hour photoperiod under a photosynthetic photon flux density (PPFD) of ~30 µM $m^{-2}s^{-1}$ light conditions supplied by 21 W fluorescent lamps. High sucrose preconditioning varied from the control only in its sucrose content (180 mM sucrose in the media). The two light stress preconditioning regimes were different to the control culture only in their light levels with ~17 and ~93 µM light $m^{-2}s^{-1}$ for low and high light preconditioning regimes, respectively. The two temperature preconditioning stress regimes were different to

the control culture only in temperature: high temperature (high temp, 35°C with a 12 hour photoperiod) and alternating temperature (alt temp, 25/5°C with a 12 hour photoperiod). *In vitro* shoots, after four weeks on BM, were separated into individual shoots and trimmed to ~50 mm before being placed onto fresh BM. After 3 weeks preconditioning, the number of new shoots (from a starting point of 9 shoots / jar), weight of each shoot (g), height of each shoot (mm), colour of the leaves, and the amount of dead leaves around the shoots was recorded in the *in vitro* shoots.

Electrolyte leakage and calculation of LT_{50}

Lomandra sonderi leaf material exposed to the various preconditioning stress treatments was analysed for electrolyte leakage and LT_{50} as described in detail by Menon et al. (2014). Leaf material in 200 µl deionised water was gradually cooled at a rate of 1.5°C h^{-1} from -1°C to -10°C using a cooling bath (PolySciences Inc) and samples were taken out each hour and stored on ice. After 24 h, samples were diluted in 5 ml deionised water. 1 ml was transferred to 10 ml deionised water and electric conductivity was measured (C1) using a 900-C Conductivity Meter (TPS Australia). Total electrolyte loss was obtained by boiling the samples (100°C for 30 min) and then measuring electrolyte conductivity (C2) as described above. The percentage loss of electrolytes (% loss of electrolytes = (C1/C2) * 100) with zero correction to account for any other electrolyte leakage that may have occurred (Zero correction = % leakage

Table 3.1. Preconditioning treatments of *L. sonderi in vitro* shoots applied 3 weeks prior to the cryopreservation experiment, determination of electrolyte leakage or antioxidant analysis

Preconditioning treatment	Stress	Abbreviation	Temperature	Light conditions	Sucrose conc.
Room temperature (control)	None	RT	24 ± 1°C	~ 30 µM m^{-2} s^{-1}	60 mM
Alternating temperature	Low alternating temperature	AT	25/5°C	~ 30 µM m^{-2} s^{-1}	60 mM
High temperature	Extreme temperature	HT	35°C	~ 30 µM m^{-2} s^{-1}	60 mM
High sucrose	Water stress	HS	24 ± 1°C	~ 30 µM m^{-2} s^{-1}	180 mM
Low light	Sub optimal light conditions	LL	24 ± 1°C	~17 µM m^{-2} s^{-1}	60 mM
High Light	Supra optimal light conditions	HL	24 ± 1°C	~93 µM m^{-2} s^{-1}	60 mM

of sample – % leakage of control) was then normalised to maximum percentage electrolyte leakage (Normalised value = Zero correction / highest % leakage value * 100). This normalised value was used to construct sigmoidal curves for the calculation of LT_{50}, the temperature at which 50% of electrolytes have leaked out of the sample (Menon et al. 2014) using GraphPad Prism (version 5.03, GraphPad Software, San Diego USA).

Cryopreservation

Droplet-vitrification, rewarming and recovery as described by Menon et al. (2012) were followed to cryopreserve shoot tips of *L. sonderi*. Briefly, after the various preconditioning regimes, shoot tips were isolated onto the BM and incubated for 48h at 25°C in darkness. The shoot tips were transferred to a loading solution (LS: 2 M glycerol and 0.4 M sucrose in liquid BM) for 20 min at 23°C and then treated in Plant Vitrification Solution 2 (PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 0.4 M sucrose, 15% (w/v) dimethyl sulfoxide (Sakai et al. 1990), with half strength MS salts at pH 6.0) for 10 min at 23˚C. Each shoot tip was placed onto a sterile aluminium foil strip along with a 1 µl droplet of PVS2. This foil was placed in a sterile cryovial and the cryovial was plunged into liquid nitrogen (LN). After one hour, the cryovials were warmed in a 40°C water bath for 10 seconds and the shoot tips were then transferred into a washing solution (WS: 1 M sucrose in liquid BM) to rinse for 20 min at 23°C. The shoot tips were then placed onto recovery medium (RM: solid BM containing 1.0 µM zeatin and 1.0 µM gibberellic acid) and incubated at 25°C for 2 weeks in darkness, after which they were placed at 23°C with a 12 h photoperiod (photon flux density ~30 µM $m^{-2}s^{-1}$). Survival data was collected 6 weeks after the cryopreservation experiment.

Antioxidant analysis

The antioxidant activities of SOD, CAT, GPx and GR were measured in shoot tips to determine if the stresses experienced during the various preconditioning regimes altered the antioxidant capacity of the plants. Shoot tips were then analysed for antioxidant activity (CAT, GPx and GR activity) comparing two preconditioning regimes (room temperature and alternating temperature) after preconditioning, preculture, one day on recovery medium (1 d R) and seven days on recovery medium (7 d R) after cryopreservation. The shoot tips were not analysed during the cryopreservation protocol (such as after exposure to PVS2) due to the possible interference of the cryoprotective agents with the analysis. Commercially available BioVision assay kits were used for detection and quantification of the antioxidant

enzymes SOD, GR, GPx and CAT. To determine antioxidant enzyme levels, approximately 20 mg of shoot tip material was homogenised in the assay kit specified assay buffer and centrifuged at 10,000 x g for 15 min at 4°C with the supernatant collected for assay. Absorbance was measured using a microplate reader (MultiPlate Reader, PerkinElmer analysed using EnSpire Manager v 4.10). Briefly, SOD activity was measured by detecting the absorbance of formazine dye (OD 450 nm) produced from the reduction of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium monosodium salt) by the superoxide anion. SOD from the sample inhibits the formation of superoxide anions produced by xanthine oxidase, resulting in a measured percentage inhibition in comparison to the control. GR activity was measured by detecting the formation of TNB^{2-} (2-nitro-5-thiobenzoic acid) (OD 405 nm) formed when GSH reacts with DNTB (5,5'-dithio-bis(2-nitrobenzoic acid)), as per Ellman's reagent, as GR from the sample catalyses the formation of GSH from glutathione disulphide (GSSG). One unit is defined as the amount of enzyme activity to generate 1 µmol TNB min^{-1} at 25°C. GPx activity was measured by detecting the reduction of NADPH (OD 340 nm) in the samples as NADPH is consumed by GR catalysing the conversion of GSSG to GSH, and GSSG is formed as GPx reduces cumene hydroperoxide. One unit is defined as the amount of enzyme activity that causes oxidation of 1 µmol NADPH to $NADP^+$ min^{-1} at 25°C. CAT activity was measured by detecting the coloured product formed when unconverted H_2O_2 reacts with the OxiRed probe (OD 570 nm). One unit is defined as the amount of enzyme activity that decomposes 1 µmol H_2O_2 min^{-1} at 25°C pH 4.5. External standards over a linear absorbance range for TNB ($R^2 = 0.995$), NADPH ($R^2 = 0.994$) and H_2O_2 ($R^2 = 0.998$) were used for the GR, GPx and CAT assays, respectively.

Statistical analysis

Samples were collected in five replicates (20 shoot tips) for each cryopreservation experiment. Three replicates for each electrolyte leakage and antioxidant enzyme tests were carried out, with electrolyte leakage data analysed using GraphPad Prism5 (version 5.0.3.477). Statistical comparison of the antioxidant, electrolyte leakage (LT_{50}) and cryopreservation experiments was done using analysis of variance (ANOVA) with $p<0.05$ using the Tukey's test using the software SigmaPlot (Version 11.0, 2008). Statistical analysis of correlations was done using the Pearson correlation test.

Results

Preconditioning effect on in vitro shoot growth

Table 3.2 shows the effect that the preconditioning regimes had on the *in vitro* shoot growth. Low light and alternating temperature preconditioning were the most successful regimes with 6 new shoots per jar, followed by the room temperature, high light, high sucrose and then the high temperature regimes (with 5, 4, 3 and 2 new shoots per jar, respectively). The weight and height of each *in vitro* shoot did not differ significantly between the preconditioning regimes. The colour of the shoots did not differ except in the high temperature preconditioned shoots, which had light green coloured leaves (Fig 3.2F), while the high temperature preconditioned shoots also had a large number of dead leaves. The high sucrose and high light preconditioning regimes also had some dead leaves in comparison to the other preconditioning regimes. The room temperature and alternating temperature regimes were very similar with soft shoots and very few dead leaves, while the low light preconditioning regime had the softest shoots and no dead leaves were observed.

Table 3.2. Effect of 3 weeks preconditioning regimes. Comparison of formation of new shoots across the various preconditioning regimes. Asterisks indicate the frequency of dead leaves occurring (No asterisk = none, * = low, ** = medium, *** = high). Different letters denote significant differences across the preconditioning regimes ($P < 0.05$).

Treatment	New shoots / jar	Weight / shoot (g)	Shoot height (mm)	Colour of shoots	Frequency of dead leaves
Room Temperature (control)	4.75 ± 0.48^{ab}	0.15 ± 0.01	78.89 ± 6.97	green	
High Sucrose	2.75 ± 0.48^{ab}	0.16 ± 0.02	60.63 ± 4.62	green	**
Low Light	6.25 ± 1.49^{a}	0.12 ± 0.01	76.67 ± 5.01	green	
High Light	3.75 ± 0.85^{ab}	0.14 ± 0.02	76.56 ± 7.88	green	**
Alternating Temperature	6 ± 1.35^{a}	0.16 ± 0.02	67.33 ± 4.43	green	*
High Temperature	1.75 ± 0.25^{b}	0.13 ± 0.03	65.5 ± 8.68	light green	***

Post-cryogenic survival

Seven preconditioning regimes were tested to assess their effect on control (-LN) and post-cryogenic (+LN, Fig 3.3) survival. The different abiotic stress preconditioning regimes listed in Table 3.1 resulted in substantial differences in post-cryogenic survival. For example, the control room temperature preconditioning regime resulted in the highest cryogenic survival rate (56%), although this was not significantly different from the next highest treatment (survival rate in the low light preconditioning regime: 46%). The high temperature and alternating temperature preconditioning regimes showed similar post-cryogenic survival rates with 28% and 29%, respectively. The high sucrose and high light preconditioning regimes resulted in the lowest post-cryogenic survival rates observed, with 19% and 14%, respectively. Little difference was observed in the control survival; however, the high temperature

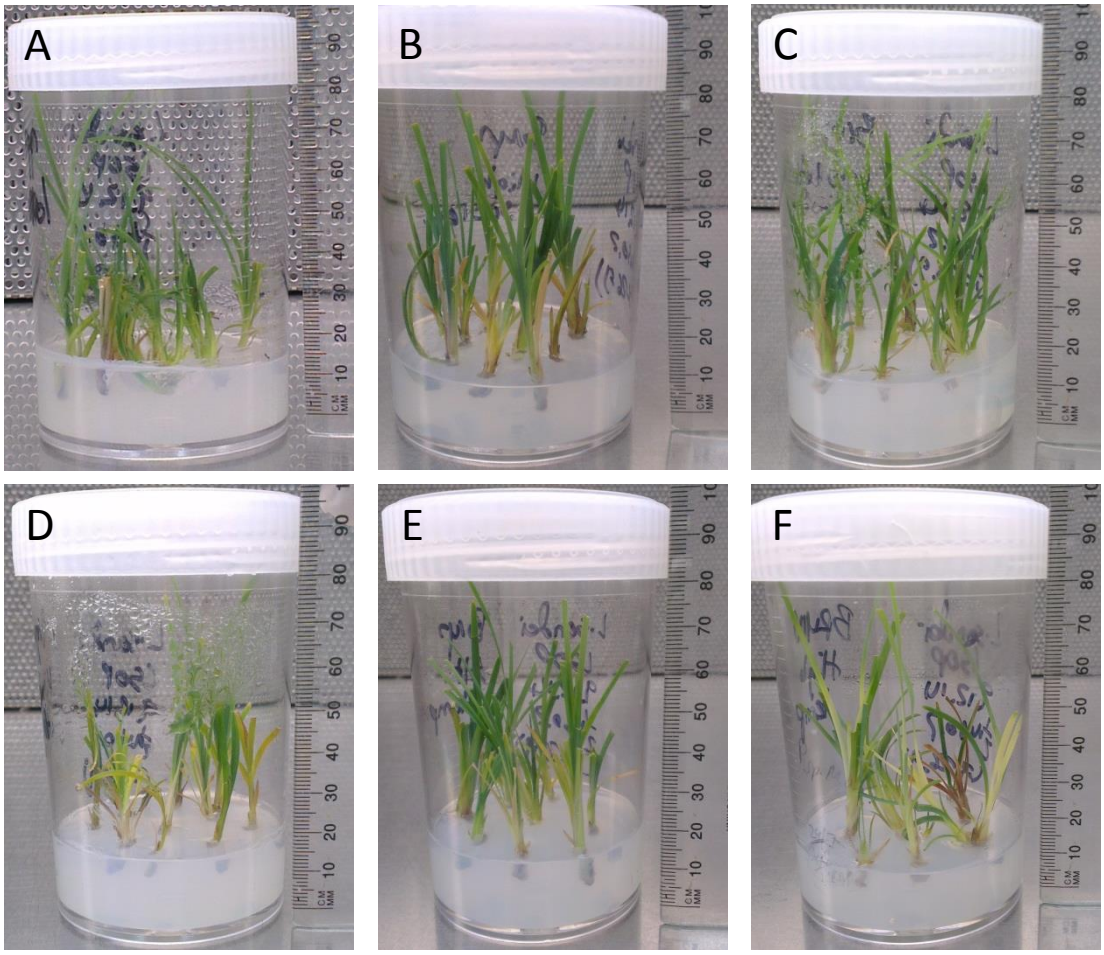

Figure 3.2. Representative preconditioned *in vitro* shoots. *In vitro* shoots of *Lomandra sonderi* exposed to room temperature (A), high sucrose (B), low light (C), high light (D), alternating temperature (E), high temperature (F) preconditioning regimes for 3 weeks.

preconditioned shoot tips did show a significantly lower survival rate (66%) compared to the room temperature and alternating temperature preconditioning regimes (with survival rates of 97% and 96%, respectively). No correlation was observed comparing control survival to post-cryogenic survival (Fig 3.5A, $R^2 = 0.04$).

Electrolyte leakage

As seen in Table 3.3, the preconditioned plants showed slight differences in cell membrane resistance to freezing temperatures, with the highest LT_{50} of -2.95°C in the control, followed by high light (-3.2°C), low light (-3.3°C), high temperature (-3.67°C), whilst the greatest resistance was observed in the alternating temperature preconditioning regime (-4.71°C). However, no statistically significant differences in LT_{50} were detected between the preconditioning regimes. The high sucrose preconditioned leaf material did not show a sigmoidal response to the freezing temperatures, which may be due to the large amount of dead leaf material observed after three weeks. The LT_{50} also showed no correlation to post-cryogenic survival (Fig 3.5B, $R^2 = 0.12$).

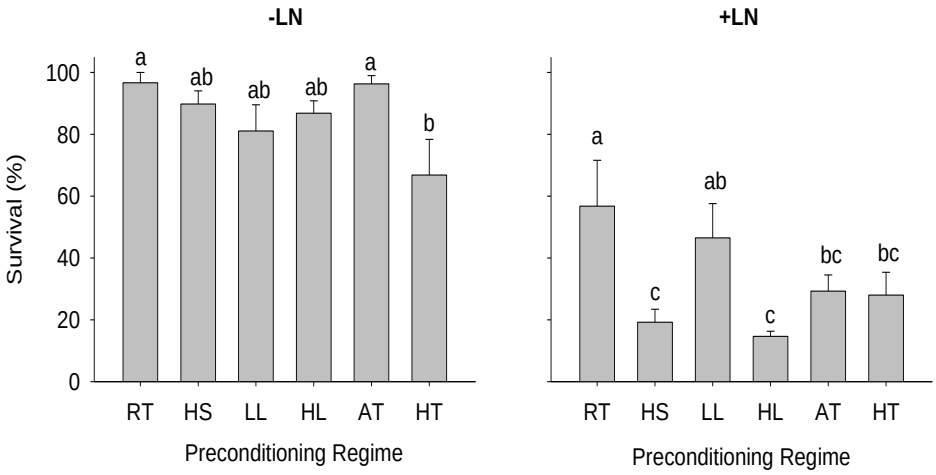

Figure 3.3. Survival of *L. sonderi* shoot tips. Effect of various preconditioning regimes under control (-LN) conditions. (B) Effect of droplet-vitrification protocol (+LN). Preconditioning regimes: RT, room temperature; AT, alternating temperature; HT, high temperature; LL, low light; HL, high light; HS, high sucrose. The bars represent the standard error of the means. Bars labelled with different letters have significant differences across the preconditioning regimes ($P < 0.05$).

Antioxidant analysis of preconditioned shoot tips

The activity of SOD was measured by quantifying the enzymatic inhibition of the formation of the superoxide radical, with increased inhibition indicating increased antioxidant activity in the samples. SOD activity was determined to be greatest in the shoot tips exposed to the room temperature preconditioning regime (39%), and was significantly higher than in the high temperature, high sucrose and alternating temperature preconditioning regimes (26%, 21% and 18% activities, respectively, as shown in Fig 3.4A). The low light and high light preconditioning regimes (36% and 31% activities, respectively) showed no significant difference to the room temperature preconditioning regime. A positive correlation was observed between SOD activity and post-cryogenic survival (Fig 3.5C, $R^2 = 0.46$, $P = 0.02$).

GR activity was measured by detecting the formation of TNB^{2-} when GSH reacts with DNTB. The highest activity was detected with the high light preconditioning regime (313 mU g^{-1} FW), and was significantly higher than that in the high temperature preconditioning regime, which showed the lowest activity (136 mU g^{-1} FW, as shown in Fig 3.4B). No significant differences were observed between the other preconditioning regimes and no correlation was detected between GR activity and post-cryogenic survival (Fig 3.5D, $R^2 = 0.03$).

Table 3.3. Effects of preconditioning regimes on LT_{50} temperature (°C ± SE).

PRECONDITIONING	**LT_{50} (°C)**
Room Temperature	-2.95 ± 1.72
High Sucrose	NA
Low Light	-3.30 ± 1.61
High Light	-3.20 ± 2.71
Alternating Temperature	-4.71 ± 1.36
High Temperature	-3.67 ± 2.19

GPx activity was measured by detecting the reduction of NADPH in the samples as it was consumed during the catalytical conversion of GSSG to GSH, since GSSG is formed upon the reduction of cumene hydroperoxide by GPx. The results of this analysis suggested that the larger the extent of NADPH reduction, the greater the activity of GPx. The largest activity was detected in the high light preconditioning regime (4379 mU g^{-1} FW) followed by the room temperature, low light, high sucrose, alternating temperature and high temperature preconditioning regimes (4360, 3914, 3041, 2331 and 1675 mU g^{-1} FW, respectively, as shown in Fig 3.4C). Nonetheless, no statistically significant differences were detected between any of the preconditioning regimes. A weak positive correlation was detected between GPx activity and post-cryogenic survival (Fig 3.5E, $R^2 = 0.10$).

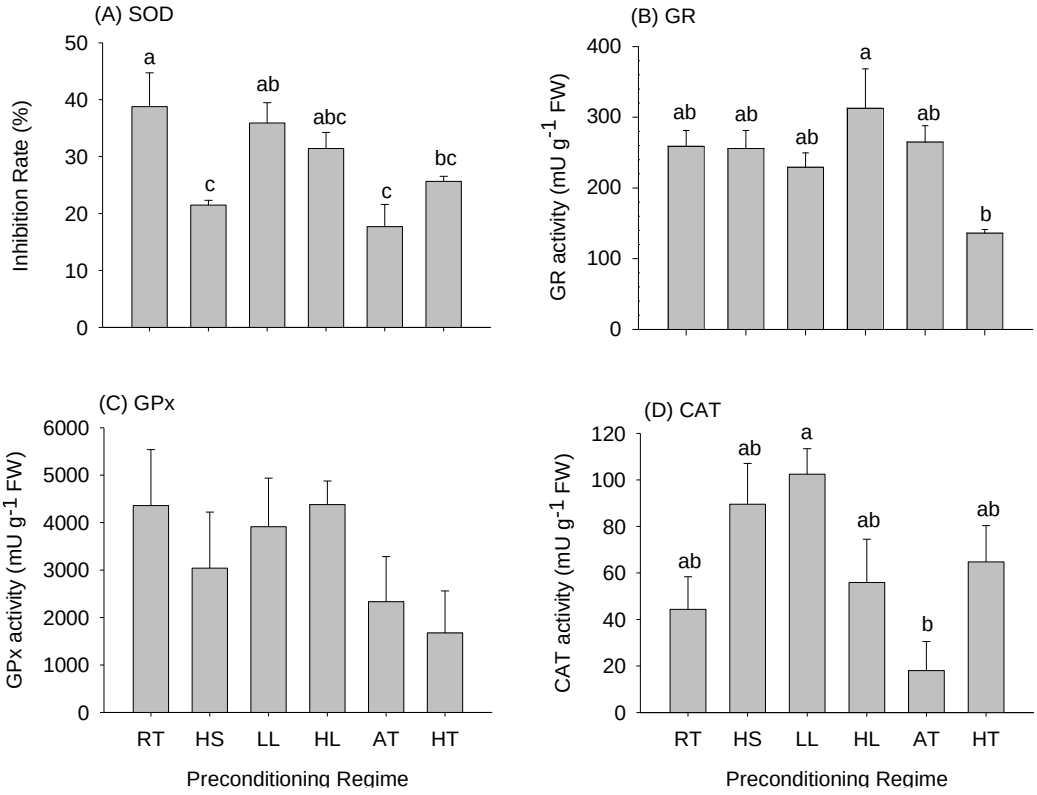

Figure 3.4. Effects of various preconditioning regimes on the antioxidant enzyme activity of *L. sonderi*. The bars represent the standard error of the means Preconditioning regimes: RT, room temperature; AT, alternating temperature; HT, high temperature; LL, low light; HL, high light; HS, high sucrose.

CAT activity was measured by detecting the coloured product formed when unconverted H_2O_2 reacts with the OxiRed probe. CAT activity was highest in the low light preconditioning regime (102 mU g^{-1} FW, as shown in Fig 3.4D). This was significantly higher than in the alternating temperature preconditioning regime (18 mU g^{-1} FW). No statistically significant differences were detected in the other preconditioning regimes, and no correlation was detected between CAT activity and post-cryogenic survival (Fig 3.5F, $R^2 = 0.00$).

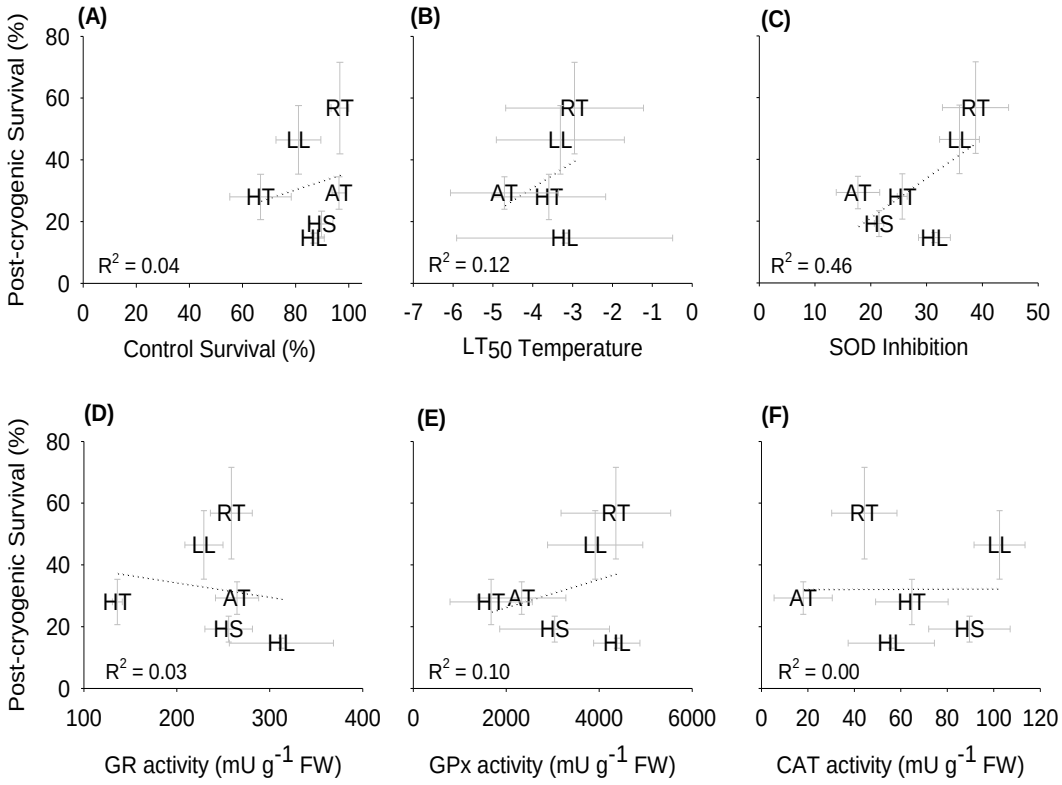

Figure 3.5. Scatter plot comparing control survival, electrolyte leakage and antioxidant activity to post-cryogenic survival. Preconditioning regimes: RT, room temperature; AT, alternating temperature; HT, high temperature; LL, low light; HL, high light; HS, high sucrose.

Comparison of antioxidant analysis of shoot tips during stages of the cryopreservation protocol

Shoot tips were also analysed for GR, GPx and CAT activity after preconditioning, preculture, and 1 and 7 days post-cryopreservation (1 d R and 7 d R, respectively). The activity of CAT showed no significant difference between the preconditioned, precultured, 1 day recovered shoot tips and 7 day recovered shoot tips (see Fig 3.6A), however, both preconditioning regimes showed increased CAT activity after the preculture stage. The room temperature preconditioned shoot tips showed decreased CAT activity after cryopreservation, while the alternating temperature preconditioned shoot tips showed an increase in the 1 day recovered shoot tips, which then reduced for the 7 day recovered shoot tips. Fig (3.6B) shows that the activity of GPx in the room temperature preconditioned shoot tips decreased significantly once the shoot tips had been cryopreserved (from 4207 to 1317 mU g^{-1} FW), whilst in the alternating temperature preconditioned shoot tips the activity of GPx increased from 2331 to 3110 to 3562 mU g^{-1} FW for the preconditioned, precultured and 1 day recovered shoot tips, respectively. This activity then fell for the 7 day recovered shoots to 2082 mU g^{-1} FW, which was just slightly higher than the room temperature 7 day recovered shoot tips. No statistically significant differences were observed between the various cryopreservation stages in the alternating temperature preconditioned shoot tips. In the case of GR (as seen in Fig 3.6C), its activity changed significantly throughout the cryopreservation protocol, increasing significantly during the 2 day preculture in comparison to the preconditioned shoot tips (from 259 to 419 mU g^{-1} FW in the case of the room temperature preconditioning regime). GR activity then decreased significantly after cryopreservation, with 1 d R shoot tips showing an activity of 93 mU g^{-1} FW in the room temperature preconditioning regime. GR activity continued to drop during the recovery phase to 10 mU g^{-1} FW after 7 days in the room temperature preconditioning regime. Comparison between the room and alternating temperature preconditioning regimes showed no significant differences between these two regimes throughout the cryopreservation process, with GR activity being, in fact, very similar in both regimes.

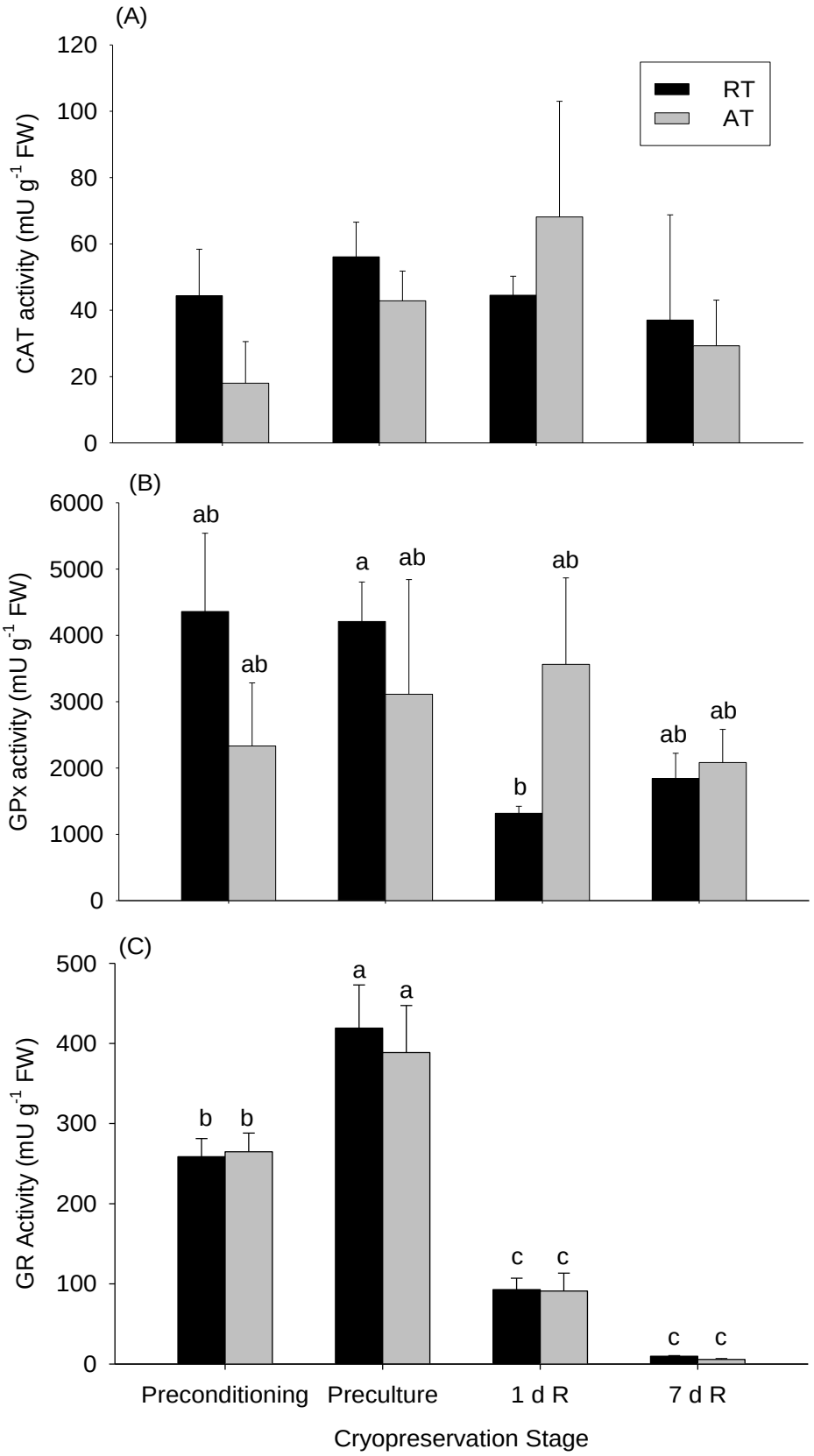

Figure 3.6. Antioxidant activity of CAT, GPx and GR through the cryopreservation protocol comparing between the 3 week room temperature (RT, black bars) or alternating temperature (AT, grey bars) preconditioning regime.

Discussion

This study aimed at measuring and understanding the influence of abiotic stress factors on the post-cryogenic survival of the south-west Western Australian Mediterranean species *L. sonderi*. A variety of stresses were investigated during the preconditioning regime, such as high temperature stress, osmotic stress and high and low light stress, with a view to determining the effects of these different abiotic conditions on post-cryogenic survival, electrolyte leakage and antioxidant activity (Table 3.1).

Stress preconditioning commonly uses a low non-freezing cold temperature stress to alter cellular compositions, such as membrane structure and fluidity, antioxidant concentrations and accumulation of solutes, to give the sample material the greatest possibility of surviving the stresses of cryopreservation (Funnekotter et al. 2013; Menon et al. 2014; Baek and Skinner 2003). The application of a cold temperature preconditioning regime prior to cryopreservation has indeed been shown to increase post-cryogenic survival in *L. sonderi* (Menon et al. 2014). Interestingly in this study, the application of an abiotic stress preconditioning regime did not appear to increase post-cryogenic survival of *L. sonderi*, with the highest survival rate achieved observed in the room temperature treatment regime, which showed more than 60% survival following cryogenic storage (Fig 3.3). The assessment of control survival (-LN) was carried out to separate the toxic effects of cryoprotective agents from any possible harmful effects of the preconditioning regimes. For these non-cryopreserved treatments the high temperature preconditioning regime was the only treatment that showed statistically significantly lower survival (67%) compared to all the other treatments. This high temperature (35°C) preconditioning regime may have likely damaged the plant before the shoot tips were isolated. In addition, the increased dead leaves of the plant (Table 3.2) made shoot tip excision more difficult as the tissue was more fibrous, thus possibly resulting in more damage to the shoot tips during excision.

The effect of the preconditioning regimes on post-cryogenic survival appears to have been detrimental, with the exception of the low light preconditioning regime, which showed slightly lower post-cryogenic survival (no statistically significance difference to the room temperature preconditioning). The high sucrose and high light preconditioning regimes resulted in the lowest observed cryogenic survival, with both below 20%. Plants in both of these preconditioning regimes were more fibrous and had more dead leaves compared to those in the room temperature preconditioning treatment. The alternating temperature and high temperature regimes also resulted in significantly lower post-cryogenic survival in comparison to the room temperature

preconditioning. The application of a cold alternating temperature (20/-1°C) preconditioning regime has previously been shown to increase post-cryogenic survival in *L. sonderi* (Menon et al. 2014), and it is not clear as to why it did not have a similar effect in this study. This may be due to the less extreme temperatures used compared to those used by Menon et al. (2014), although the post-cryogenic survival rates were similar when the alternating temperature preconditioning regime was applied: 32% as reported by Menon et al. (2014) and 29% in this study. Interestingly, survival rates in the room temperature preconditioning regime differed substantially, with 56% found in this study compared to 14% in the study of Menon et al. (2014). This difference may also be attributed to the influence of the operator on shoot tip extraction or the additional age of the *in vitro* shoots as the same clonal line is used in this study.

Electrolyte leakage (LT_{50})

The process of cold-acclimation can stabilise cell membranes against cellular dehydration induced by freezing (Thomashow et al. 1997), however not all plant species show this response. The extent of cell membrane damage can be quantified by measuring electrolyte leakage, as the larger the extent of damage to the integrity of membranes, the larger the amount of electrolytes that will leak out of cells. The preconditioning regimes did result in non-significant alterations to the LT_{50}, where the alternating temperature preconditioned shoots showed the greatest resistance to cold temperatures. Previously the application of an alternating cold temperature preconditioning regime was reported to decrease the LT_{50} (from -2.5 to -5.5°C); however, the chill tolerance achieved was not as great (Menon et al. 2014). This provides further confirmation that the temperature of -1°C used previously in the alternating temperature preconditioning regime applied by Menon et al. (2014) led to a greater cold acclimation response in *L. sonderi*.

Cold hardening of plants by the application of a high sucrose medium has shown similar effects to cold acclimation in date palm (Fki et al. 2013); however, due to the non-sigmoidal curve observed in our determinations, it was not possible to determine if the high sucrose preculture resulted in increased freezing tolerance. Light levels (both high and low light) during preconditioning had little effect on LT_{50} in comparison to the control preconditioning regime. These preconditioning regimes were not expected to alter the chilling tolerance of *L. sonderi,* as light should have little effect on cell membrane composition and stability unless excessive photo-oxidation occurs, which may lead to lipid peroxidation and damage to critical membranes. The high

temperature preconditioning regime resulted in an increased resistance of cell membranes to freezing temperatures but, like in the case of the high sucrose preconditioning regime, this may be due to the high amount of dead leaf material observed in the *in vitro* shoots after the preconditioning regime. Collection of any dead or dying leaf material for electrolyte leakage analysis is unlikely to give consistent measurements between the replicates.

Whilst the preconditioning regimes did alter the electrolyte leakage observed, the LT_{50} temperatures determined were not found to correlate with increased post-cryogenic survival. For example, leaf material from the room temperature preconditioning regime showed the least resistance to freezing temperatures but exhibited the greatest post-cryogenic survival, whilst leaf material from the alternating temperature preconditioning regime showed the greatest resistance to freezing temperature, but exhibited only average survival after cryopreservation. There have been many reports that have linked cold acclimation, defined as increased tolerance to freezing temperatures after exposure to low non-freezing temperatures (Hincha and Zuther 2014), to increased cryopreservation survival; however, the cold acclimation response of *L. sonderi* is limited, with only a small change in LT_{50} (2-3°C). Species that show a greater change in LT_{50} (>10°C), such as peach (Arora et al. 1992), may exhibit a better correlation to post-cryogenic survival. The advantage of cold acclimation in regards to improving post-cryogenic survival may not be directly linked to increased cell membrane freezing tolerance alone, but rather to other benefits such as increased sugar and solute content and greater antioxidant activity, both of which may protect the shoot tips throughout the cryopreservation protocol.

Antioxidants

Oxidative stress caused by increased ROS production can be initiated by a variety of abiotic stresses, such as desiccation, light (UV stress), temperature, salinity, heavy metals and ozone stress (Agarwal 2007; Chaitanya et al. 2002; Romero-Puertas et al. 2006; Sumithra et al. 2006; Zgallaï et al. 2006; Rao and Reddy 2008; McKersie et al. 1993). The ROS superoxide is commonly produced during mitochondrial electron transport and during photosynthesis. SOD is the major defence against the superoxide radical (Bowler et al. 1992). Exposure to high light levels has been shown to increase SOD production and activity in response to increased superoxide formation (Tsang et al. 1991). Increased SOD activity along with increased GR activity have also been observed in drought tolerant tomatoes and maize (Malan et al. 1990; Bowler et al. 1992). In this study, the level of SOD activity was determined to be

highest in the room temperature preconditioned shoot tips. The low light, high light and high temperature preconditioning regimes resulted in no significant changes in SOD activity, but showed reduced activity in comparison to the room temperature preconditioned shoot tips. High light exposure (5000 lux) has been shown to increase SOD transcription (Tsang et al. 1991); however, an increase in SOD activity was not observed in this study, and this may be due to the light intensity being too low to induce a response, as the photosynthetic apparatus will not be over-excited resulting in the formation of the superoxide radical. Alternatively, increased SOD production may have indeed occurred in leaf material whilst shoot tips showed little change due to the low levels of photosynthesis that occur in them, thus limiting the increase in the levels of SOD required to protect the cells from ROS. The main source of ROS would then come from respiration, and this may be the reason why room temperature preconditioned shoot tips showed the highest level of SOD activity, as they have the highest growth of all the preconditioning regimes, whilst the alternating temperature preconditioning regime exhibited stunted growth and the lowest level of SOD activity.

Large amounts of H_2O_2 can be produced during photosynthesis (Asada 2006). Removal of this H_2O_2 is vital since otherwise the more damaging hydroxyl radical may be produced through Fenton's reaction (Halliwell and Gutteridge 2007). CAT protects cells from ROS damage as it decomposes H_2O_2 into water and oxygen. Little significant differences in CAT activity were detected between the preconditioning regimes. CAT activity differed significantly only between the low light and alternating temperature preconditioned shoot tips (Fig 4D), and showed no correlation to post-cryogenic survival (Fig 5F). GPx is also involved in protecting the cells from H_2O_2 as well as organic peroxides (Navrot et al. 2006). GPx activity showed no significant differences between the preconditioning regimes (Fig 4C), and a weak positive correlation to post-cryogenic survival was observed (Fig 5E). GR is important for maintaining the reduced sacrificial antioxidant GSH, which is an important antioxidant and multifunctional metabolite. The high light preconditioned shoot tips showed the greatest GR activity, significantly higher than the high temperature preconditioned shoot tips. No other differences were observed between the rest of the preconditioning regimes.

The application of the high light preconditioning regime differed from the rest of the preconditioning regimes, as revealed by the higher SOD, GR and GPx antioxidant activities than would be expected in view of the rates of post-cryogenic survival (Fig 3.5). These observations suggest that antioxidant activity of SOD, GR, GPx and

possibly CAT in the high light preconditioned shoot tips is unlikely to be the reason for their low post-cryogenic survival as the antioxidant activity is not compromised.

Changes in antioxidants activity throughout the cryopreservation protocol

Whilst the analysis of antioxidant activity provided insight into the effects that preconditioning can have on *in vitro* shoots, it appears that the antioxidant activity observed in the preconditioned shoots had large variations throughout the cryopreservation protocol, as determined by the levels of activity of GR, GPx and CAT measured after the 2 day preculture as well as 1 and 7 days following cryopreservation and placement on recovery medium (Fig 6). While GR activity in the preconditioned shoot tips did not show any correlation to post-cryogenic survival, the activity of GR showed the greatest variation through the cryopreservation stages. Its activity increased after the preculture stage, possibly in relation to the excision damage as the shoot tips recover during this period. GR activity showed a large reduction in activity after the cryopreservation protocol and continued to decline during 7 days following cryopreservation. The low level of GR activity may be due to the high amount of dead tissue present in the recovering shoot tips, as it is expected that over 40% of the shoot tips will not survive the cryopreservation process due to cryo-injury (60% survival was observed in the room temperature preconditioned shoot tips). These dead shoot tips would affect the apparent enzymatic activity, leading to the lower level of GR activity detected. However, GR activity showed a greater sensitivity to cryo-injury in comparison to GPx and CAT, which did not show a large reduction in activity after cryopreservation. GR is vital for maintaining the redox balance within the cells, not just for GSH but also for ascorbate. Without sufficient levels of GR activity the cells can become oxidised, which may lead to excessive damage and a reduction in post-cryogenic survival or in slowed regrowth of shoot tips as they attempt to repair the oxidative damage. Continued examination of GR activity may be useful to determine if GR activity can return to the rate observed in the preconditioned shoot tips, as declining antioxidant enzyme activity has been linked to senescence (Bowler et al. 1992). GPx is an important antioxidant enzyme involved in scavenging H_2O_2 and reducing lipid peroxides into their respective alcohols. Little significant differences were observed in GPx activity throughout the cryopreservation protocol. The only change noted was a reduction in GPx activity after cryopreservation in the control preconditioned shoot tips, similarly to the reduction in GR activity. Whilst GPx activity decreased in the 7 day old post-cryogenic shoot tips, the activity was not significantly different to that in the preconditioned shoot tips. CAT activity showed the least change throughout the cryopreservation protocol. The control preconditioning

regime showed a greater CAT activity compared to the alternating temperature preconditioning regime; however, this difference was greatest in the preconditioned shoot tips.

Conclusions

Six preconditioning regimes were investigated in this study and room temperature preconditioned shoot tips showed the greatest post-cryogenic survival, followed by low light preconditioning, alternating temperature, high temperature, high sucrose and high light preconditioning, as shoots showed progressively lower post-cryogenic survival rates than in the room temperature regime. Analysis of the freezing tolerance showed little correlation to post-cryogenic survival; however, *L. sonderi* showed only limited increase in freezing tolerance (LT_{50}). The resistance of plants to the deleterious effects of cryopreservation may be enhanced by increasing the availability of antioxidants in the tissue before exposure to the stresses of cryopreservation.

The antioxidant activities of SOD and GPx in the preconditioned shoot tips showed a positive correlation to post-cryogenic survival, whilst GR and CAT activities showed no correlation. Analysis of the antioxidants showed that GR activity significantly decreased after cryopreservation, whilst GPx and CAT did not change. The high light preconditioned shoot tips showed increased antioxidant activity, exhibiting the greatest GR and GPx activities of all the preconditioning regimes, but nonetheless having the lowest post-cryogenic survival rate. It is not clear why the high light preconditioning regime led to such low survival rates; however, there are many other factors that can influence post-cryogenic survival, including the many other antioxidants such as ascorbate peroxidases and the major sacrificial antioxidant ascorbic acid.

References

Agarwal, S. 2007. "Increased Antioxidant Activity in *Cassia* Seedlings under Uv-B Radiation." *Biologia Plantarum* 51 (1): 157-160

Apel, K., and H. Hirt. 2004. "Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction." *Annual Review of Plant Biology* 55: 373-399

Arora, R., M.E. Wisniewski, and R. Scorza. 1992. "Cold Acclimation in Genetically Related (Sibling) Deciduous and Evergreen Peach (*Prunus Persica* [L.] Batsch) I. Seasonal Changes in Cold Hardiness and Polypeptides of Bark and Xylem Tissues." *Plant Physiology* 99 (4): 1562-1568

Asada, K. 2006. "Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions." *Plant Physiology* 141 (2): 391-396

Baek, K.H., and D.Z. Skinner. 2003. "Alteration of Antioxidant Enzyme Gene Expression During Cold Acclimation of near-Isogenic Wheat Lines." *Plant Science* 165 (6): 1221-1227

Bajaj, Y.P.S. 1995. "Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity." In *Biotechnology in Agriculture and Forestry 32*, ed. Y.P.S. Bajaj, 1-18. New York: Springer.

Benson, E.E. 2008. "Cryopreservation Theory." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 15-32. New York: Springer.

Bowler, C., M. van Montagu, and D. Inze. 1992. "Superoxide Dismutase and Stress Tolerance." *Annual Review of Plant Biology* 43 (1): 83-116

Chaitanya, K., D. Sundar, S. Masilamani, and A.R. Reddy. 2002. "Variation in Heat Stress-Induced Antioxidant Enzyme Activities among Three Mulberry Cultivars." *Plant growth regulation* 36 (2): 175-180

Chang, Y., R.E. Barker, and B.M. Reed. 2000. "Cold Acclimation Improves Recovery of Cryopreserved Grass *(Zoysia* and *Lolium* Sp.)." *CryoLetters* 21 (2): 107-116

Crowe, J.H., L.M. Crowe, J.F. Carpenter, A. Rudolph, C.A. Wistrom, B. Spargo, and T. Anchordoguy. 1988. "Interactions of Sugars with Membranes." *Biochimica et Biophysica Acta* 947 (2): 367-384

Fki, L., N. Bouaziz, O. Chkir, R. Benjemaa-Masmoudi, A. Rival, R. Swennen, N. Drira, and B. Panis. 2013. "Cold Hardening and Sucrose Treatment Improve

Cryopreservation of Date Palm Meristems." *Biologia Plantarum* 57 (2): 375-379

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Gill, S.S., and N. Tuteja. 2010. "Reactive Oxygen Species and Antioxidant Machinery in Abiotic Stress Tolerance in Crop Plants." *Plant Physiology and Biochemistry* 48 (12): 909-930

Gratão, P.L., A. Polle, P.J. Lea, and R.A. Azevedo. 2005. "Making the Life of Heavy Metal-Stressed Plants a Little Easier." *Functional Plant Biology* 32 (6): 481-494

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Hincha, D., and E. Zuther. 2014. "Introduction: Plant Cold Acclimation and Freezing Tolerance." In *Plant Cold Acclimation*, eds Dirk K. Hincha and Ellen Zuther, 1-6. Springer New York.

Huner, N., G. Öquist, and F. Sarhan. 1998. "Energy Balance and Acclimation to Light and Cold." *Trends in Plant Science* 3 (6): 224-230

Kaczmarczyk, A., N. Shvachko, Y. Lupysheva, M.R. Hajirezaei, and E.R. Keller. 2008. "Influence of Alternating Temperature Preculture on Cryopreservation Results for Potato Shoot Tips." *Plant Cell Reports* 27 (9): 1551-8

Kaczmarczyk, A., S.R. Turner, E. Bunn, R.L. Mancera, and K.W. Dixon. 2011b. "Cryopreservation of Threatened Native Australian Species—What Have We Learned and Where to from Here?" *In Vitro Cellular & Developmental Biology - Plant* 47 (1): 17-25

Koch, J.M., and G.P. Samsa. 2007. "Restoring Jarrah Forest Trees after Bauxite Mining in Western Australia." *Restoration Ecology* 15: S17-S25

Kranner, I., and S. Birtić. 2005. "A Modulating Role for Antioxidants in Desiccation Tolerance." *Integrative and Comparative Biology* 45 (5): 734-740

Malan, C., M.M. Greyling, and J. Gressel. 1990. "Correlation between Cuzn Superoxide Dismutase and Glutathione Reductase, and Environmental and Xenobiotic Stress Tolerance in Maize Inbreds." *Plant Science* 69 (2): 157-166

McKersie, B.D., Y. Chen, M. de Beus, S.R. Bowley, C. Bowler, D. Inzé, K. D'Halluin, and J. Botterman. 1993. "Superoxide Dismutase Enhances Tolerance of Freezing Stress in Transgenic Alfalfa (*Medicago Sativa* L.)." *Plant Physiology* 103 (4): 1155-1163

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Mishra, N.P., R.K. Mishra, and G.S. Singhal. 1993. "Changes in the Activities of Anti-Oxidant Enzymes During Exposure of Intact Wheat Leaves to Strong Visible Light at Different Temperatures in the Presence of Protein Synthesis Inhibitors." *Plant Physiology* 102 (3): 903-910

Murashige, T., and F. Skoog. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473-497

Navrot, N., V. Collin, J. Gualberto, E. Gelhaye, M. Hirasawa, P. Rey, D.B. Knaff, E. Issakidis, J.-P. Jacquot, and N. Rouhier. 2006. "Plant Glutathione Peroxidases Are Functional Peroxiredoxins Distributed in Several Subcellular Compartments and Regulated During Biotic and Abiotic Stresses." *Plant Physiology* 142 (4): 1364-1379

Noctor, G., and C.H. Foyer. 1998. "Ascorbate and Glutathione: Keeping Active Oxygen under Control." *Annual Review of Plant Biology* 49 (1): 249-279

Noctor, G., G. Queval, A. Mhamdi, S. Chaouch, and C.H. Foyer. 2011. "Glutathione." *The Arabidopsis Book / American Society of Plant Biologists* 9: e0142. doi:10.1199/tab.0142

Paczkowska, G. 1998. *Lomandra Sonderi*. Western Australian Herbarium (FloraBase). Accessed 13 March, http://florabase.dec.wa.gov.au/browse/profile/1244.

Panis, B., and M. Lambardi. 2006. "Status of Cryopreservation Technologies in Plants (Crops and Forest Trees)." In *The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*, eds J. Ruane and A. Sonnino, 61-78. Rome, Italy: United Nations Food and Agriculture Organization (FAO).

Plummer, J., A. Crawford, and S. Taylor. 1995. "Germination of *Lomandra Sonderi* (Dasypogonaceae) Promoted by Pericarp Removal and Chemical-Stimulation of the Embryo." *Australian Journal of Botany* 43 (2): 223-230

Pritchard, H., B. Grout, and K. Short. 1986. "Osmotic Stress as a Pregrowth Procedure for Cryopreservation: 1. Growth and Ultrastructure of Sycamore and Soybean Cell Suspensions." *Annals of Botany* 57 (1): 41-48

Rao, A.C., and A.R. Reddy. 2008. "Glutathione Reductase: A Putative Redox Regulatory System in Plant Cells." In *Sulfur Assimilation and Abiotic Stress in Plants*, 111-147. Springer.

Reed, B. 2011. "Choosing and Applying Cryopreservation Protocols to New Plant Species or Tissues." *Acta Horticulturae* 908: 363-372

Romero-Puertas, M.C., F.J. Corpas, L.M. Sandalio, M. Leterrier, M. Rodríguez-Serrano, L.A. Del Río, and J.M. Palma. 2006. "Glutathione Reductase from Pea Leaves: Response to Abiotic Stress and Characterization of the Peroxisomal Isozyme." *New Phytologist* 170 (1): 43-52

Sakai, A., S. Kobayashi, and I. Oiyama. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb. Var. Brasiliensis Tanaka) by Vitrification." *Plant Cell Reports* 9 (1): 30-33

Smallwood, M., and D.J. Bowles. 2002. "Plants in a Cold Climate." *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 357 (1423): 831-847

Sumithra, K., P. Jutur, B.D. Carmel, and A.R. Reddy. 2006. "Salinity-Induced Changes in Two Cultivars of *Vigna Radiata*: Responses of Antioxidative and Proline Metabolism." *Plant Growth Regulation* 50 (1): 11-22

Tausz, M., H. Šircelj, and D. Grill. 2004. "The Glutathione System as a Stress Marker in Plant Ecophysiology: Is a Stress-Response Concept Valid?" *Journal of Experimental Botany* 55 (404): 1955-1962

Thomashow, M.F., E.J. Stockinger, K.R. Jaglo-Ottosen, S.J. Gilmour, and D.G. Zarka. 1997. "Function and Regulation of *Arabidopsis Thaliana* Cor (Cold-Regulated) Genes." *Acta Physiologiae Plantarum* 19 (4): 497-504

Tsang, E., C. Bowler, D. Hérouart, W. Van Camp, R. Villarroel, C. Genetello, M. Van Montagu, and D. Inzé. 1991. "Differential Regulation of Superoxide Dismutases in Plants Exposed to Environmental Stress." *The Plant Cell Online* 3 (8): 783-792

Uemura, M., R.A. Joseph, and P.L. Steponkus. 1995. "Cold Acclimation of Arabidopsis Thaliana (Effect on Plasma Membrane Lipid Composition and Freeze-Induced Lesions)." *Plant Physiology* 109 (1): 15-30

Uemura, M., Y. Tominaga, C. Nakagawara, S. Shigematsu, A. Minami, and Y. Kawamura. 2006. "Responses of the Plasma Membrane to Low Temperatures." *Physiologia Plantarum* 126 (1): 81-89

Vandenbussche, B., S. Leurdian, V. Verdoodt, M. Gysemberg, and M. De Proft. 1999. "Changes in Sugar Content and Fatty Acid Composition of in Vitro Sugar Beet Shoots after Cold Acclimation: Influence on Survival after Cryopreservation." *Plant Growth Regulation* 28 (3): 157-163

Whitaker, C., R.P. Beckett, F.V. Minibayeva, and I. Kranner. 2010. "Production of Reactive Oxygen Species in Excised, Desiccated and Cryopreserved Explants of *Trichilia Dregeana* Sond." *South African Journal of Botany* 76 (1): 112-118

Willyams, D. 2005. "Tissue Culture of Geophytic Rush and Sedge Species for Revegetation of Bauxite Mine Sites in the Northern Jarrah Forest of Western Australia." In *Contributing to a sustainable future. Proceedings of the Australian Branch of the International Association for Plant Tissue Culture & Biotechnology. IAPTC&B, Perth, Western Australia*, 226–241.

Zgallaï, H., K. Steppe, and R. Lemeur. 2006. "Effects of Different Levels of Water Stress on Leaf Water Potential, Stomatal Resistance, Protein and Chlorophyll Content and Certain Anti - Oxidative Enzymes in Tomato Plants." *Journal of Integrative Plant Biology* 48 (6): 679-685

Chapter 4 – Antioxidant analysis of low-molecular weight thiols and ascorbic acid during cryopreservation of native Australian plant shoot tips

Abstract

Low molecular weight (LMW) thiols (cysteine, *gamma*-glutamylcysteine and glutathione) and ascorbic acid, which represent, the two major water soluble antioxidants in plants, were analysed throughout the cryopreservation protocol of three native Australian plant species (*Anigozanthos viridis, Lomandra sonderi* and *Loxocarya cinerea*). Post-cryogenic regeneration was greatest in *A. viridis* (78%), then *L. sonderi* (50%), whilst *L. cinerea* did not show any post-cryogenic regeneration. The application of a 3-week 5°C preconditioning regime resulted in significantly lower post-cryogenic regeneration for *A. viridis* (33%) but had little effect on the other two species. Total antioxidant concentration decreased significantly with each step throughout the cryopreservation protocol, particularly in the cryoprotection and washing stages. Antioxidant levels then increased during the subsequent 7-day post-cryopreservation recovery period, with the greatest increase measured in *A. viridis* shoot tips. Concentrations of LMW thiols and their corresponding disulphides were used to calculate the corresponding half-cell reduction potentials. Results suggest that the ability of these plant species to maintain a strong reducing environment throughout the cryopreservation protocol was found to correlate with post-cryogenic survival.

Introduction

The application of cryopreservation for the conservation of plant species allows the long-term storage of a wide range of plant material, usually in liquid nitrogen (LN) (Bajaj 1995). Cryopreservation relies on the ability of specimens to regenerate after rewarming from ultra-low (-196°C) temperature storage. Cryopreservation typically involves a multi-stage process, including the propagation of the plant species in tissue culture, excision of shoot tips, preculture, exposure to cryoprotective agents (CPAs), ultra-cooling, rewarming, washing (also known as unloading) and recovery stages (Kaczmarczyk et al. 2012). There is a wide variation in cryo-capability, particularly between species, but it also appears within clonal lines of species as well (Reed

2008). Understanding why some species exhibit low survival and regeneration after cryopreservation is vital for advancing the techniques currently used in this process.

The stresses involved in the cryopreservation process can lead to the formation of reactive oxygen species (ROS) that can disrupt the delicate redox balance of cells, leading to oxidative stress and reduced regeneration of plant samples after cryopreservation (Benson and Bremner 2004). ROS include damaging free radicals such as superoxide and hydroxyl radical, as well as non-radicals such as hydrogen peroxide and singlet oxygen, all of which can interact with and damage a wide range of cellular components such as lipids, proteins and nucleic acids (Halliwell and Gutteridge 2007). Antioxidants such as glutathione and ascorbic acid (AsA) constitute a major line of defence against ROS because they are preferentially oxidised to form relatively unreactive products that are rapidly reduced or exported from the cell, a mechanism which maintains redox homeostasis within cells and prevents oxidative damage to lipids, proteins and nucleic acids (Benson and Bremner 2004; Halliwell and Gutteridge 2007; Foyer and Noctor 2011).

Glutathione is a tripeptide (γ-glutamylcysteinylglycine) found in most eukaryotes and some prokaryotes. Along with other low-molecular weight (LMW) thiols, such as *gamma*-glutamylcysteine (γ-Glu-Cys) and cysteine (Cys), glutathione contains a sulphhydryl group that can act as a strong reducing agent. In addition to their antioxidant roles, LMW thiols are important for the transport of reduced sulphur (Tausz et al. 2004). Reduced glutathione (GSH) is a direct scavenger of ROS including $OH^{\bullet}$, $^{1}O_2$, HOCl and H_2O_2, and also removes H_2O_2 indirectly by acting as a co-substrate for the glutathione peroxidase enzyme. GSH also protects protein-SH groups from oxidation, acts as a signalling molecule and participates in the ascorbate-glutathione cycle (Halliwell and Gutteridge 2007; Noctor et al. 2012; Foyer et al. 2008). The ratio of reduced GSH to glutathione disulphide (GSSG, oxidised glutathione) is usually very high (>80%), and GSSG is rapidly reduced back to GSH by glutathione reductase. Due to the high levels of GSH present in cells, the ratio of GSH to GSSG is a large contributor to the 'redox state', and the half-cell reduction potential of the glutathione/glutathione disulphide redox couple is used as an indicator of the cellular redox state (Schafer and Buettner 2001; Foyer et al. 2008). Cys and γ-Glu-Cys are precursors of glutathione. The first step in the glutathione biosynthesis pathway is catalysed by gamma-glutamate-cysteine ligase to form γ-Glu-Cys, and the second step is catalysed by glutathione synthetase to form GSH (Noctor et al. 2012).

AsA, also known as vitamin C or ascorbate, is found in almost all eukaryotic cells and has a wide range of functions for protecting the cells from ROS damage. AsA has

been shown to scavenge $O_2^{\bullet-}$, $HO_2^{\bullet}$, $OH^{\bullet}$, $^1O_2$, HOCl and reduce alpha-tocopheroxyl to alpha-tocopherol, and is also an important co-factor for ascorbate-dependant peroxidases (Halliwell and Gutteridge 2007). AsA is the major water soluble antioxidant in plants and is particularly abundant in chloroplasts, with concentrations of up to 20 mM compared to around 3-5 mM for glutathione (Foyer et al. 2008; Smirnoff and Wheeler 2000). However, AsA may have a pro-oxidant effect in the presence of transition metals such as iron and copper, resulting in the formation of the hydroxyl radical. These pro-oxidant effects are not limited to AsA as GSH, tocopherol, NAD(P)H and several plant phenolics have also demonstrated similar effects, which must be taken into consideration as a possible source of ROS production, particularly since iron is a component of the propagation medium used for plant tissue culture (Antony et al. 2013; Uchendu et al. 2010a).

Little is known about how antioxidants are affected throughout the cryopreservation protocol or indeed whether they can be utilised to inform or improve current cryogenic approaches. Whitaker et al. (2010) observed the production of superoxide in *Trichilia dregeana* embryonic axes at multiple stages in the cryopreservation protocol and suggested that excessive ROS damage was the cause of low survival. Increased levels of volatile gases were detected in carrot cells after cryopreservation and thawing, indicating the occurrence of extensive membrane damage due to lipid peroxidation (Benson and Withers 1987). As antioxidants are the natural defence against the accumulation of ROS and their by-products, plant material with intrinsically high antioxidant capacity may be more stress tolerant. The application of exogenous antioxidants during the cryopreservation protocol has been shown to increase survival and regeneration post-cryopreservation in some studies (Uchendu et al. 2010a; Uchendu et al. 2010b; Wang and Deng 2004), but does not always improve survival (Canepa et al. 2011).

Preconditioning prior to cryopreservation improves post-cryogenic survival for a wide range of species (Chang et al. 2000; Funnekotter et al. 2013; Kaczmarczyk et al. 2008; Leunufna and Keller 2005; Gupta and Reed 2006; Menon et al. 2012). Preconditioning typically involves exposing the *in vitro* shoots to low non-freezing temperatures to induce an acclimation response which results in better survival when shoots are subsequently exposed to freezing temperatures (Hincha and Zuther 2014; Thomashow 1998). Preconditioned shoot tips are more resistant to temperature stresses due to a variety of cellular changes, such as increased content of sugars and solutes, altered fatty acid compositions which make membranes more resistant to leakage, and increased antioxidant capacity (Funnekotter et al. 2013; Fujikawa et al.

1999; Hincha and Zuther 2014; Kamata and Uemura 2004; Menon et al. 2014; Sasaki et al. 1996). Cold acclimated plant species also have decreased lipid peroxidation (O'Kane et al. 1996), increased glutathione production (Kocsy et al. 2000) and increased antioxidant activity (Prasad 1996). Some tropical species may, however, be susceptible to chilling injury when exposed to low temperature. Fadzillah *et al.* (1996) reported rice shoot cultures were unable to acclimate to low temperatures of 4°C and displayed decreased growth rates, increased H_2O_2 accumulation, reduced glutathione reductase and catalase activity, reduced glutathione content, and a lower ratio of GSH: GSSG compared to cultures at 25°C. Similar reductions in antioxidant activity have been reported with maize seedlings that were not able to cold acclimate (Prasad 1996). Exposure to low temperatures generally reduces the scavenging ability of antioxidants leading to ROS accumulation, which can operate as a signal for cold acclimation (Suzuki and Mittler 2006). It is suggested that plants that are best able to cold acclimate have a greater ability to mitigate this increased ROS production through increased production of antioxidants before the ROS titre becomes too high, whereas cold-sensitive species do not produce sufficient antioxidants to neutralise the increase of ROS resulting in lethal oxidative damage (Prasad 1996).

Australia contains a diverse range of plant species with over 70% found nowhere else in the world. South-west Western Australia is considered a biodiversity hotspot with over 8000 species, of which around 2600 species require some form of conservation and 420 of these currently considered to be threatened (Kaczmarczyk et al. 2011b; Department of Parks and Wildlife 2014). Three endemic Western Australian species were considered in this study: *Anigozanthos viridis* ssp. *terrapectans* (Haemodoraceae), *Loxocarya cinerea* (Restionaceae) and *Lomandra sonderi* (Asparagaceae). *Anigozanthos viridis* ssp. *terrapectans* is a small rhizomatous short-lived perennial herb that has a restricted range and is listed as a threatened species by the Western Australian Department on Parks and Wildlife (FloraBase, Western Australian Herbarium). *Loxocarya cinerea* and *Lomandra sonderi* are dioecious rhizomatous perennial herbs, which are important in post-mining restoration (Kaczmarczyk et al. 2013; Menon et al. 2012). These species are considered recalcitrant for post-mining rehabilitation efforts, due to their low seed production and complex dormancy issues. Thus these species are micropropagated under nursery conditions and planted back to mine restoration sites by hand to reinstall species diversity in the restored sites to levels reflective of the pre-mining environment. Prior research has indicated that *A. viridis* and *L. sonderi* can be cryopreserved using vitrification protocols, while *L. cinerea* has proven extremely difficult with only very low

and sporadic regeneration from shoot tips after cryostorage in liquid nitrogen (Menon et al. 2012; Kaczmarczyk et al. 2013; Turner et al. 2000).

This study aimed to determine if antioxidant homeostatic capacity of shoot material could be artificially improved (via temperature preconditioning treatments), and if this might, in turn, improve stress tolerance and consequently show greater recovery from cryogenic conditions. The effect of preconditioning on post-cryogenic survival and antioxidant content of apical shoot tips was investigated in three Australian species subject to three different temperature regimes. LMW thiols and their corresponding half-cell reduction potentials as well as AsA content were analysed to determine (a) how the concentrations of antioxidants change and, (b) whether differences in antioxidant capacity (reduction potential of antioxidants) between species are related to their ability to survive stresses incurred during the cryopreservation process.

Materials and methods

Plant material

Anigozanthos viridis and *L. sonderi in vitro* shoots were obtained from Kings Park and Botanic Gardens. *Loxocarya cinerea in vitro* shoots were obtained from Alcoa of Australia's Marrinup Nursery in Western Australia. All three species were subcultured on a half strength Murashige and Skoog basal medium (BM) as previously described (Funnekotter et al. 2013; Kaczmarczyk et al. 2013; Menon et al. 2012). *Anigozanthos viridis* and *L. cinerea* were maintained on BM containing 60 mM sucrose, 0.8% (w/v) agar and 0.5 µM 6-benzylaminopurine (BA). *Lomandra sonderi* was maintained on the same medium described above except with 0.2 µM BA. All species were subcultured every 3-4 weeks.

Preconditioning

Three different temperature preconditioning regimes were applied for 3 weeks prior to cryopreservation and antioxidant analysis. *In vitro* shoots from all three species were placed at either a control temperature of 25°C, an alternating temperature regime of 25/5°C day/night cycle or a low temperature of 5°C, all with a 12 h photoperiod (photosynthetic photon flux density of ~30 µmol $m^{-2}s^{-1}$ at culture level, supplied via 2 x 21 W fluorescent lamps per 60 x 90 cm shelf) (Fig 1).

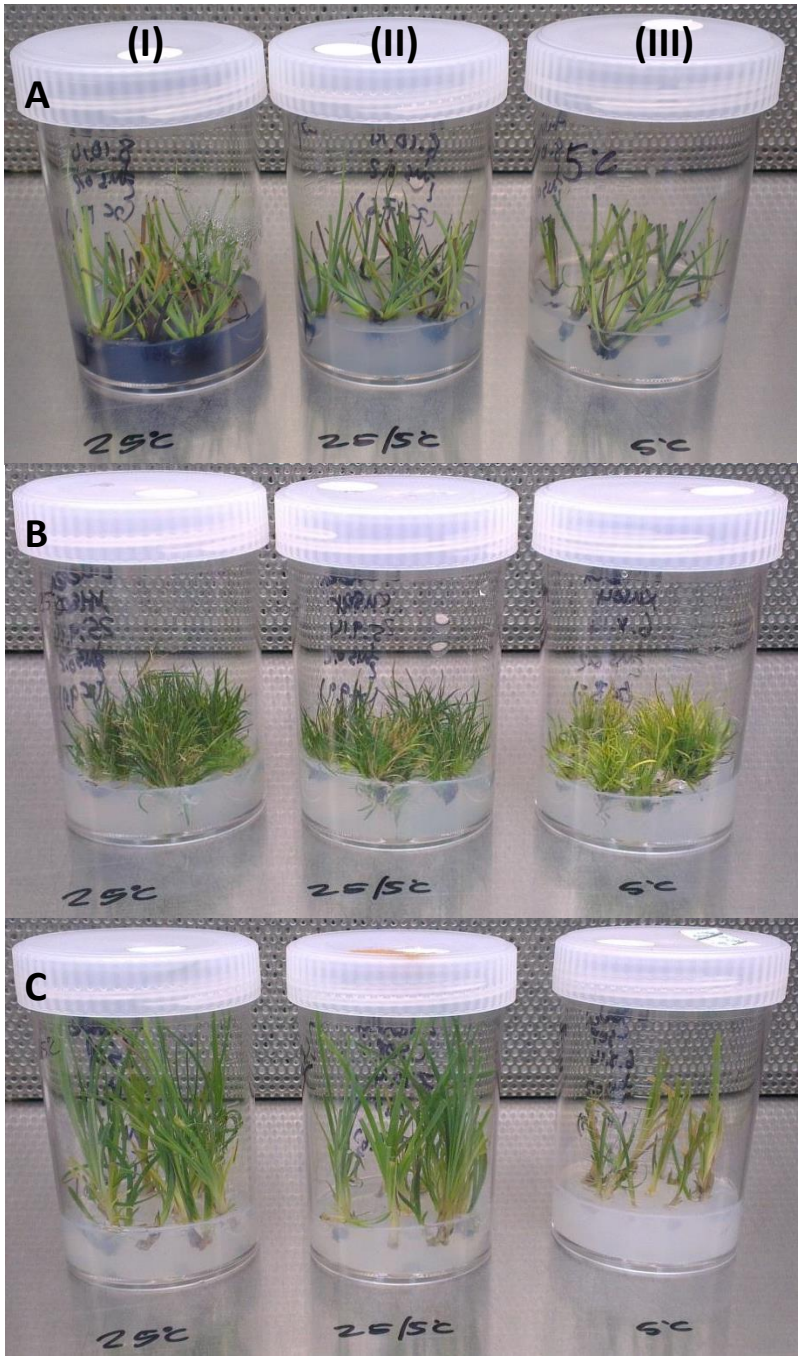

Figure 4.1. Preconditioned *in vitro* shoots. Preconditioned *in vitro* shoots of *Anigozanthos viridis* (A), *Loxocarya cinerea* (B) and *Lomandra sonderi* (C) exposed to 25°C (I), 25/5°C day/night cycle (II) or 5°C (III) preconditioning temperatures (with a 12 h photoperiod, PFFD of ~30 µmol $m^{-2}s^{-1}$) after 3 weeks.

Cryoprotectant solutions

Preculture medium (PM) consisted of BM, 0.8% (w/v) agar, and either 60 mM sucrose, 0.2 M sucrose or 0.4 M sucrose for *L. sonderi*, *L. cinerea* and *A. viridis,* respectively (Menon et al. 2012; Kaczmarczyk et al. 2013; Turner et al. 2000). Loading solution (LS) consisted of BM with 2 M glycerol and 0.4 M sucrose. Plant vitrification solution 2 (PVS2) consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide and 0.4 M sucrose all in BM (Sakai et al. 1990). Washing solution (WS) consisted of BM and 0.75 M sucrose. Recovery medium (RM) consisted of BM with 60 mM sucrose, 2 mM choline chloride, 1 µM zeatin, 1 µM gibberellic acid and 0.8% (w/v) agar.

Cryopreservation technique

Following preconditioning, isolated shoot tips were placed on to their respective PM and incubated at the previously applied preconditioning regime (25°C, 25/5°C or 5°C) for 48 h in the dark. Shoot tips were incubated in LS at room temperature for 20 min prior to transfer to PVS2. Previously optimised cryopreservation CPA incubation times were used to cryopreserve the three species: *A. viridis* and *L. cinerea* were incubated in PVS2 on ice for 30 and 20 min, respectively, while *L. sonderi* was incubated for 10 min in PVS2 at room temperature (Kaczmarczyk et al. 2013; Menon et al. 2012; Turner et al. 2000). After PVS2 incubation shoot tips were placed into 1 µl droplets of PVS2 on aluminium foil strips, transferred to a cryo-vial and plunged into liquid nitrogen (LN). After a minimum of 1 h in LN, cryo-vials were rewarmed in a 40°C waterbath for 10 seconds, and shoot tips were incubated in WS for 20 min at room temperature. Shoot tips treated as described above excluding exposure to LN were used as controls for determination of potential toxic side effects of the CPAs alone as distinct from the effects of LN exposure.

Analysis of survival and regeneration of shoot tips

After the WS incubation, shoot tips were placed onto RM and incubated at 25°C in darkness for 4 weeks. Shoot tips were then transferred to fresh RM and incubated for a further 4 weeks at 25°C under low light conditions (12 h photoperiod, ~15 µmol $m^{-2}s^{-1}$). Survival was recorded where shoot tips were green and swollen or if callusing occurred. Regeneration was recorded where normal shoot re-growth was observed.

Antioxidant extraction

Analysis of LMW thiols and AsA in shoot tips exposed to the three different preconditioning regimes was performed after the preconditioning, preculturing, cryoprotection, washing and recovery stages of the protocol (Fig 4.2). Between 15 and 25 mg of shoot tips (~30 shoot tips) were homogenised in LN with 2 ml 0.1 M HCl.

HPLC analysis of LMW thiols

LMW thiols were analysed using a modified method as described by Kranner (1998). Briefly, total thiol content was determined through reduction of disulphides using dithiothreitol (DTT) prior to fluorescent labelling of thiols with monobromobimane. For the analysis of oxidised thiols, N-ethylmaleimide (NEM) was used to block thiols, prior

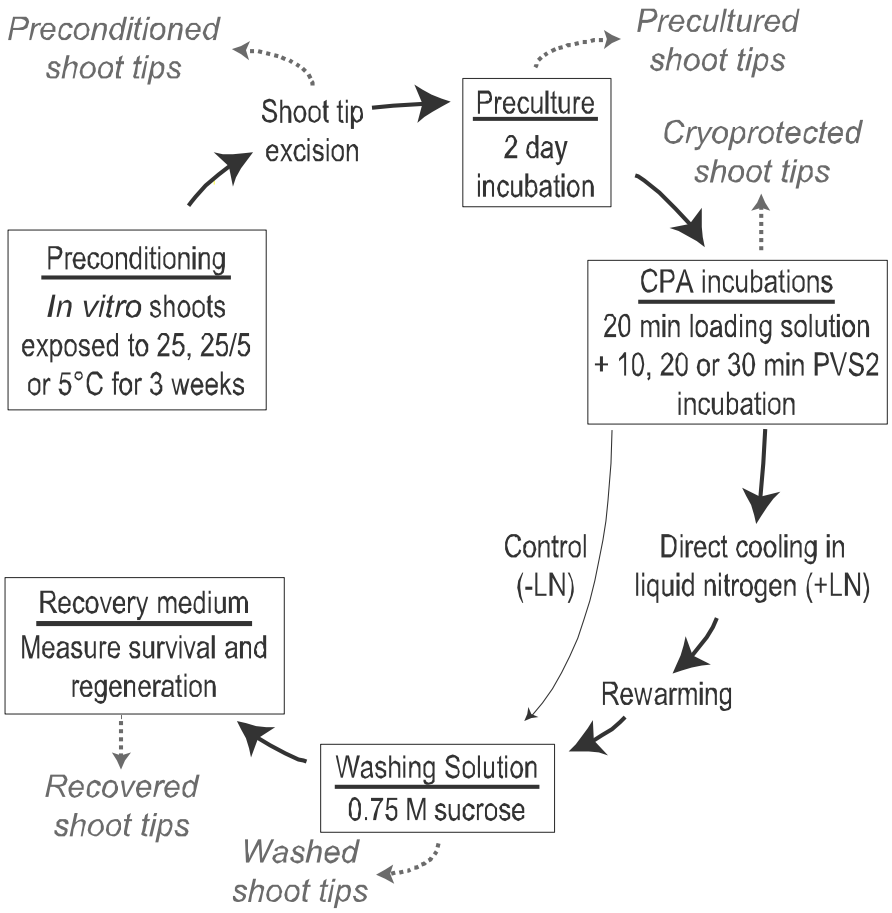

Figure 4.2. Cryopreservation protocol. Dotted arrows signify where shoot tips were analysed for antioxidant content. Adapted from Kaczmarczyk et al. (2012); originally published under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/32860

to the reduction of disulphides with DTT and labelling with monobromobimane. Labelled thiols were separated by reversed-phase HPLC (Jasco, UK) using a HiQ Sil C18HS column (2.1 mm x 250 mm; KyaTech) with gradient elution comprising 0.25% acetic acid (v/v) pH 3.9 and methanol at a flow rate of 0.4 mL min^{-1}, and detected using a fluorescence detector (excitation wavelength of 380 nm, emission wavelength of 480 nm). Peak areas were calibrated against analytical standards of GSH, γ-Glu-Cys and Cys (Sigma-Aldrich). The half-cell reduction potentials of the LMW thiol-disulphide redox couples ($E_{LMW\ disulphide\ /\ 2\ LMW\ thiol}$) were calculated using the Nernst equation (Birtic et al. 2011; Kranner et al. 2006; Schafer and Buettner 2001):

$$E_{\ LMW\ disulphide\ /\ 2\ LMW\ thiol} = E^{0\prime} - \frac{R\,T}{n\,F}\ \ln\frac{[\text{thiol}]^2}{[\text{disulphide}]}$$

Where $E^{0'}$ is the standard half-cell reduction potential at pH 7 (-240 mV for $E_{GSSG/2GSH}$, and -226 mV for $E_{cystine/2Cys}$ and $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$), R is the gas constant (8.314 J K^{-1} mol^{-1}), T the temperature in K, n is the number of electrons transferred, and F is the Faraday constant (9.6485 x 10^4 C mol^{-1}). The molar concentrations of LMW thiols and disulphides were calculated based on the water content of the shoot tips, which was estimated by subtracting the mass of dry shoot tips from that of fresh shoot tips and using the density of water (1 g/mL) to calculate the volume of water available for dissolution. The overall redox environment ($E_{thiol\text{-}disulphide}$) was calculated according to Birtic et al. (2011):

$$Redox\ environment = \sum_{i-1}^{n\ (couple)} E_i \times [reduced\ species]_i$$

Where E_i and $[\text{reduced species}]_i$ are the half-cell reduction potential and the concentration of the reduced species of each individual redox couple.

HPLC analysis of AsA

For AsA analysis, 50 µl of the 0.1 M HCl extract was diluted with 450 µl 0.1 M HCl and analysed by HPLC for reduced AsA content. For total AsA (AsA + dehydroascorbic acid [DHA]) content, 50 µl of extract was incubated with 450 µl 100 mM $NaPO_4$ containing 5 mM tris(2-carboxyethyl)phosphine (TCEP) at pH 5.6 for 4 h

to reduce all DHA to AsA. Ascorbic acid was analysed by reversed-phase HPLC using a HiQ Sil C83W column (4.6 mm x 250 mm; KYA Tech) with a 30 mM potassium phosphate (pH 2.8) mobile phase and a flow rate of 1 mL min^{-1}. Ascorbic acid was detected at 248 nm using a photodiode array detector and calibrated against an AsA analytical standard.

Statistics

All antioxidant experiments consisted of a minimum of 3 replicates. Cryopreservation experiments contained 20 shoot tips per replicate. Statistical analysis was performed using SigmaPlot (Version 12.0). Post-hoc tests were completed using the Tukey test ($P<0.05$) for statistical significance.

Results

Post-cryogenic survival and regeneration

Anigozanthos viridis showed the greatest cryo-capability with 78% regeneration after cryopreservation, followed by *L. sonderi* with 50% regeneration. For both species the best regeneration was obtained following preconditioning at 25°C. *Loxocarya cinerea* showed no regeneration post-cryopreservation but post-cryogenic survival was observed in all three preconditioning regimes with a maximum 43% survival after preconditioning at 5°C (Fig 4.3). The application of a 5°C preconditioning regime resulted in significantly lower regeneration in the cryopreserved *A. viridis* shoot tips (Fig 4.3), with no significant differences in shoot survival or regeneration observed between the 25/5°C and 25°C preconditioning regimes. Preconditioning had little effect on the survival and regeneration of *L. cinerea* and *L. sonderi* shoot tips (Fig 4.3).

The preconditioning regimes had a noticeable effect on the *in vitro* growth of shoots of the three species. The application of the 5°C preconditioning resulted in visibly stunted growth after 3 weeks for *A. viridis* and *L. sonderi*, whilst *L. cinerea* was a lighter green colour when incubated at 5°C compared to shoots grown at 25°C. No difference in growth was seen between the 25/5°C and 25°C preconditioning regimes in all 3 species (Fig 4.1).

LMW thiol analysis

LMW thiols (Cys, γ-Glu-Cys and glutathione) were measured during 5 stages of the cryopreservation protocol (preconditioned, precultured, cryoprotected, washed and

recovered shoot tips) in *A. viridis* (Fig 4.4), *L. cinerea* (Fig 4.5) and *L. sonderi* (Fig 4.6). GSH was the most abundant LMW thiol in all species and at all stages of the cryopreservation protocol. In preconditioned shoot tips the highest levels of GSH were in *L. sonderi* followed by *L. cinerea*. In both species GSH concentrations were highest in shoot tips that had been preconditioned at 5°C and lowest in shoot tips preconditioned at 25°C.

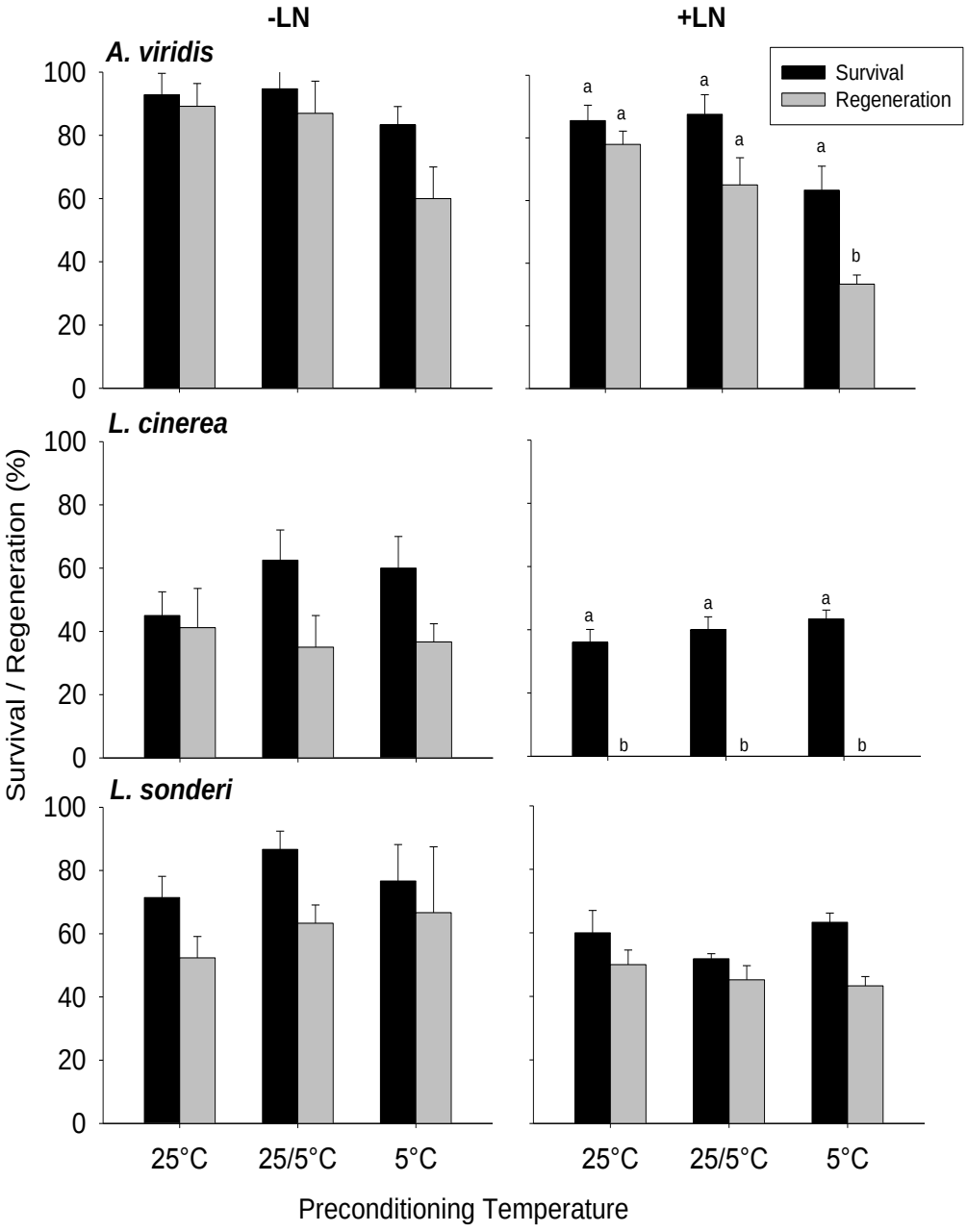

Figure 4.3. Survival and regeneration of *Anigozanthos viridis, Loxocarya cinerea* and *Lomandra sonderi* following cryopreservation. Survival (black bars) and regeneration (grey bars) of shoot tips before (-LN) and after (+LN) cryopreservation in *A. viridis*, *L. cinerea* and *L. sonderi*. Data shows mean ± SE. Different letters donate significant differences (One-way ANOVA Tukey's Test $P < 0.05$).

In contrast, the concentration of GSH in 5°C preconditioned *A. viridis* shoot tips was much lower, at around one third of the concentration in the other two species, and the highest GSH concentration was instead observed following 25/5°C preconditioning (Fig 4.4). The difference in total glutathione (GSH+GSSG) concentrations between the preconditioning treatments was greatest for *L. cinerea*, which had 2- to 3-fold higher total glutathione concentrations in 5°C preconditioned shoot tips compared to shoot tips preconditioned at 25°C and 25/5°C. In comparison, the total glutathione concentration in 5°C preconditioned shoot tips of *L. sonderi* was 1.5-fold higher than in 25°C preconditioned shoot tips. The higher total glutathione concentration observed in the 5°C preconditioned shoot tips of *L. cinerea* compared to 25°C and 25/5°C preconditioned shoot tips was maintained throughout the cryopreservation protocol until the recovery stage, where all *L. cinerea* shoot tips contained similar amounts of total glutathione regardless of the preconditioning regime to which they were subjected (Fig 4.5).

As seen in Fig 4.5 and 4.6, the concentration of total glutathione in the shoot tips of *L. sonderi* and *L. cinerea* was highest during preconditioning, and subsequently declined during the preculture, cryoprotection and washing stages before increasing during the recovery stage. The shoot tips of *A. viridis* showed a similar pattern, but total glutathione concentrations were maintained at preconditioning levels during preculture. All three species showed a similar increase in total glutathione levels during the recovery stage in relation to the glutathione concentrations during preconditioning. The greatest increase was observed during recovery of 25°C preconditioned shoot tips of *L. sonderi* and *A. viridis*, and 25°C and 25/5°C preconditioned shoot tips of *L. cinerea*, with total glutathione concentrations reaching 60% of the concentrations observed during 25°C preconditioning compared to 30-40% of the concentrations observed during 5°C preconditioning ($p = 0.04$).

In the preconditioned shoot tips of all three species the majority (>98%) of total glutathione comprised GSH. This was maintained until the washing and recovery stages, when the ratio of GSH to GSSG decreased slightly in *L. cinerea* and *L. sonderi*. In *L. sonderi* 90% - 93% of total glutathione was comprised of GSH during the washing and recovery stages, whilst in *L. cinerea* ca. 88% comprised GSH. *Anigozanthos viridis* maintained ca. 97% of the total glutathione pool as GSH throughout washing and recovery for shoot tips preconditioned at 25°C, while shoot tips that had been preconditioned at 5°C had 90% and 94% of the total glutathione pool in the reduced form following washing and recovery, respectively.

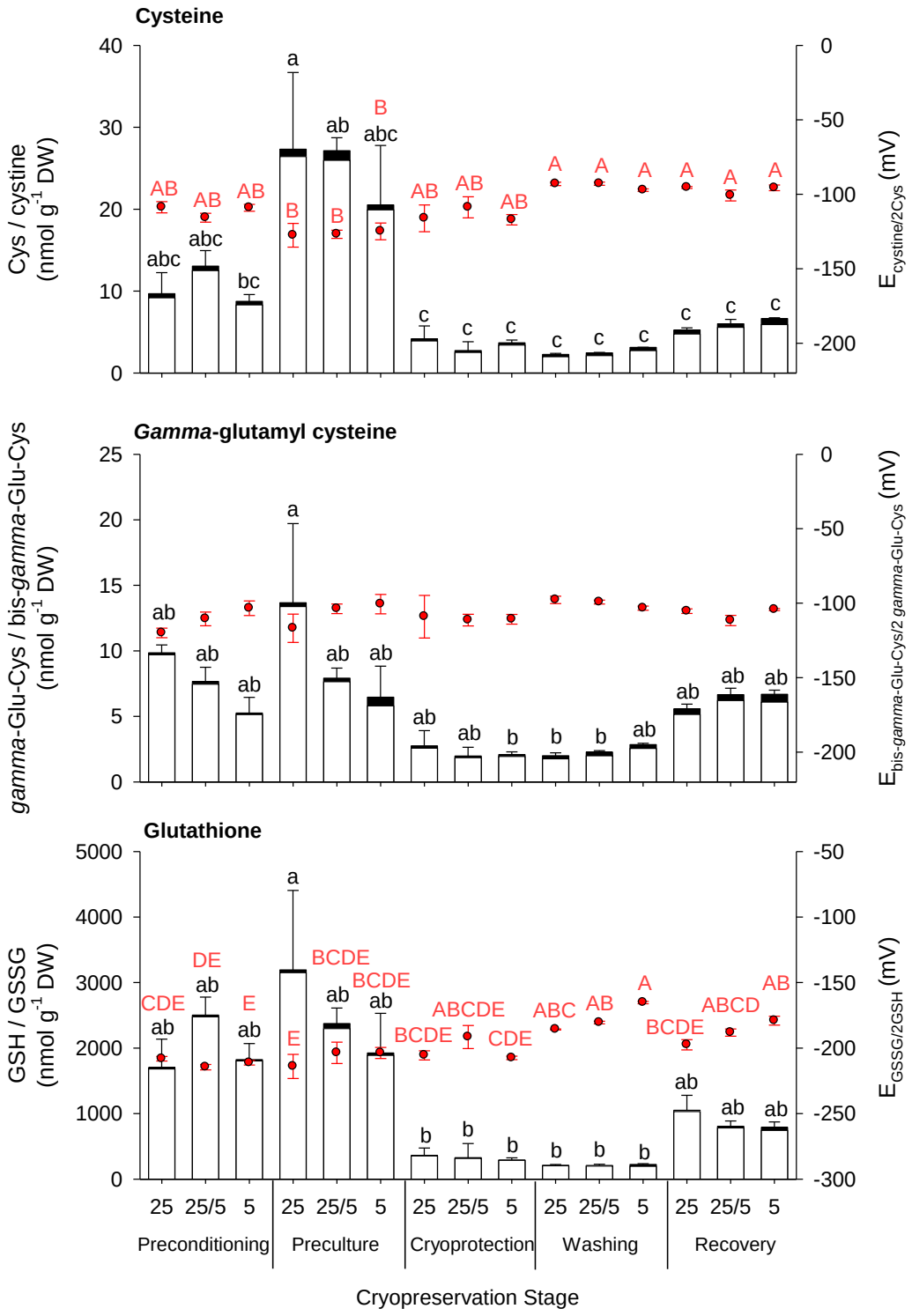

Figure 4.4. Analysis of total concentration of LMW thiols cysteine, *gamma*-glutamyl cysteine and glutathione for *Anigozanthos viridis*. White bars show reduced thiol concentration and black bars show corresponding disulphide thiol concentration ± SE of total thiol concentration. Red circles show the respective half-cell reduction potential of each redox couple ($E_{cystine/2Cys}$, $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$ and $E_{GSSG/2GSH}$) ± SE (right hand y-axis). Different letters indicate significant differences between treatments: black lower case letters for total thiol concentration and red upper case letters for half-cell reduction potentials (One-way ANOVA Tukey's Test $P < 0.05$).

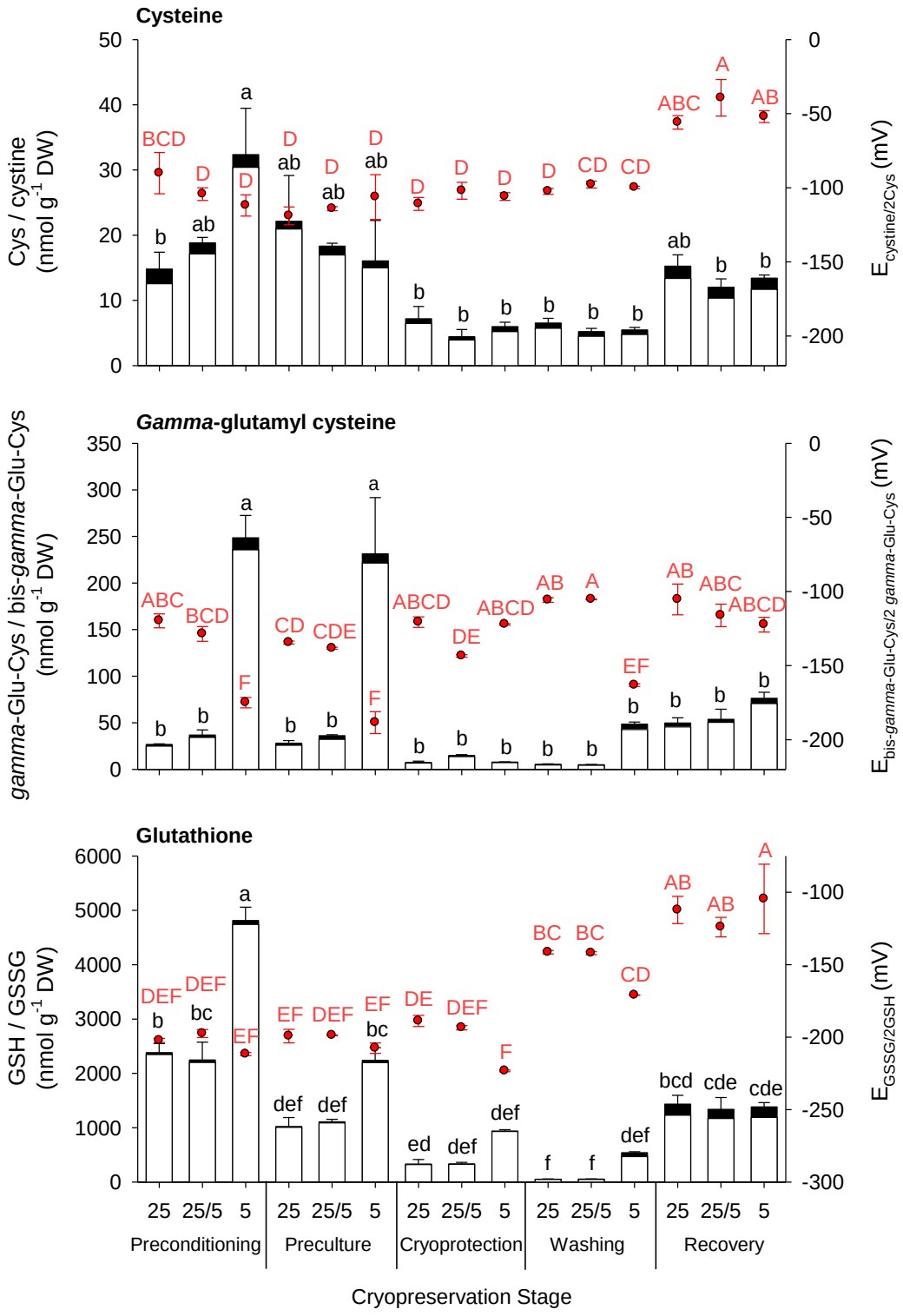

Figure 4.5. Analysis of total concentration of LMW thiols cysteine, *gamma*-glutamyl cysteine and glutathione for *Loxocarya cinerea*. White bars show reduced thiol concentration and black bars show corresponding disulphide thiol concentration ± SE of total thiol concentration. Red circles show the respective half-cell reduction potential of each redox couple ($E_{cystine/2Cys}$, $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$ and $E_{GSSG/2GSH}$) ± SE (right hand y-axis). Different letters indicate significant differences between treatments: black lower case letters for total thiol concentration and red upper case letters for half-cell reduction potentials (One-way ANOVA Tukey's Test $P < 0.05$).

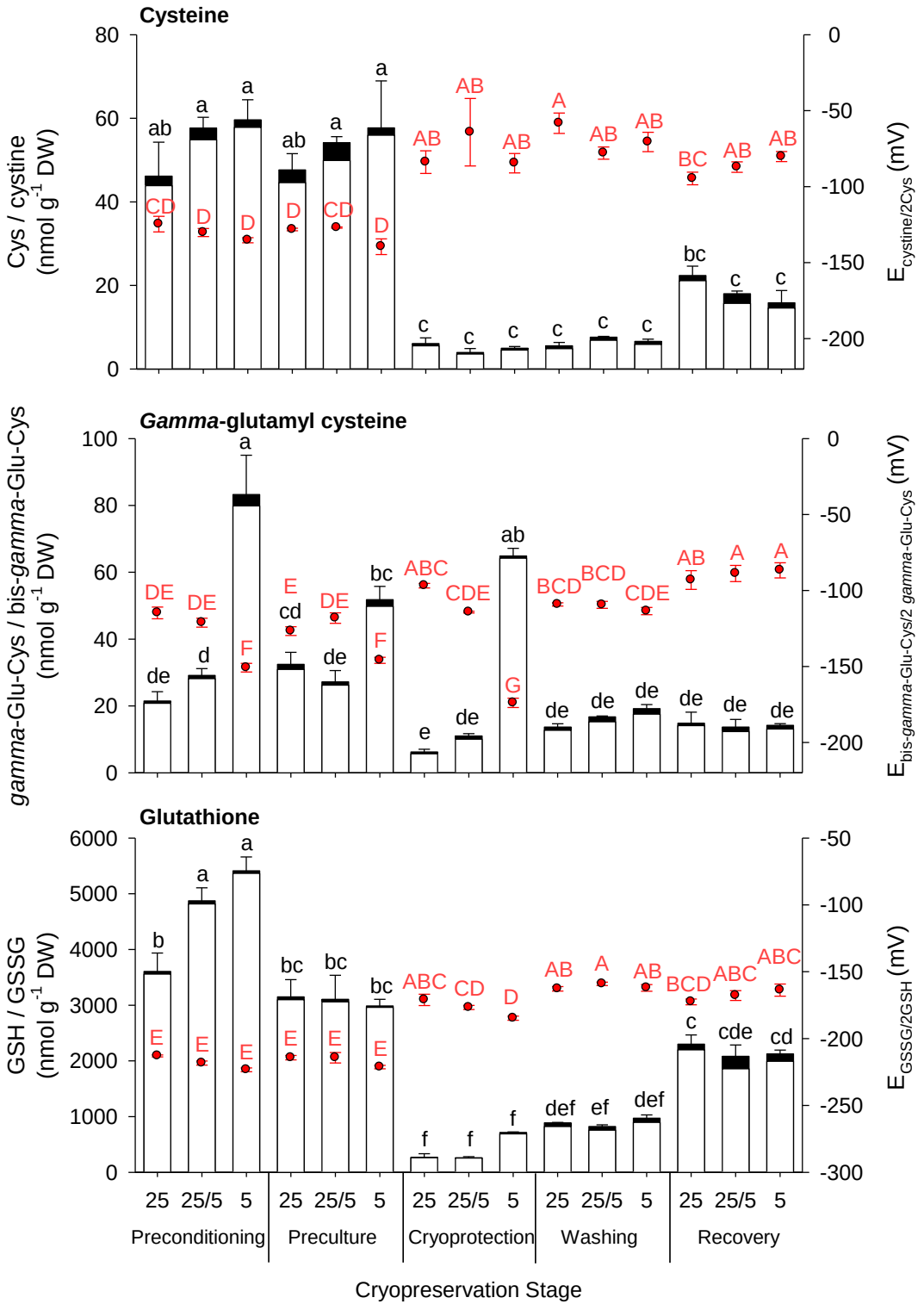

Figure 4.6. Analysis of total concentration of LMW thiols cysteine, *gamma*-glutamyl cysteine and glutathione for *Lomandra sonderi*. White bars show reduced thiol concentration and black bars show corresponding disulphide thiol concentration ± SE of total thiol concentration. Red circles show the respective half-cell reduction potential of each redox couple ($E_{cystine/2Cys}$, $E_{bis\text{-}\gamma\text{-}GC/2\gamma\text{-}GC}$ and $E_{GSSG/2GSH}$) ± SE (right hand y-axis). Different letters indicate significant differences between treatments: black lower case letters for total thiol concentration and red upper case letters for half-cell reduction potentials (One-way ANOVA Tukey's Test $P < 0.05$).

The redox state of GSH is best described by the half-cell reduction potential ($E_{GSSG/2\ GSH}$), which takes into account not only the ratio of GSH: GSSG, but also the concentration of GSH. A more positive $E_{GSSG/2\ GSH}$ indicates less reducing conditions within the cells. During preconditioning the $E_{GSSG/2\ GSH}$ ranged from -223 mV to -213 mV in *L. sonderi* shoot tips, -212 to -198 mV in *L. cinerea* shoot tips, and -215 to -208 mV in *A. viridis* shoot tips (Fig 4.4). In both *L. cinerea* and *L. sonderi* the $E_{GSSG/2\ GSH}$ was most reducing (most negative) in 5°C preconditioned shoot tips, whilst in *A. viridis* it was most reducing in 25/5°C preconditioned shoot tips. $E_{GSSG/2\ GSH}$ was less reducing (most positive) in 25°C preconditioned shoot tips of *A. viridis* and *L. sonderi*. There were no significant correlations between $E_{GSSG/2\ GSH}$ in preconditioned shoot tips and survival or regeneration post-cryopreservation. $E_{GSSG/2\ GSH}$ shifted towards less reducing (more positive) values during the washing stage. The shift was greatest for *L. cinerea*. There was, nonetheless, a negative correlation between $E_{GSSG/2\ GSH}$ during recovery and regeneration ($R^2 = 0.91$, $p < .001$; not shown) and survival ($R^2 = 0.75$, $p = 0.001$; Fig 4.7), i.e. survival and regeneration were greater in the shoot tips with the most reducing (lowest) $E_{GSSG/2\ GSH}$ value.

Cys and γ-Glu-Cys were far less abundant than GSH, with each generally representing less than 2% of the total LMW thiols in *A. viridis* and *L. sonderi* shoot tips. Cys and γ-Glu-Cys were present at similar concentrations in *L. sonderi* and *A. viridis*, but γ-Glu-Cys was more abundant than Cys in shoot tips of *L. cinerea* and represented ca. 4% of the total LMW thiols. Both Cys and γ-Glu-Cys followed a similar pattern to GSH during the cryopreservation protocol, with a marked decline during the cryoprotection and washing stages. Similar to glutathione, total γ-Glu-Cys concentrations were higher in *L. cinerea* shoot tips following preconditioning at 5°C compared to preconditioning at 25°C and 25/5°C. The half-cell reduction potentials were calculated for the Cys/cystine and γ-Glu-Cys/bis-γ-Glu-Cys redox couples. $E_{cystine/2\ Cys}$ during recovery showed a significant inverse relationship with survival ($R^2 = 0.65$, $p = 0.006$; Fig 4.7) and regeneration ($R^2 = 0.81$, $p < 0.001$; not shown). In contrast, $E_{bis\text{-}\gamma\text{-}Glu\text{-}Cys/2\ \gamma\text{-}Glu\text{-}Cys}$ showed no relationship with survival or regeneration. The overall LMW thiol-based redox environment was calculated and showed a shift towards more positive (oxidising) values throughout the cryopreservation protocol (Fig 4.7). Following 25°C and 25/5°C preconditioning, *L. sonderi* tended to have the most negative (reducing) redox environment, and *L. cinerea* the most positive (oxidising), but following 5°C preconditioning the shoot tips of *A. viridis* had the most positive redox environment. The redox environment during the recovery stage showed a negative correlation with regeneration ($R^2 = 0.73$, $p = 0.003$; not shown),

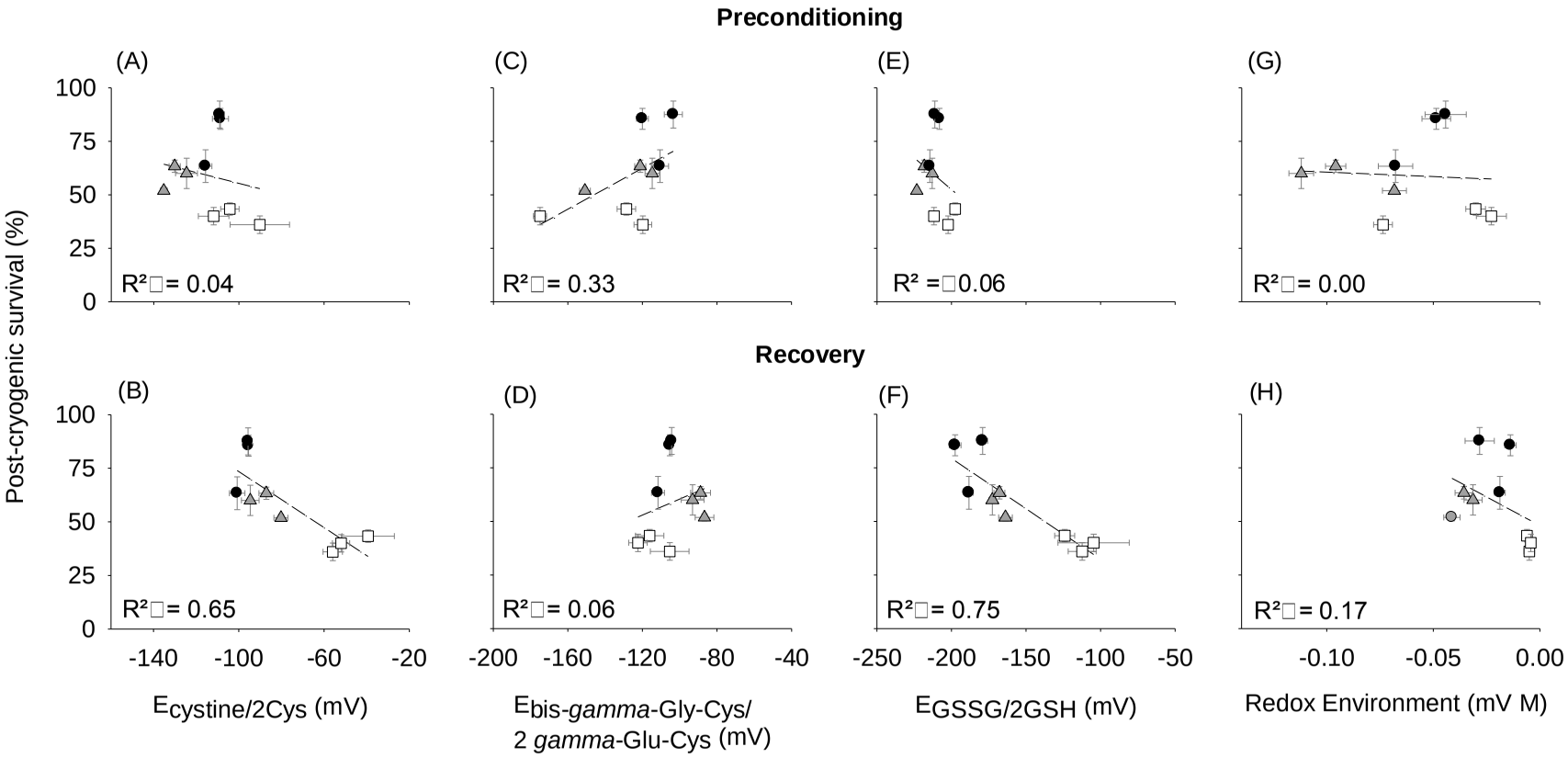

Figure 4.7. Scatterplots comparing half-cell reduction potential of the thiols $E_{cystine/2Cys}$ (A, B), $E_{bis\text{-}\gamma\text{-}Glu\text{-}Cys/2\gamma\text{-}Glu\text{-}Cys}$ (C, D), $E_{GSSG/2GSH}$ (E, F) and the overall redox environment (G, H) in the preconditioned shoot tips (A, C, E, G) and the recovered shoot tips (B, D, F, H) vs post-cryogenic survival in *Anigozanthos viridis* (black circles), *Loxocarya cinerea* (white squares) and *Lomandra sonderi* (grey triangles). Data shows mean ± SE.

but there was no significant correlation with survival (Fig 4.7). However, all the species showed a shift to a more oxidised environment in the recovered shoot tips in comparison to the preconditioned shoot tips (Fig 4.7).

Ascorbic acid analysis

Loxocarya cinerea had the highest concentrations of ascorbic acid of the 3 species, with over 1.6 mmol g^{-1} DW in preconditioned shoot tips compared with 1 and 0.5 mmol g^{-1} DW for *L. sonderi* and *A. viridis*, respectively (Fig 4.8). A large decrease in the total AsA content (-73%) of *L. cinerea* shoot tips occurred during the preculture stage, and the AsA content continued to decrease during the cryoprotection and washing stages. The lowest AsA concentration of 0.09 mmol g^{-1} DW was observed during the washing stage of 25°C preconditioned shoot tips, and represented a 94% reduction in total AsA concentration compared to the concentration after the preconditioning stage. A similar reduction was observed in *L. sonderi* shoot tips, whilst *A. viridis* showed a reduction of around 90%. Total AsA concentrations increased during the recovery stage for all species. The highest level of increase was in *A. viridis*, which showed between 450% and 740% increase in total AsA in 25/5°C and 25°C preconditioned shoot tips, respectively, during recovery compared to washed shoot tips. This increase during recovery restored total AsA in 25°C preconditioned shoot tips to the levels observed during preconditioning, but in 25/5°C and 5°C preconditioned shoot tips total AsA was restored to only around 50% of the preconditioning levels. The increase in total AsA concentrations in *L. cinerea* during recovery ranged from 150% in 25/5°C preconditioned shoot tips to 270% in 25°C preconditioned shoot tips in relation to concentrations after the washing stage. This resulted in total AsA concentrations in recovered shoot tips that were only around 20% of the total AsA concentrations during preconditioning. Restoration of total AsA levels during recovery of *L. sonderi* shoot tips was slightly better, with increases of between 300% (25/5°C preconditioned shoot tips) and 560% (5°C preconditioned shoot tips) in relation to the concentrations in washed shoot tips, which resulted in total AsA concentrations of between 30 and 45% of the preconditioning values.

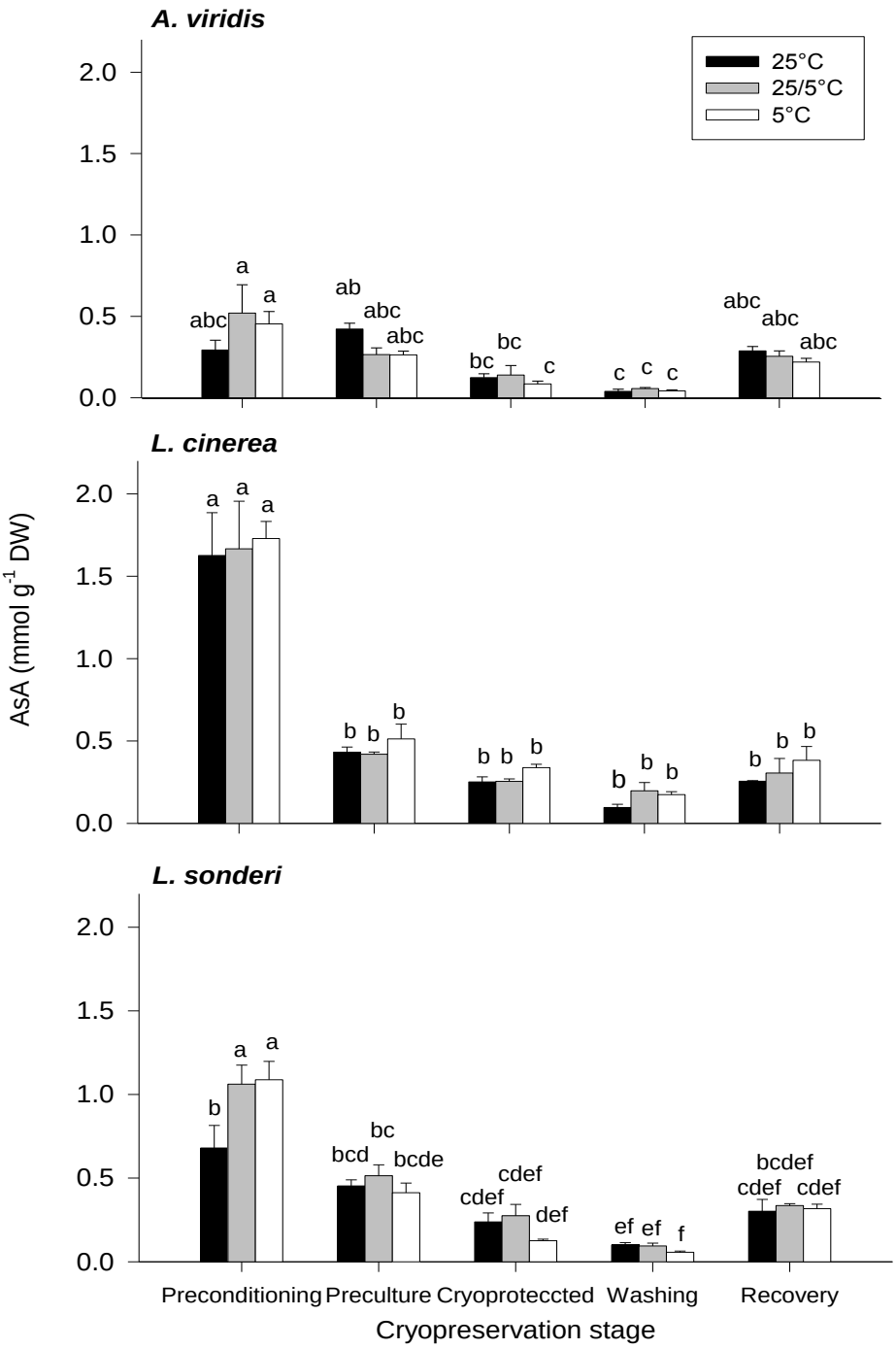

Figure 4.8. Analysis of total ascorbic acid concentration after different steps of the cryopreservation protocol in *Anigozanthos viridis* (A), *Loxocarya cinerea* (B) and *Lomandra sonderi* (C). 25°C (black bars), 25/5°C (grey bars) and 5°C (white bars) preconditioned shoot tips at the respective cryopreservation stages shown in each graph. Data represents mean ± SE, different letters denote significant differences (One-way ANOVA, Tukey's Test $P < 0.05$).

There was no significant difference between the total AsA concentrations in the shoot tips of the three species during recovery (Fig 4.9A), and, therefore, no correlation between total AsA concentration during recovery, and survival or regeneration of the shoot tip (R^2 = 0.12). However, negative correlations between total AsA concentrations during preconditioning and survival (R^2 = 0.79, p = 0.0014) and regeneration were observed (R^2 = 0.81, p < .001).

Comparing the change in AsA concentration between the preconditioned shoot tips and recovered shoot tips, *A. viridis* showed the least difference (-1.9%) while *L. cinerea* showed the greatest difference (-84.3%). As seen in Fig 4.9B, a positive correlation was observed between this change in AsA concentration and post-cryogenic survival (R^2 = 0.69) and regeneration (R^2 = 0.68).

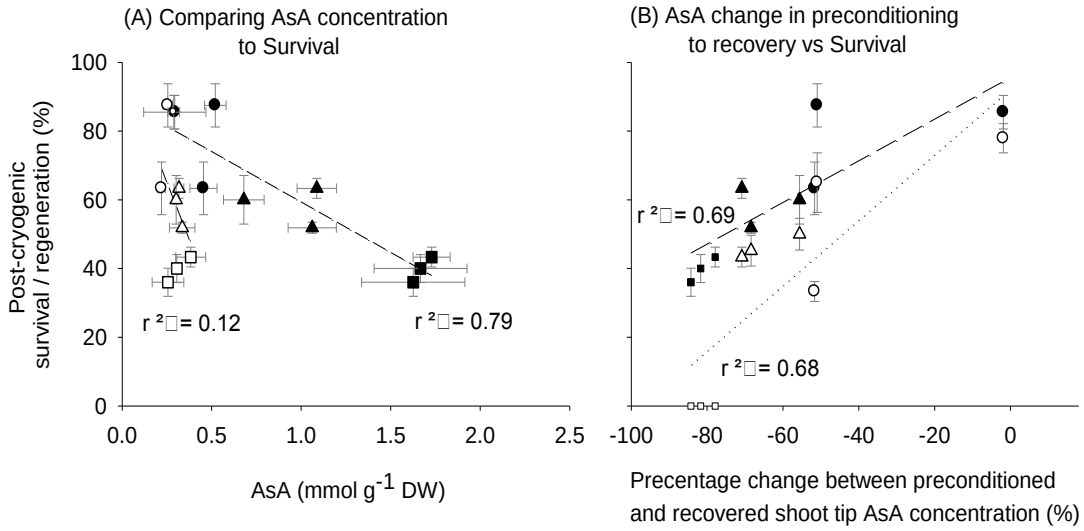

Figure 4.9. Scatterplot comparing ascorbic acid (AsA) concentration to post-cryogenic survival and regeneration. Graph (A) shows post-cryogenic survival vs AsA concentration in the preconditioned shoot tips (closed symbols) or post-cryogenic survival vs AsA concentration in the recovered shoot tips (open symbols). Graph (B) shows the percentage change in AsA concentration between the preconditioning and recovery stages vs either post-cryogenic survival (closed symbols) or post- cryogenic regeneration (open symbols). Results show *Anigozanthos viridis* (circles), *Loxocarya cinerea* (squares) and *Lomandra sonderi* (triangles) ± SE, dotted lines show regression line with regression values indicated.

Discussion

Cryopreservation

Anigozanthos viridis, *L. sonderi* and *L. cinerea* were chosen for this study as native Australian plant species with previously described cryopreservation protocols and quite variable post-cryogenic survival and regeneration (Kaczmarczyk et al. 2013; Menon et al. 2012; Turner et al. 2000). These three species display a wide range of cryo-capability, allowing comparison between them and, therefore, imparting a greater understanding of the stresses experienced by plants during cryopreservation and in particular the different stages leading up to LN immersion.

Little is known about the ability of Australian plants to cold acclimate. Previous work on *L. sonderi* showed some increased tolerance to freezing temperatures when the plants were preconditioned (Menon et al. 2012). The application of an alternating cold temperature preconditioning regime has also shown benefits for the Australian species *Grevillea scapigera* (Funnekotter et al. 2013). Cold acclimation has been linked to increased levels of antioxidants and greater antioxidant enzyme activity (Kocsy et al. 2000), which helps to maintain the redox homeostasis through the stresses of the cryopreservation protocol. Assessment of survival and regeneration of the shoot tips from the three species after 8 weeks on RM showed considerable differences between their cryo-capabilities. *Anigozanthos viridis* exhibited the greatest regeneration rate with 78% regrowth, followed by *L. sonderi* with 50% regrowth whilst *L. cinerea* showed no post-cryogenic regeneration (although 43% of the shoot tips showed post-cryogenic survival). Previous work had shown cold preconditioning (5°C and 25/5°C) to be beneficial for *L. sonderi*, with post-cryogenic survival increasing by over 15% points (Menon et al. 2012), but in this study preconditioning did not have as large an effect on cryopreservation survival and regeneration as expected. However, a more extreme temperature difference (20/-1°C) in the 25/5°C preconditioning regime and a slightly different temperature (23°C) in the control regime were used in the previous study instead of the temperature regimes used here, which may account for these observed differences. Previous work on *L. cinerea* showed no significant influence of cold preconditioning on shoot tip survival (Funnekotter et al. 2013), with similar results observed in this study. In contrast, the application of 5°C preconditioning was detrimental to post-cryopreservation regeneration of *A. viridis*, resulting in 33% of shoot tips regenerating compared to 78% of 25°C preconditioned shoot tips. The *in vitro* shoots of *A. viridis* showed stunted growth with the 5°C preconditioning regime in comparison to the 25/5 and 25°C preconditioning regimes

(Fig 4.1A), indicating the presence of stressful conditions to the shoots; however, in this case the low temperature may have been too extreme for this species to result in the beneficial effects of cold acclimation, and therefore resulting in reduced post-cryogenic regeneration.

Antioxidant analysis

The application of the 5°C preconditioning regime resulted in significantly higher amounts of GSH in comparison to the 25°C preconditioning regime for *L. sonderi* and *L. cinerea*. Increased antioxidants in plants are a common response during cold acclimation (Prasad 1996; Kocsy et al. 2001). The fact that *A. viridis* did not show this response is an indication that the preconditioning temperature did not result in cold acclimation. This lack of a response is a likely contributing factor to the observed lower post-cryogenic survival in the 5°C preconditioning regime.

Total LMW thiol content decreased significantly throughout the cryopreservation protocol in all three molecules measured (glutathione, Cys, γ-Glu-Cys). In particular, a large reduction in thiol content was seen in the cryoprotected and washed stages (up to 97% reduction) in comparison to the preconditioned and precultured stages. Analysis of shoot tips at the recovery stage typically showed an increase in thiol concentrations compared to shoot tips at the washing stage, but none of the thiol contents recovered to the levels measured at the preconditioning stage. It is difficult to make use of the total glutathione, Cys and/or γ-Glu-Cys thiol content trends to explain why *A. viridis* has the best cryo-capability, as it did not have the greatest pool of thiols or maintain a higher amount of thiols throughout the cryopreservation protocol. However, it should be noted that *L. cinerea* showed the greatest reduction (-51 to -64%) in glutathione content in shoot tips at the preculture stage, whilst *A. viridis* showed an increase of 87% in glutathione for the 25°C conditioned shoot tips at the preculture stage and displayed the greatest post-cryogenic regeneration. This may indicate the inability of *L. cinerea* to recover from the damages of excision; however, more work is required to tease this factor out further. With the decline of glutathione in the cryoprotected and washed shoot tips an accumulation of Cys would be expected if glutathione was degraded. However, this was not seen in our findings, possibly indicating that the thiols were bound to proteins during protein thiolation (Dixon et al. 2005), which may account for the increase in thiols in the recovered shoot tips as protein thiolation is a reversible process. The reduction may also be due to the thiols being exported from the cells, which is most probable in the washed shoot tips if membrane pores formed either from the action of CPAs (Hughes and Mancera

2013; Hughes and Mancera 2014; Hughes et al. 2012) or ice crystallisation damage (Steponkus 1984).

The sensitivity of antioxidants to oxidation facilitates the analysis of the redox state of cells. GSH is commonly used as an indicator of redox state by measuring the half-cell reduction potential. Increased oxidative stress will result in the oxidation of GSH to GSSG. If the production of these oxidised antioxidants exceeds the ability of plants to reduce them, then the reduction potential will increase, indicating excessive ROS formation. The half-cell reduction potential is commonly used in seed storage experiments, linking the reduction of germination to a change in the half-cell reduction potential as the stored seeds become more oxidised over time (Colville and Kranner 2010; Birtic et al. 2011; Chen et al. 2013; Nagel et al. 2014). $E_{GSSG/2GSH}$ was more positive (oxidising) in recovered shoot tips compared to preconditioned shoot tips of all three species, and the greatest shift in $E_{GSSG/2GSH}$ was observed in *L. cinerea*. The higher oxidation of *L. cinerea* shoot tips is a likely reason why no regeneration was observed despite the observation of shoot tip survival, since GSH and the GSH/GSSG ratio have been linked to cellular proliferation (Sánchez-Fernández et al. 1997).

Similar to the levels of thiols observed throughout the cryopreservation process, measurements of AsA levels showed reductions in the concentrations throughout the cryopreservation protocol stages from the preconditioned through to the washing stage; however, this was a more gradual decrease at each stage rather than the large reduction detected in the cryoprotected shoot tips with the LMW thiols. The levels of AsA increased as the shoot tips were placed on the recovery medium. Similar to the thiols, *A. viridis* 25°C preconditioned shoot tips were an exception, as levels of AsA in shoot tips at the preculture stage were greater than at the preconditioning stage, whilst *L. cinerea* had the largest reduction in AsA levels in the precultured shoot tips. *Anigozanthos viridis* had the lowest AsA concentration in the preconditioned shoot tips of the three species, yet exhibited the highest post-cryogenic regeneration. Our findings indicate that there is an inverse relationship between preconditioned AsA concentration and post-cryogenic survival (Fig 4.9A). However, it is unlikely that increased AsA concentration in the shoot tips should result in reduced regeneration after cryopreservation. Comparison of the AsA concentration in the recovered shoot tips showed no correlation to post-cryogenic survival, with all species showing similar concentrations of AsA in the shoot tips. However, the change in AsA concentration between preconditioned and the recovered shoot tips showed a positive correlation to post-cryogenic survival and regeneration (Fig 4.9B), suggesting that *A. viridis* was better able to recover and resynthesise AsA to levels that confer protection from ROS.

It is also possible that other antioxidants are able to be recruited to compensate for the low levels of AsA detected in *A. viridis*, which may in turn suggest a degree of redundancy with respect to options for antioxidant defence in *A. viridis* that may not exist in *L. cinerea*.

Conclusions

This study characterised changes in the concentration of the water-soluble antioxidants, LMW thiols and AsA, throughout the cryopreservation protocol to further our understanding of the stresses that cryopreservation imposes on the shoot tips of three native Australian species. Antioxidant pools were determined to decrease throughout the cryopreservation protocol; however, the total content of antioxidants does not appear to be sufficient or effective at protecting shoot tips from cryo-injury. Understanding the precise role that each antioxidant plays in protecting cells and tissues during the cryopreservation process remains a challenging prospect. These antioxidants have a wide range of interactions and signalling processes, and it may be unrealistic to expect that the presence of an increased pool or a higher proportion of reduced antioxidants will always result in greater cryo-capability of a plant species. Indeed, analysis of the half-cell reduction potential showed the greatest change in *L. cinerea*, particularly in the washed and recovered shoot tips. We postulate that this elevated oxidation, possibly due to the diminished pool of antioxidants after cryopreservation, is a likely cause for the lack of post-cryogenic regeneration.

Antioxidants are, however, unlikely to be the only factor that influences post-cryogenic regeneration. Many other factors can influence the ability of plants to regenerate, such as membrane damage, protein uncoupling, DNA damage, metabolic activity and osmotic stress, all of which will have some effect on plant regeneration. This study has nonetheless provided further evidence that antioxidants have a critical role to play in successful cryopreservation, but that the way this occurs appears to be complex and almost certainly species-dependent.

References

Antony, J.J.J., C.L. Keng, M. Mahmood, and S. Subramaniam. 2013. "Effects of Ascorbic Acid on Pvs2 Cryopreservation of *Dendrobium* Bobby Messina's Plbs Supported with Sem Analysis." *Applied Biochemistry and Biotechnology* 171 (2): 315-329

Bajaj, Y.P.S. 1995. "Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity." In *Biotechnology in Agriculture and Forestry 32*, ed. Y.P.S. Bajaj, 1-18. New York: Springer.

Benson, E.E., and D. Bremner. 2004. "Oxidative Stress in the Frozen Plant: A Free Radical Point of View." In *Life in the Frozen State*, eds B.J. Fuller, N. Lane and E.E. Benson, 206–241. Boca Raton, Florida: CRC Press.

Benson, E.E., and L.A. Withers. 1987. "Gas Chromatographic Analysis of Volatile Hydrocarbon Production by Cryopreserved Plant Tissue Cultures: A Non-Destructive Method for Assessing Stability." *CryoLetters* 8: 35-46

Birtic, S., L. Colville, H.W. Pritchard, S.R. Pearce, and I. Kranner. 2011. "Mathematically Combined Half-Cell Reduction Potentials of Low-Molecular-Weight Thiols as Markers of Seed Ageing." *Free Radical Research* 45 (9): 1093-1102

Canepa, P., A. Panta, and D. Tay. 2011. "The Effect of Antioxidants on the Cryopreservation Recovery of Two Potato Cultivars Following Post-Thawing Culture." In *International Symposium on Cryopreservation in Horticultural Species*, 101-105. Acta Hort. (ISHS).

Chang, Y., R.E. Barker, and B.M. Reed. 2000. "Cold Acclimation Improves Recovery of Cryopreserved Grass *(Zoysia* and *Lolium* Sp.)." *CryoLetters* 21 (2): 107-116

Chen, H., D. Osuna, L. Colville, O. Lorenzo, K. Graeber, H. Küster, G. Leubner-Metzger, and I. Kranner. 2013. "Transcriptome-Wide Mapping of Pea Seed Ageing Reveals a Pivotal Role for Genes Related to Oxidative Stress and Programmed Cell Death." *PloS One* 8 (10): e78471

Colville, L., and I. Kranner. 2010. "Desiccation Tolerant Plants as Model Systems to Study Redox Regulation of Protein Thiols." *Plant Growth Regulation* 62 (3): 241-255

Department of Parks and Wildlife. 2014. Wildelife Conservation (Rare Flora) Notice. Accessed 20 Jan 2015, http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/threatened-plants.

Dixon, D.P., M. Skipsey, N.M. Grundy, and R. Edwards. 2005. "Stress-Induced Protein S-Glutathionylation in *Arabidopsis*." *Plant Physiology* 138 (4): 2233-2244

Fadzillah, N.M., V. Gill, R.P. Finch, and R.H. Burdon. 1996. "Chilling, Oxidative Stress and Antioxidant Responses in Shoot Cultures of Rice." *Planta* 199 (4): 552-556

Foyer, C.H., L.D. Gomez, and D.R. van Heerden. 2008. "Glutathione." In *Antioxidants and Reactive Oxygen Species in Plants*, ed. N. Smirnoff, 1-24. Wiley.

Foyer, C.H., and G. Noctor. 2011. "Ascorbate and Glutathione: The Heart of the Redox Hub." *Plant Physiology* 155 (1): 2-18

Fujikawa, S., Y. Jitsuyama, and K. Kuroda. 1999. "Determination of the Role of Cold Acclimation-Induced Diverse Changes in Plant Cells from the Viewpoint of Avoidance of Freezing Injury." *Journal of Plant Research* 112 (2): 237-244

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Gupta, S., and B.M. Reed. 2006. "Cryopreservation of Shoot Tips of Blackberry and Raspberry by Encapsulation-Dehydration and Vitrification." *CryoLetters* 27 (1): 29-42

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Hincha, D., and E. Zuther. 2014. "Introduction: Plant Cold Acclimation and Freezing Tolerance." In *Plant Cold Acclimation*, eds Dirk K. Hincha and Ellen Zuther, 1-6. Springer New York.

Hughes, Z.E., and R.L. Mancera. 2013. "Molecular Dynamics Simulations of Mixed Dopc−B-Sitosterol Bilayers and Their Interactions with Dmso." *Soft Matter* 9: 2920-2935

Hughes, Z.E., and R.L. Mancera. 2014. "Molecular Mechanism of the Synergistic Effects of Vitrification Solutions on the Stability of Phospholipid Bilayers." *Biophysical Journal* 106 (12): 2617-2624

Hughes, Z.E., A.E. Mark, and R.L. Mancera. 2012. "Molecular Dynamics Simulations of the Interactions of Dmso with Dppc and Dopc Phospholipid Membranes." *The Journal of Physical Chemistry B* 116 (39): 11911-11923

Kaczmarczyk, A., B. Funnekotter, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., B. Funnekotter, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for *Loxocarya Cinerea*-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519

Kaczmarczyk, A., N. Shvachko, Y. Lupysheva, M.R. Hajirezaei, and E.R. Keller. 2008. "Influence of Alternating Temperature Preculture on Cryopreservation Results for Potato Shoot Tips." *Plant Cell Reports* 27 (9): 1551-8

Kaczmarczyk, A., S.R. Turner, E. Bunn, R.L. Mancera, and K.W. Dixon. 2011b. "Cryopreservation of Threatened Native Australian Species—What Have We Learned and Where to from Here?" *In Vitro Cellular & Developmental Biology - Plant* 47 (1): 17-25

Kamata, T., and M. Uemura. 2004. "Solute Accumulation in Wheat Seedlings During Cold Acclimation: Contribution to Increased Freezing Tolerance." *CryoLetters* 25 (5): 311-322

Kocsy, G., G. Galiba, and C. Brunold. 2001. "Role of Glutathione in Adaptation and Signalling During Chilling and Cold Acclimation in Plants." *Physiologia Plantarum* 113 (2): 158-164

Kocsy, G., G. Szalai, A. Vágújfalvi, L. Stéhli, G. Orosz, and G. Galiba. 2000. "Genetic Study of Glutathione Accumulation During Cold Hardening in Wheat." *Planta* 210 (2): 295-301

Kranner, I. 1998. "Determination of Glutathione, Glutathione Disulphide and Two Related Enzymes, Glutathione Reductase and Glucose-6-Phosphate

Dehydrogenase, in Fungal and Plant Cells." In *Mycorrhiza Manual*, ed. Ajit Varma, 227-241. Springer Berlin Heidelberg.

Kranner, I., S. Birtic, K.M. Anderson, and H.W. Pritchard. 2006. "Glutathione Half-Cell Reduction Potential: A Universal Stress Marker and Modulator of Programmed Cell Death?" *Free Radical Biology and Medicine* 40 (12): 2155-2165

Leunufna, S., and E.R. Keller. 2005. "Cryopreservation of Yams Using Vitrification Modified by Including Droplet Method: Effects of Cold Acclimation and Sucrose." *CryoLetters* 26 (2): 93-102

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Nagel, M., I. Kranner, K. Neumann, H. Rolletschek, C.E. Seal, L. Colville, B. Fernández-Marín, and A. Börner. 2014. "Genome-Wide Association Mapping and Biochemical Markers Reveal That Seed Ageing and Longevity Are Intricately Affected by Genetic Background and Developmental and Environmental Conditions in Barley." *Plant, Cell & Environment*: doi: 10.1111/pce.12474

Noctor, G., A. Mhamdi, S. Chaouch, Y. Han, J. Neukermans, B. Marquez‐Garcia, G. Queval, and C.H. Foyer. 2012. "Glutathione in Plants: An Integrated Overview." *Plant, Cell & Environment* 35 (2): 454-484

O'Kane, D., V. Gill, P. Boyd, and R. Burdon. 1996. "Chilling, Oxidative Stress and Antioxidant Responses in *Arabidopsis Thaliana* Callus." *Planta* 198 (3): 371-377

Prasad, T.K. 1996. "Mechanisms of Chilling‐Induced Oxidative Stress Injury and Tolerance in Developing Maize Seedlings: Changes in Antioxidant System, Oxidation of Proteins and Lipids, and Protease Activities." *The Plant Journal* 10 (6): 1017-1026

Reed, B.M. 2008. "Cryopreservation - Practical Considerations." In *Plant Cryopreservation. A Practical Guide*, ed. B.M. Reed, 3-13. New York: Springer.

Sakai, A., S. Kobayashi, and I. Oiyama. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb. Var. Brasiliensis Tanaka) by Vitrification." *Plant Cell Reports* 9 (1): 30-33

Sánchez-Fernández, R., M. Fricker, L.B. Corben, N.S. White, N. Sheard, C.J. Leaver, M. Van Montagu, D. Inzé, and M.J. May. 1997. "Cell Proliferation and Hair Tip Growth in the *Arabidopsis* Root Are under Mechanistically Different Forms of Redox Control." *Proceedings of the National Academy of Sciences* 94 (6): 2745-2750

Sasaki, H., K. Ichimura, and M. Oda. 1996. "Changes in Sugar Content During Cold Acclimation and Deacclimation of Cabbage Seedlings." *Annals of Botany* 78 (3): 365-369

Schafer, F.Q., and G.R. Buettner. 2001. "Redox Environment of the Cell as Viewed through the Redox State of the Glutathione Disulfide/Glutathione Couple." *Free Radical Biology and Medicine* 30 (11): 1191-1212

Smirnoff, N., and G.L. Wheeler. 2000. "Ascorbic Acid in Plants: Biosynthesis and Function." *Critical Reviews in Biochemistry and Molecular Biology* 35 (4): 291-314

Steponkus, P.L. 1984. "Role of the Plasma Membrane in Freezing Injury and Cold Acclimation." *Annual Review of Plant Physiology* 35 (1): 543-584

Suzuki, N., and R. Mittler. 2006. "Reactive Oxygen Species and Temperature Stresses: A Delicate Balance between Signaling and Destruction." *Physiologia Plantarum* 126 (1): 45-51

Tausz, M., H. Šircelj, and D. Grill. 2004. "The Glutathione System as a Stress Marker in Plant Ecophysiology: Is a Stress-Response Concept Valid?" *Journal of Experimental Botany* 55 (404): 1955-1962

Thomashow, M.F. 1998. "Role of Cold-Responsive Genes in Plant Freezing Tolerance." *Plant Physiology* 118 (1): 1-7

Turner, S., D. Touchell, K. Dixon, and B. Tan. 2000. "Cryopreservation of *Anigozanthos Viridis* Ssp. *Viridis* and Related Taxa from the South-West of Western Australia." *Australian Journal of Botany* 48 (6): 739-744

Uchendu, E.E., S.W. Leonard, M.G. Traber, and B.M. Reed. 2010a. "Vitamins C and E Improve Regrowth and Reduce Lipid Peroxidation of Blackberry Shoot Tips Following Cryopreservation." *Plant Cell Reports* 29 (1): 25-35

Uchendu, E.E., M. Muminova, S. Gupta, and B.M. Reed. 2010b. "Antioxidant and Anti-Stress Compounds Improve Regrowth of Cryopreserved *Rubus* Shoot Tips." *In Vitro Cellular & Developmental Biology-Plant*: 1-8

Wang, Z.C., and X.X. Deng. 2004. "Cryopreservation of Shoot-Tips of Citrus Using Vitrification: Effect of Reduced Form of Glutathione." *CryoLetters* 25 (1): 43-50

Whitaker, C., R.P. Beckett, F.V. Minibayeva, and I. Kranner. 2010. "Production of Reactive Oxygen Species in Excised, Desiccated and Cryopreserved Explants of *Trichilia Dregeana* Sond." *South African Journal of Botany* 76 (1): 112-118

Chapter 5 – Evaluation of the new vacuum infiltration vitrification (VIV) cryopreservation technique for native Australian plant shoot tips

Preface

The following chapter has been accepted for publication in the *CryoLetters* journal.

Manuscript was submitted to *CryoLetters* (13/11/2014), accepted for publication after revision (19/12/2014), revised manuscript was submitted with a response to reviewers (see appendix, 08/01/2015) and manuscript was accepted in the *CryoLetters* journal (15/01/2015).

Abstract

BACKGROUND: The application of a vacuum during the incubations in cryoprotective agents such as PVS2 allows for increased penetration, reducing total incubation times required before vitrification and post-cryopreservation regeneration is achieved. **OBJECTIVE:** This study compared a conventional droplet-vitrification protocol to the new vacuum infiltration vitrification protocol in four Australian plant species. **MATERIALS AND METHODS:** The new vacuum infiltration vitrification applied an 80 kPa vacuum during incubations in loading solution and PVS2. Infiltration of the cryoprotective agents into shoot tips was determined by differential scanning calorimetry measuring ice formation in the thermographs comparing a range of loading solution and PVS2 incubation times. **RESULTS:** The application of the vacuum infiltration vitrification technique resulted in a significantly reduced PVS2 incubation time for cryogenic survival and regeneration for all four species, reducing the time needed to adequately protect shoot tips by half to a quarter when compared to a conventional droplet-vitrification technique.

Introduction

Advances in cryopreservation biotechnology have allowed for efficient long-term storage methods for the preservation of genetically important plant material. Cryopreservation techniques have been developed for a wide range of species and plant material (dormant buds, seeds, embryos, somatic embryos, shoot tips and callus cultures) (Bajaj 1995). The progression of new techniques such as vitrification (Sakai et al. 1990), then droplet vitrification (modified from the droplet freezing technique

(Kartha et al. 1982)) and currently novel vacuum infiltration vitrification (VIV) and V-cryo-plate techniques have reportedly improved post-cryostorage survival while simplifying and accelerating critical stages of cryopreservation (Nadarajan and Pritchard 2014; Yamamoto et al. 2012).

Avoiding detrimental intra- and extra-cellular ice formation during cryopreservation is a key issue for the development of successful cryogenic techniques (Sakai and Yoshida 1967). Exposure of plant material to cryoprotective agents (CPAs) such as dimethyl sulfoxide, glycerol, ethylene glycol and a variety of sugars can result in the formation of vitrified water (Mandumpal et al. 2011), thus preserving cellular structure during cooling to cryogenic temperatures (Benson 2008; Sakai and Engelmann 2007; Sakai et al. 1990). Cryopreservation for many plants requires lengthy incubation in CPAs.

The use of a vacuum to speed the infiltration of chemicals into various plant tissues is a common practice for gene transformation (agroinfiltration) and increasing storage life of fruit (Bechtold and Pelletier 1998; Ponappa et al. 1993). The application of vacuum during incubation in solutions is thought to reduce and eliminate trapped air bubbles within the microtopographical features of the plant tissues (such as the leaf primordium and trichomes), resulting in greater contact of the solutions with the surfaces of the samples. This improved contact is credited with gaining a more uniform infiltration as well as removing intercellular air in vacuoles and apoplastic compartments, allowing the influx of CPAs at a much greater rate (Nadarajan and Pritchard 2014). Initial cryopreservation experiments using rat tissue showed positive results for increasing CPA infiltration into tissues when utilising a vacuum (Soejima et al. 2012). This technique was also successfully applied to the zygotic embryos of three plant species (*Carica papaya*, *Laurus nobilis* and *Passiflora edulis*) greatly reducing the incubation times required (from 30 to 60 min down to 1 to 5 min) during PVS2 exposure to achieve corresponding post-cryopreservation growth (Nadarajan and Pritchard 2014).

The purpose of this study is to advance biotechnology research for *ex situ* conservation to help stem the loss of plant biodiversity in a biodiversity hotspot, the south-west of Western Australia (Kaczmarczyk et al. 2011b). Four native species including two threatened taxa were used to test the new VIV cryopreservation technique, *Androcalva perlaria* (Malvaceae), *Anigozanthos viridis* ssp. *terrapectans* (Haemodoraceae), *Lomandra sonderi* (Asparagaceae) and *Loxocarya cinerea* (Restionaceae). In this study both conventional droplet-vitrification and the new vacuum infiltration vitrification cryopreservation techniques were assessed and the

post-cryopreservation survival and regeneration of shoot tips of the four study species compared. Thermal analysis was undertaken to establish both the extent of ice formation and infiltration of CPAs in cryopreserved tissues, in response to the use of both cryogenic approaches.

Materials and methods

Plant material and in vitro conditions

Loxocarya cinerea (clone XH804) *in vitro* shoots were provided for experimental analysis from Alcoa of Australia's Marrinup Nursery, Western Australia. *Anigozanthos viridis*, *Lomandra sonderi* (clone LSOP) and *Androcalva perlaria* (clone 96) *in vitro* shoots were provided by Kings Park and Botanic Garden, Western Australia.

Basal medium (BM) consisting of half strength Murashige and Skoog (Murashige 1962) macro- and micronutrients with 100 µM NaFeEDTA), 1 µM thiamine hydrochloride, 2.5 µM pyridoxine, 4 µM nicotinic acid, 500 µM myo-inositol and 500 µM 4-morpholineethanesulfonic acid (MES) was used in all subculturing media and cryopreservation solutions.

In vitro shoots of *A. viridis*, *L. cinerea* and *L. sonderi* were maintained on BM with 60 mM sucrose, 0.8% w/v agar and 0.2 µM 6-benzylaminopurine (BA), at pH 6.0 and autoclaved at 121°C for 20 min. *In vitro* shoots of *A. perlaria* were maintained on the same medium described above except with 1.25 µM kinetin and 0.125 µM BA. All shoots were subcultured on a 3-4 weekly interval and maintained at 22-25°C with a 12 h light/12 h dark photoperiod (PPFD approximately 30 µmol $m^{-2}s^{-1}$ at culture level).

Cryopreservation solutions

Preculture medium (PM-1) consisting of BM with 0.2 M sucrose and 0.8% w/v agar was used for *A. viridis*, *L. cinerea* and *L. sonderi*. For *A. perlaria* PM-2 consisted of BM with 1.2 M glycerol and 0.8% w/v agar. Loading solution (LS) consisted of BM with 2 M glycerol and 0.4 M sucrose. Plant Vitrification Solution 2 (PVS2), consisted of BM with 0.4 M sucrose, 30% w/v glycerol and 15% w/v ethylene glycol and dimethyl sulfoxide at 15% w/v (Sakai et al. 1990). Washing solution (WS) consisted of BM with 0.75 M sucrose. Recovery medium (RM) consisted of BM with 60 mM sucrose, 2 mM choline chloride, 1 µM zeatin, 1 µM gibberellic acid and 0.8% w/v agar.

Conventional droplet-vitrification (CV) cryopreservation technique

Shoot tips for *A. viridis*, *L. cinerea* and *L. sonderi* (~1.5 x 0.5 mm, 1.0 x 0.3 mm and 1.2 x 0.5 mm respectively) were isolated onto PM-1 for 48 h incubation at 25°C with no exposure to light as previously described (Funnekotter et al. 2013; Kaczmarczyk et al. 2013; Menon et al. 2012), *A. perlaria* shoot tips (~1.0 x 0.5 mm) were isolated onto PM-2 and maintained under the same conditions as the *in vitro* shoots for 48 h. Shoot tips were placed in 2 ml LS for 20 min at room temperature (22-25°C) and then transferred to 2 ml PVS2 for various incubation durations at room temperature. After PVS2 incubation the shoot tips were placed into 1 µl PVS2 droplets on aluminium foils, transferred to a cryo-vial and cryo-vial was plunged into LN. Cryo-vials were rewarmed after 1 h in a 40°C water-bath for 10 s then shoot tips on foils were removed from the cryo-vials and incubated in WS for 20 min at room temperature.

Vacuum infiltration vitrification (VIV) cryopreservation technique

VIV cryopreservation technique was the same as above except for exposing the shoots to a vacuum during the 5 min LS and various PVS2 solution incubations using a 200 mm ø Kartell vacuum chamber. Vacuum was applied using a Rietschle Thomas vacuum pump (model 905CD23-194) with 80 kPa vacuum for the duration of the incubation. Vacuum was released over a 30 s period at the end of the incubation period.

Survival and regeneration assessment post-cryopreservation

After the WS incubation, shoot tips were placed onto RM for 4 weeks at 25°C with no light as done previously with *L. cinerea* and *L. sonderi* (Kaczmarczyk et al. 2013; Menon et al. 2012). Shoot tips were then transferred to fresh RM and cultured at 22-25°C with a 12 h light/12 h dark photoperiod (PPFD, approximately 30 µmol $m^{-2}s^{-1}$). Survival and regeneration of shoot tips post-cryopreservation were determined after 8 weeks, with survival recorded when shoot tips were green and swollen, or if callusing occurred (i.e. development and growth of non-specialised plant cells) that was also counted as survival. Regeneration was recorded if the original material showed actual shoot development, i.e. resumed normal shoot growth (Fig 5.1).

Thermal analysis

Thermal analysis of the shoot tips to identify the formation of ice crystals was done using differential scanning calorimetry (DSC). A Perkin-Elmer DSC 8000 instrument with a Perkin-Elmer CLN2 controlled LN accessory was used and controlled by Pyris V10.1 software, with the DSC instrument calibrated using an indium standard. Two shoot tips weighing 0.5-1 mg were placed in 30 µl DSC pans and hermetically sealed. Samples were held at 30°C for 1 min then cooled to -60°C at a rate of 10°C/min. Samples were then held at -60°C for 1 min and then rewarmed to 30°C at a rate of 50°C/min. Peak areas from the cooling curve were normalised to sample weight and the peak temperature was calculated using the Pyris 10.1 software when detected.

Statistics

Thermal analysis experiments were replicated three times for each treatment condition tested. Cryopreservation experiments involved 20 shoot tips for each treatment replicated four times. One-way-ANOVAs were used to determine statistical differences between treatments using Sigmaplot (Version 12.0, 2011, Systat Software Inc.). Where required, post hoc tests were completed using Fishers LSD to determine specific treatment differences.

Results

Thermal analysis

As expected all species showed large ice peaks after the preculture and LS treatments (Table 5.1). Progressively longer incubations in PVS2 were applied until no ice peak was detected in all replicates. The shoot tips of *A. viridis* required at least 20 min LS + 15 min PVS2 CV incubation before no discernible ice peak was

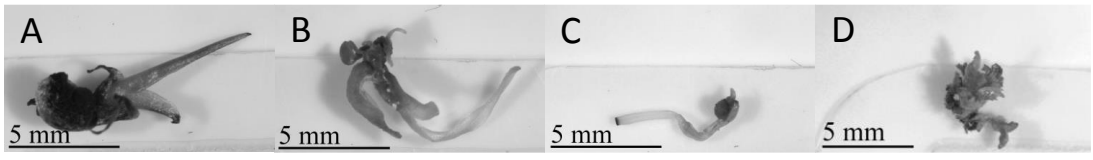

Figure 5.1. Post-cryogenic regeneration. Representative shoot tip showing post-cryogenic regeneration of *Anigozanthos viridis* (A), *Lomandra sonderi* (B), *Loxocarya cinerea* (C) and *Androcalva perlaria* (D).

Table 5.1. Comparison of ice formation in DSC themographs. CV – conventional vitrification technique, VIV – vacuum infiltration vitrification technique, LS – loading solution (2 M glycerol + 0.4 M sucrose), PVS2 – plant vitrification solution 2 (30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO + 0.4 M sucrose), ND – not detected. VIV incubation applied at room temperature with 80 kPa vacuum tested on four plant species: *Anigozanthos viridis, Lomandra sonderi, Loxocarya cinerea* and *Androcalva perlaria* (n = 3).

Species	Technique	Treatment	Peak Area (J/g ± SE)	Peak Temperature
A. viridis		0.2 M sucrose preculture	166.6 ± 43.8	-19.2 ± 0.2
	CV	20 min LS	98 ± 4	-32 ± 2.1
	VIV	5 min LS	95.5 ± 31.6	-25.9 ± 1
	CV	20 min LS + 5 min PVS2	33.4 ± 14	-35.9 ± 1.9
	CV	20 min LS + 10 min PVS2	0.3 ± 0.3	-35.7 ± 14.6
	CV	20 min LS + 15 min PVS2	ND	ND
	VIV	5 min LS + 2.5 min PVS2	21.1 ± 8.5	-29.1 ± 0.9
	VIV	5 min LS + 5 min PVS2	9 ± 7.9	-30.3 ± 2.5
	VIV	5 min LS + 7.5 min PVS2	1.4 ± 1	-36.8 ± 0.8
	VIV	5 min LS + 10 min PVS2	ND	ND
L. sonderi		0.2 M sucrose preculture	231.9 ± 20.9	-17.7 ± 1.6
	CV	20 min LS	129.9 ± 29	-22.8 ± 0.8
	VIV	5 min LS	134.6 ± 45.4	-20.5 ± 2.6
	CV	20 min LS + 2.5 min PVS2	31.8 ± 23.5	-44.5 ± 2.5
	CV	20 min LS + 5 min PVS2	8.3 ± 1.4	-39.8 ± 1.8
	CV	20 min LS + 10 min PVS2	0.2 ± 0.2	-33.5 ± 0
	VIV	5 min LS + 2.5 min PVS2	25.9 ± 14.8	-37.7 ± 5.4
	VIV	5 min LS + 5 min PVS2	0.3 ± 0.2	-33.2 ± 0.8
	VIV	5 min LS + 7.5 min PVS2	ND	ND
L. cinerea		0.2 M sucrose preculture	177.4 ± 35.6	-14.6 ± 0.9
	VIV	5 min LS	104.3 ± 9.1	-17 ± 1.4
	CV	20 min LS + 5 min PVS2	24.2 ± 1.2	-33.1 ± 1.3
	CV	20 min LS + 10 min PVS2	6.5 ± 5	-39.2 ± 3.7
	CV	20 min LS + 15 min PVS2	0.9 ± 0.3	-29 ± 2.7
	CV	20 min LS + 20 min PVS2	1.1 ± 0	-29 ± 0
	CV	20 min LS + 25 min PVS2	ND	ND
	VIV	5 min LS + 2.5 min PVS2	41.2 ± 4.4	-19.1 ± 1.5
	VIV	5 min LS + 5 min PVS2	14.9 ± 10.5	-28.9 ± 1.1
	VIV	5 min LS + 7.5 min PVS2	5.7 ± 2.7	-37.2 ± 4.3
	VIV	5 min LS + 10 min PVS2	3.2 ± 1.3	-34.7 ± 5.2
	VIV	5 min LS + 12.5 min PVS2	5.9 ± 3	-26.9 ± 1.9
	VIV	5 min LS + 15 min PVS2	6.7 ± 4.9	-35.8 ± 5.7
	VIV	5 min LS + 17.5 min PVS2	ND	ND
	VIV	5 min LS + 20 min PVS2	ND	ND
A. perlaria		1.2 M glycerol preculture	65.7 ± 14.6	-25.9 ± 1.4
	VIV	5 min LS + 5 min PVS2	6.9 ± 2.1	-30.4 ± 0.9
	VIV	5 min LS + 7.5 min PVS2	6.6 ± 5.3	-15.6 ± 0
	VIV	5 min LS + 10 min PVS2	0.5 ± 0.4	-19.4 ± 0

detected (Table 5.1). By comparison, no ice peak was detected after a 5 min LS + 10 min PVS2 with VIV applied (Table 5.1). *Lomandra sonderi* and *A. perlaria* showed similar ice peak reductions with increased incubation time of PVS2 (Table 5.1). However *L. cinerea* required the longest incubation time of all the species before no ice peak was detected, with 20 min LS + 25 min PVS2 CV incubation or 5 min LS + 17.5 min PVS2 VIV incubation (Fig 1C+D). Ice crystallization peak areas for *L. cinerea* did not decrease with increased PVS2 incubation times, with an extension of the incubation time from 7.5 min up to 15 min using the VIV technique showing similar small ice peaks (Table 5.1).

Cryopreservation

The shoot tips of three species were cryopreserved to compare the CV technique with the new VIV technique. Shoot tips were measured for survival and regeneration for both control (-LN) and cryopreserved (+LN) treatments. No apparent difference in post-cryogenic regeneration of the shoots was detected between the CV and VIV techniques. Thermal analysis results suggested that applying 5 min VIV LS was similar to the 20 min CV LS incubation (Table 5.1), thus 5 min VIV LS incubations were applied for all VIV cryopreservation experiments. Various VIV PVS2 incubation times were applied to reflect the thermal analysis results for comparison between detectable ice peaks and cryopreserved survival rates of the four study species.

Anigozanthos viridis showed significantly higher shoot tip survival with the VIV technique (66%) compared to the CV technique (30%) after cryopreservation; however, shoot tip regeneration did not differ significantly ($P > 0.05$) between the two techniques (31% and 20% respectively). Regeneration of cryopreserved shoot tips required a minimum of 7.5 min incubation time in PVS2 with the CV technique, whilst the use of the VIV technique reduced the minimum incubation time to 2.5 min before cryopreserved regeneration was observed (Fig 5.2).

CV cryopreservation of *L. sonderi* achieved a maximum 50% regeneration utilising 10 min PVS2 incubation with at least 7.5 min PVS2 incubation required before regeneration (20%) of shoot tips was observed (Fig 5.3). In comparison, VIV cryopreservation achieved regeneration of 27% after only 5 min PVS2 incubation, with a maximum cryopreserved regeneration rate of 42% after 10 min in PVS2. *Loxocarya cinerea* showed no regeneration of shoot tips using CV despite high levels of cryogenic survival (up to 85%). VIV cryopreservation did achieve shoot tip regeneration in *L. cinerea* when 5 or 10 min PVS2 incubation times were utilised,

with 7 and 10% regeneration respectively (Fig 5.4). However, regeneration was variable and not observed in all replicate treatments. *Androcalva perlaria* showed the highest rates of cryogenic survival and regeneration of all species tested, with 40% regeneration using 5 min VIV PVS2 incubation and increasing to 75% when the incubation period was increased to 10 min (Fig 5.5). All shoot tips that survived cryopreservation underwent regeneration to form shoots.

Comparison of potential CPA toxicity with the control treatment (-LN) showed no significant differences in survival and regeneration between the CV and VIV techniques for the same incubation times for both *A. viridis* and *L. cinerea*. *Lomandra sonderi* showed an apparent increase in CPA toxicity when the VIV technique was applied at 2.5 and 5 min, with shoot regeneration being significantly lower in comparison to the CV technique (86% to 55% and 92% to 51%, respectively). Overall, survival and regeneration of *L. sonderi* shoot tips appeared to

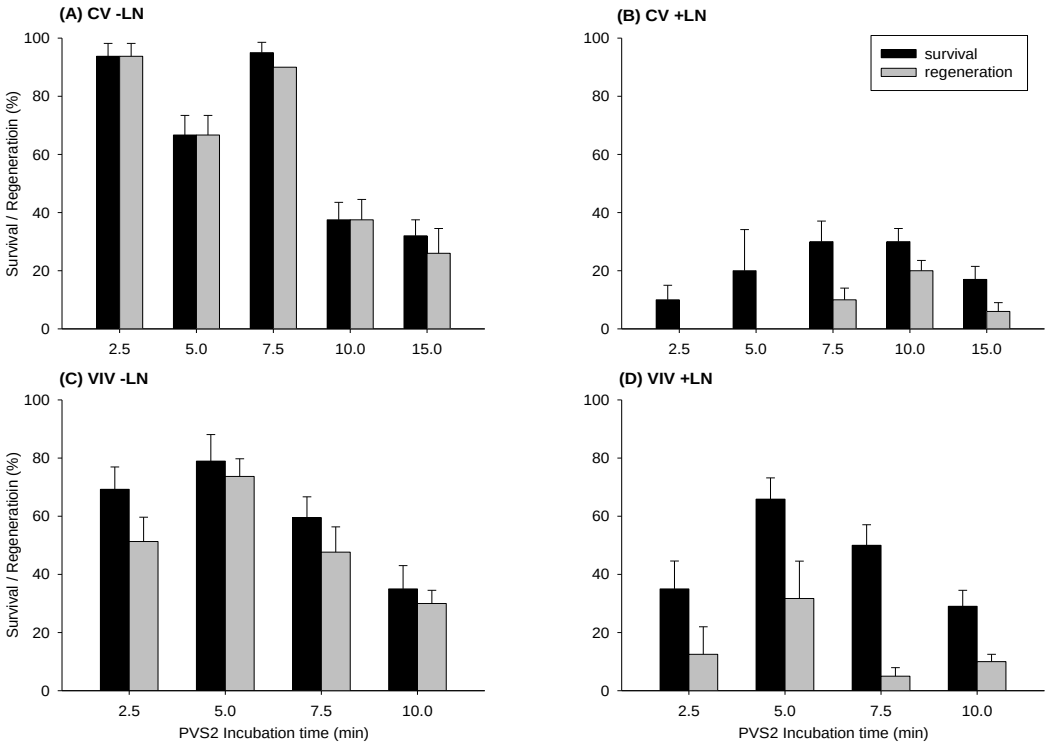

Figure 5.2. Survival and regeneration of *Anigozanthos viridis* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A and C) and after (+LN, B and D) cryopreservation comparing conventional (A and B) and vacuum infiltration (C and D) vitrification techniques. A 20 min loading solution (LS) was applied to CV treatments, while a 5 min LS +vacuum infiltration step was applied to VIV treatments. Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE.

be more consistent with the VIV method without exposure to LN, with very little variation from 2.5 to 10 min exposure times.

Discussion

CPAs have two main actions: they suppress the formation of ice crystals and protect the cells when they are dehydrated. CPAs suppress ice formation by increasing the viscosity of free liquid water in cells and tissues, thus interfering with ice nucleation and allowing water to supercool until a vitrified state is achieved (Kauzmann 1948; Mishima and Stanley 1998). The application of CPAs is vital if successful cryopreservation techniques are to be developed; however, some CPAs are phytotoxic including DMSO and glycerol, especially at the concentrations required to fully protect plant cells and tissues (Volk et al. 2006). It is therefore important to

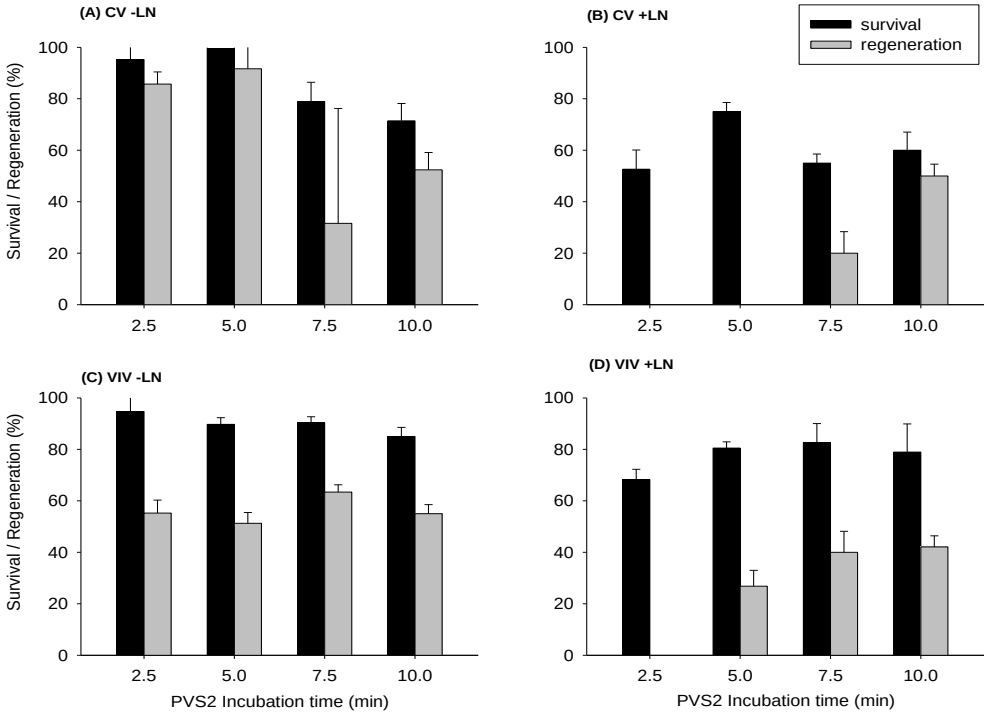

Figure 5.3. Survival and regeneration of *Lomandra sonderi* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A and C) and after (+LN, B and D) cryopreservation comparing conventional (A and B) and vacuum infiltration (C and D) vitrification techniques. A 20 min loading solution (LS) was applied to CV treatments, while a 5 min LS +vacuum infiltration step was applied to VIV treatments. Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE.

achieve the right balance between the provision of sufficient protection from ice damage and the avoidance of excessive dehydration and phytotoxicity. As all species differ morphologically, physiologically and biochemically, achieving an optimal internal CPA concentration for maximum post-cryopreservation regeneration can require extensive testing for individual species. The type of plant material, culture conditions of the plant material, the composition of the CPAs, exposure time and temperature during exposure to the CPAs, as well as the specific cryopreservation procedure used will all have a large cumulative effect on the regeneration capacity of plant tissues following cryopreservation (Funnekotter et al. 2013; Kaczmarczyk et al. 2012; Kaczmarczyk et al. 2011a; Kim et al. 2009a; Kim et al. 2009b; Kulus and Zalewska 2014; Menon et al. 2014; Sakai and Engelmann 2007).

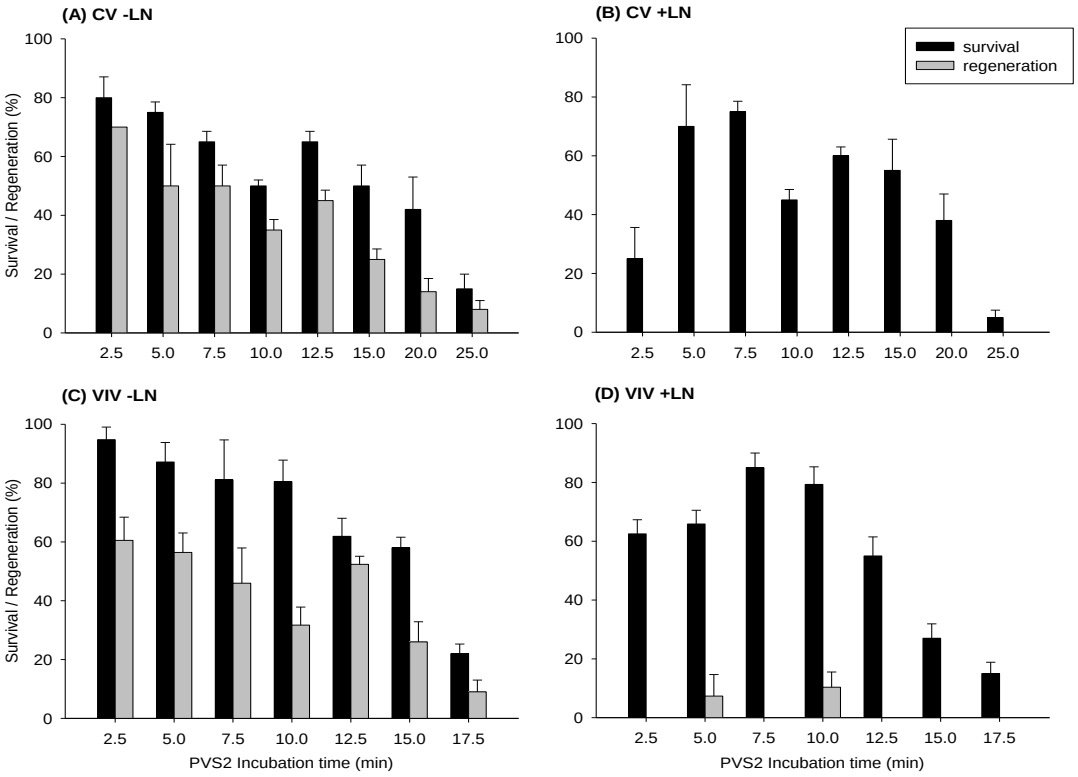

Figure 5.4. Survival and regeneration of *Loxocarya cinerea* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A and C) and after (+LN, B and D) cryopreservation comparing conventional (A and B) and vacuum infiltration (C and D) vitrification techniques. A 20 min loading solution (LS) was applied to CV treatments, while a 5 min LS +vacuum infiltration step was applied to VIV treatments. Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE.

Post-cryopreservation regeneration of the shoot tips of four of the species tested in this study showed evidence of increased penetration rate of CPAs using the VIV technique compared to the CV technique (Table 5.1, Fig 5.2-5). Optimal internal CPA concentrations may be achieved more rapidly when applying the VIV technique, thereby enabling shorter incubation times and, consequently, more rapid processing of large numbers of plant samples.

Comparison of regeneration rates between the CV and VIV controls indicates that the VIV technique results in greater apparent toxicity to the shoot tips of only one of the test species, *L. sonderi,* when comparing similar incubation times (Fig 5.3). However this result is not unexpected as VIV results in greater surface contact of the CPAs, with a faster rate of CPA penetration of shoot tips and more rapid CPA accumulation in tissues to the point where phytotoxic damage may occur. Therefore whilst the VIV technique substantially reduces the exposure time necessary for CPA exposure, care must be taken to avoid the potential for increased toxic side effects. Incubation of shoot tips in CPAs at 0°C is commonly used to reduce phytotoxic effects, with many studies showing that the application of PVS2 solutions is much more effective at lower temperatures, although these need to be applied for considerably longer periods of time (Menon et al. 2012; Volk et al. 2006). At lower temperatures (such as 0°C),

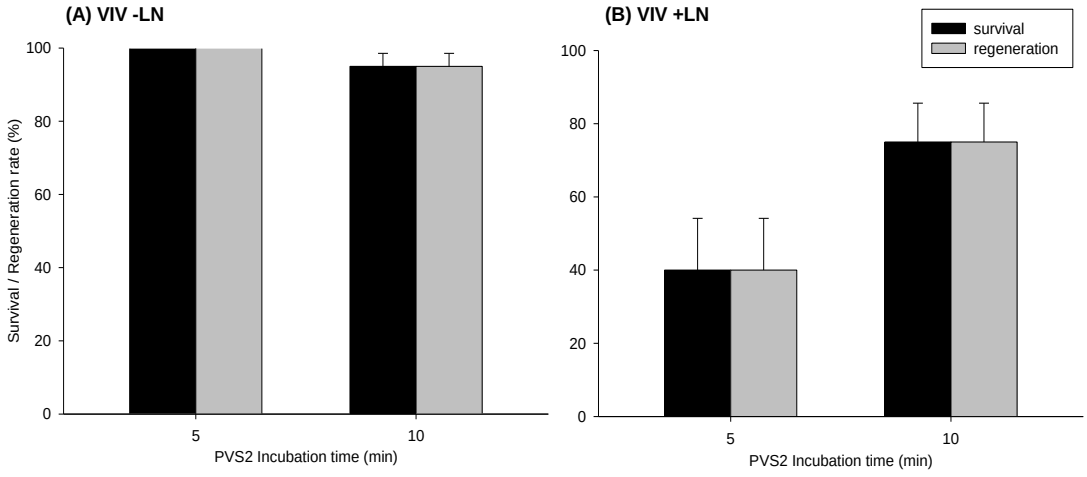

Figure 5.5. Survival and regeneration of *Androcalva perlaria* shoot tips following cryopreservation. Survival and regeneration of shoot tips before (-LN, A) and after (+LN, B) cryopreservation utilising the vacuum infiltration vitrification technique (+5 min LS step) Black bars represent survival of the shoot tips and grey bars represent regeneration of the shoot tips following normal development. Data shows mean ± SE.

however, penetration of the CPAs is also reduced (due to increased viscosity and reduced molecular diffusion), therefore requiring longer incubation times (Cho et al. 2002; Matsumoto et al. 1994). Application of the CPAs at 0°C or the use of diluted PVS2 may benefit the VIV technique by reducing phytotoxic effects whilst lengthy incubation times will still be avoided. Whilst this is beneficial for many species, tropical species in particular suffer when exposed to CPAs for long durations at low temperatures and show a better capacity for regeneration when a higher temperature (room temperature) coupled with a shorter incubation time is used (Sakai et al. 2008). In this study the application of room temperature incubation for all cryogenic solutions was utilised to simplify development and comparison of the new protocol. In addition, previous work undertaken on *L. sonderi* indicated a requirement for room temperature incubation for best regeneration following cryopreservation (Menon et al. 2012).

The use of DSC for the detection of ice formation/melting peaks in the thermographs is helpful for the development of new cryopreservation protocols, with the ability to determine precisely the optimum incubation time in CPA solutions to prevent or at worst minimise ice formation (Kaczmarczyk et al. 2013). This information indicates whether or not vitrification is achieved and, therefore, excessive CPA exposure can be minimised and phytotoxic damage reduced, all of which greatly reduce the range of cryopreservation experiments needed to determine optimum incubation times. Thermal analysis confirmed that the infiltration of CPAs into the shoot tips is increased when using the VIV technique. The overall incubation times for the VIV technique were reduced by half (*L. cinerea*) down to a quarter (*L. sonderi*) of the time when compared to the CV technique. Whilst this was not as great as the ~10 fold reduction seen for isolated embryos/axes (Nadarajan and Pritchard 2014), Nadarajan *et al.* (2014) compared the two techniques with CV incubations done at 0°C, whilst the VIV incubations in this present study were done at room temperature. Comparison of thermal analysis previously done on *L. cinerea*, where PVS2 was applied at 0°C using CV showed an optimal incubation period of over 60 min in PVS2 (Kaczmarczyk et al. 2013), revealed that an incubation of 25 min in PVS2 at room temperature (25°C) was sufficient to remove the detection of an ice formation peak (Table 1), which demonstrates the substantial influence that temperature imposes on the effectiveness of PVS2 incubation times.

However, it appears that the optimum incubation time suggested from the thermal analysis data is not necessarily optimal for maximum regeneration of shoot tips after cryopreservation. The optimum PVS2 incubation suggested by the thermographs for *A. viridis* was 15 min for CV and 10 min VIV, yet the highest cryopreserved

regeneration observed resulted from an incubation period of 10 min for CV and 5 min for VIV (Fig 5.1). This was also seen in the other three species, where the optimal incubation time suggested by the thermographs was longer than the incubation time that resulted in the greatest cryopreserved regeneration. The majority of incubation times that showed regeneration had very small ice formation peak areas (less than 10 J/g), which may be suppressed even further due to the higher cooling rate achieved with the droplet-vitrification method (7800°C/min compared to the 10°C/min cooling rate inherent in the DSC procedure used here) (Kaczmarczyk et al. 2011a). Furthermore, these small peaks may simply represent the formation of ice in non-critical tissue regions and consequently the presence of this small amount of ice does not significantly impact meristem regeneration.

Previous efforts at successfully cryopreserving *L. cinerea* shoot tips have had limited success (Funnekotter et al. 2013; Kaczmarczyk et al. 2013), and limited penetration of CPAs into the shoot tips may contribute to the cryogenic recalcitrant nature of this species. Of all four species tested, *L. cinerea* took the longest incubation time in PVS2 before an ice formation peak was no longer detectable in the thermographs in both CV and VIV techniques (Fig 5.4). This confirms previous work reported where long incubation times were required for shoot tips in PVS2 at 0°C with ice formation peaks still detected after 60 min incubations (Kaczmarczyk et al. 2013). The low level of regeneration of cryopreserved *L. cinerea* shoot tips does not seem linked to the optimal incubation time suggested by the thermographs on the basis of the elimination of ice formation peaks (17.5 min PVS2 VIV incubation or 25 min PVS2 CV incubation), nor with the highest cryogenic survival achieved (with a 7.5 min PVS2 VIV incubation). Whilst previous work suggested that ice formation is an unlikely reason for the lack of regeneration after cryopreservation (Kaczmarczyk et al. 2013), the continual occurrence of ice formation peaks despite increasing incubation times suggest that penetration of CPAs in *L. cinerea* may not occur at a uniform rate across the shoot tips. At present, it is not known where this ice formation occurs within the shoot tips, but if ice formation occurs in the meristematic tissue then regeneration may be significantly compromised. This may explain the high levels of regeneration seen in the control (-LN) experiments as 50-85% of shoot tips that survived underwent successful regeneration, in comparison to cryopreserved (+LN) shoot tips that showed only 0-13% of the surviving shoot tips regenerating into actively growing new plant tissues (Fig 5.3). The high levels of post-cryogenic survival (up to 85%) indicate that the majority of the tissue in the shoot tips is sufficiently protected by the CPAs. It is therefore difficult to establish with any certainty why some shoot tips showed

regeneration and not others. Regeneration of a few shoot tips may be due to these tips having the optimum size for CPA penetration to avoid the small ice formation peaks detected in the thermographs, yet CV showed similar cryopreservation survival rates albeit with no regeneration. Thus if CPA penetration into the meristem is the issue, the application of the VIV technique may enhance penetration by allowing a more uniform and rapid distribution of the CPAs throughout the shoot tips undergoing PVS2 treatment. This aspect is worthy of further investigation as it may hold the key to optimal regeneration with difficult to cryostore species such as *L. cinerea*.

The application of LS for 5 min and 5 or 10 min incubation in PVS2 when applying the VIV technique showed promising results for *A. viridis*, *L. cinerea* and *L. sonderi*. Previous work on *A. perlaria* showed high levels of post-cryogenic regeneration up to 83% using a CV technique (Whiteley & Turner unpublished results). This result on *A. perlaria* (Fig 5.4) showed high regeneration of cryopreserved shoot tips (75%) when applying the VIV technique, confirming that the application of 5 min LS and 10 min PVS2 incubation under vacuum is a good starting point for the development of new cryopreservation protocols. The protocol used to cryopreserve *A. perlaria* in this study has previously been optimised for achieving high levels of regeneration and this may account for why the highest regeneration rates were observed in this species. Higher survival and regeneration rates may be expected if the propagation and preculture media were customised for each of the other three species (*A. viridis*, *L. cinerea* and *L. sonderi*); however, the use of identical culture conditions, and propagation and preculture media for these three species was undertaken to provide a greater understanding of the potential of the previously untried VIV technique with these species and the expected outcomes when a generic cryopreservation approach is applied to several dissimilar taxa.

This study shows the large variation in cryo-capability commonly seen when applying the same cryopreservation protocol to multiple plant species, comparison of the 10 min PVS2 incubation between the species showed the greatest post-cryogenic survival in *A. perlaria* then *L. sonderi*, both of which also showed good –LN regeneration, *A. viridis* had the lowest –LN regeneration and these three species all showed very little to no ice formation with 10 min PVS2 incubation. Thus the post-cryogenic regeneration rates are most likely linked to the plants sensitivity to the CPAs. *Loxocarya cinerea* however showed a similar sensitivity to the CPAs as *A. viridis* and the largest amount of ice formation in the shoot tips, thus the low levels of post-cryogenic regeneration for *L. cinerea* is likely due to a combined effect of CPA phyototoxicity and crystallisation damage.

Conclusions

To the best of our knowledge this is the first report utilising VIV for the cryopreservation of shoot tips of any Australian plant species. The application of the VIV technique resulted in a significantly reduced PVS2 incubation time for cryogenic survival and regeneration, reducing the time needed to adequately protect shoot tips by half to a quarter when compared to the CV technique (Table 5.1).

The increase in vacuum pressure is thought to increase CPA infiltration (Zhao and Xie 2004). Consequently higher vacuum pressures may further reduce the incubation times required for optimum CPA penetration to be achieved. Although this was not tested in this study it warrants further investigation, particularly for *L. cinerea* which showed the least improvements when applying the VIV technique to reduce incubation times. This technique will also need to be tested on a wider range of species to fully understand its advantages and potential disadvantages if not used discriminately.

References

Bajaj, Y.P.S. 1995. "Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity." In *Biotechnology in Agriculture and Forestry 32*, ed. Y.P.S. Bajaj, 1-18. New York: Springer.

Bechtold, N., and G. Pelletier. 1998. "In Planta Agrobacterium Mediated Transformation of Adult *Arabidopsis Thaliana* Plants by Vacuum Infiltration." In *Arabidopsis Protocols*, eds José Martinez-Zapater and Julio Salinas, 259-266. Humana Press.

Benson, E.E. 2008. "Cryopreservation Theory." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 15-32. New York: Springer.

Cho, E.G., Y.L. Hor, H.H. Kim, V.R. Rao, and F. Engelmann. 2002. "Cryopreservation of *Citrus Madurensis* Embryonic Axes by Vitrification: Importance of Loading and Treatment with Vitrification Solution." *CryoLetters* 23 (5): 317-324

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Kaczmarczyk, A., B. Funnekotter, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., B. Funnekotter, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for *Loxocarya Cinerea*-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519

Kaczmarczyk, A., V.-M. Rokka, and E.J. Keller. 2011a. "Potato Shoot Tip Cryopreservation. A Review." *Potato Research* 54 (1): 45-79

Kaczmarczyk, A., S.R. Turner, E. Bunn, R.L. Mancera, and K.W. Dixon. 2011b. "Cryopreservation of Threatened Native Australian Species—What Have We Learned and Where to from Here?" *In Vitro Cellular & Developmental Biology - Plant* 47 (1): 17-25

Kartha, K., N. Leung, and L. Mroginski. 1982. "*In Vitro* Growth Responses and Plant Regeneration from Cryopreserved Meristems of Cassava (*Manihot Esculenta* Crantz)." *Zeitschrift für Pflanzenphysiologie* 107 (2): 133-140

Kauzmann, W. 1948. "The Nature of the Glassy State and the Behavior of Liquids at Low Temperatures." *Chemical Reviews* 43 (2): 219-256

Kim, H.H., Y.G. Lee, S.U. Park, S.C. Lee, H.J. Baek, E.G. Cho, and F. Engelmann. 2009a. "Development of Alternative Loading Solutions in Droplet-Vitrification Procedures." *CryoLetters* 30 (4): 291-299

Kim, H.H., Y.G. Lee, D.J. Shin, H.C. Ko, J.G. Gwag, E.G. Cho, and F. Engelmann. 2009b. "Development of Alternative Plant Vitrification Solutions in Droplet-Vitrification Procedures." *CryoLetters* 30 (5): 320-34

Kulus, D., and M. Zalewska. 2014. "Cryopreservation as a Tool Used in Long-Term Storage of Ornamental Species–a Review." *Scientia Horticulturae* 168: 88-107

Mandumpal, J.B., C.A. Kreck, and R.L. Mancera. 2011. "A Molecular Mechanism of Solvent Cryoprotection in Aqueous Dmso Solutions." *Physical Chemistry Chemical Physics* 13 (9): 3839-3842

Matsumoto, T., A. Sakai, and K. Yamada. 1994. "Cryopreservation of in Vitro-Grown Apical Meristems of Wasabi (*Wasabia Japonica*) by Vitrification and Subsequent High Plant Regeneration." *Plant Cell Reports* 13 (8): 442-446

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Mishima, O., and H.E. Stanley. 1998. "The Relationship between Liquid, Supercooled and Glassy Water." *Nature* 396 (6709): 329-335

Murashige, T. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473

Nadarajan, J., and H.W. Pritchard. 2014. "Biophysical Characteristics of Successful Oilseed Embryo Cryoprotection and Cryopreservation Using Vacuum Infiltration Vitrification: An Innovation in Plant Cell Preservation." *PloS One* 9 (5): e96169

Ponappa, T., J.C. Scheerens, and A.R. Miller. 1993. "Vacuum Infiltration of Polyamines Increases Firmness of Strawberry Slices under Various Storage Conditions." *Journal of Food Science* 58 (2): 361-364

Sakai, A., and F. Engelmann. 2007. "Vitrification, Encapsulation-Vitrification and Droplet-Vitrification: A Review." *CryoLetters* 28 (3): 151-72

Sakai, A., D. Hirai, and T. Niino. 2008. "Development of Pvs-Based Vitrification and Encapsulation–Vitrification Protocols." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 33-57. New York: Springer.

Sakai, A., S. Kobayashi, and I. Oiyama. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb. Var. Brasiliensis Tanaka) by Vitrification." *Plant Cell Reports* 9 (1): 30-33

Sakai, A., and S. Yoshida. 1967. "Survival of Plant Tissue at Super-Low Temperature Vi. Effects of Cooling and Rewarming Rates on Survival." *Plant Physiology* 42 (12): 1695-1701

Soejima, K., K. Shimoda, M. Takeuchi, T. Yamaki, T. Kono, H. Sakurai, and H. Nakazawa. 2012. "Preliminary Report of Novel Technique for Cryopreservation—Vacuum-Assisted Cryoprotectant Infiltration." *Cryobiology* 65 (1): 21-26

Turner, S., D. Touchell, K. Dixon, and B. Tan. 2000. "Cryopreservation of *Anigozanthos Viridis* Ssp. *Viridis* and Related Taxa from the South-West of Western Australia." *Australian Journal of Botany* 48 (6): 739-744

Volk, G.M., J.L. Harris, and K.E. Rotindo. 2006. "Survival of Mint Shoot Tips after Exposure to Cryoprotectant Solution Components." *Cryobiology* 52 (2): 305-308

Yamamoto, S., T. Rafique, K. Fukui, K. Sekizawa, and T. Niino. 2012. "V-Cryo-Plate Procedure as an Effective Protocol for Cryobanks: Case Study of Mint Cryopreservation." *CryoLetters* 33 (1): 12-23

Zhao, Y., and J. Xie. 2004. "Practical Applications of Vacuum Impregnation in Fruit and Vegetable Processing." *Trends in Food Science & Technology* 15 (9): 434-451

Chapter 6 – Application of exogenous ascorbic acid and lipid peroxidation analysis in the improved protocol for the successful cryopreservation of Australian plant species *Loxocarya cinerea*

Abstract

Loxocarya cinerea is an important plants species in mine-site rehabilitation programs. It is currently propagated through plant tissue culture but has proven exceptionally difficult to regenerate from cryogenically stored shoot tips. To improve current cryogenic approaches on this species the use of ascorbic acid (AsA) was assessed in concert with variations in the cryogenic protocol. Extending the preculture time (from 2 to 3, 5 and 7 d) was found to significantly increase regrowth of *L. cinerea* shoot tips after cryopreservation from 0% to 9%, 37% and 35% regeneration, respectively. The addition of 0.5 mM AsA to the preculture medium further increased regrowth from 35% to 53% of shoot tips regenerating after cryopreservation of 7 day precultured shoot tips. Higher levels of malondialdehyde (MDA) were detected in recovering shoot tips that exhibited the best post-cryogenic regrowth, implicating a higher degree of lipid peroxidation where it would not otherwise be expected. The use of the C11-BODIPY$^{581/591}$ fluorophore, which shows a red to green fluorescence shift upon oxidation, indicated that increased oxidative stress occurred in shoot tips with low regeneration after cryopreservation, and this approach may be applicability to other problematic cryogenically recalcitrant species for observing oxidative stress.

Introduction

Cryopreservation allows for the cost effective long-term storage of a wide range of plant material without degradation for species that are rare and threatened, difficult to propagate via conventional means or do not grow true-to-type from seeds (Kaczmarczyk et al. 2012). *Loxocarya cinerea* (Restionaceae), an south-west Western Australian endemic plant species, is considered a recalcitrant species in post-mining rehabilitation due to complex dormancy and low seed production and, consequently, it is micropropagated and transplanted back into rehabilitation sites at present (Willyams 2005; Koch and Samsa 2007). Cryopreservation of *L. cinerea* has had little success with cryogenically stored shoot tips, showing very low regeneration and regrowth post-cryopreservation, although initial survival is reasonably high (~50%) (Funnekotter et al. 2013; Funnekotter et al. 2015; Kaczmarczyk et al. 2013).

Throughout the cryopreservation process, extracted shoots can be exposed to a wide range of stresses and the ability of plants to resist and overcome these stresses significantly influences post-cryogenic survival and regeneration. Cell membranes represent a primary site for cryo-injury, which occurs due to several different deleterious processes that occur during cryopreservation (Steponkus 1984). Membranes are susceptible to rupture through ice crystal penetration as well as deleterious phase changes that can result in the formation of non-bilayer phases and oxidative damage. All of these damages can affect the fluidity, stability, structure and active/passive cross membrane transportation systems required for proper membrane functionality (Funnekotter et al. 2013; Benson 2008; Hammerstedt et al. 1990; Steponkus 1984; Menon et al. 2014). To address these damaging processes, cryoprotective agents (CPAs) are used to protect shoot tips from excessive ice crystallisation and related cellular damage.

Lipid peroxidation (LPO) can also be highly destructive to membranes, fatty acids and membrane proteins, altering the semi-permeable characteristics and functionality of the membrane (Benson et al. 1992; Halliwell and Gutteridge 2007). Malondialdehyde (MDA) is a commonly used marker of LPO, produced during the process of LPO in polyunsaturated fatty acids (PUFAs) with more than two double bonds (Halliwell and Gutteridge 2007). MDA has been shown to alter proteins, DNA and RNA through Shiff's base additions, causing cellular damage (Esterbauer et al. 1991; Marnett 1999b). Previous work on blackberry shoot tips by Uchendu et al. (2010a) linked increased MDA formation with reduced post-cryogenic survival, with similar results observed in rice cells as well (Benson et al. 1992).

The use of oxidation-sensitive fluorophores enables analysis of oxidative stress in individual shoot tips, thus providing an advantage over other types of analysis where shoot tips are pooled. This is of particular interest in post-cryogenic shoot tips, where pooled samples will contain a mix of regenerating, surviving and dead shoot tips, making the identification of the causes and locations of specific tissue damage problematic. The fluorophore C11-BODIPY$^{581/591}$ is designed to mimic PUFAs and, upon oxidation, the fluorescence excitation and emission shift from red to green is used to perform a ratiometric comparison of oxidative stress (Drummen et al. 2004) where oxidation of the probe is comparable to or slightly quicker than LPO of PUFA (Pap et al. 1999; MacDonald et al. 2007). The application of the BODIPY$^{581/591}$ fluorophore to observe oxidative stress during cryopreservation has predominantly been used in animal spermatozoa analysis, where the stresses of cryopreservation have been shown to result in increased oxidative damage and LPO after

cryopreservation (Castellano et al. 2010; Brouwers et al. 2005; Domínguez ‐ Rebolledo et al. 2010).

The addition of exogenous antioxidants prior to, during or following the cryopreservation process has been shown to increase post-cryogenic survival. Wang and Deng (2004) observed increased survival of citrus with the addition of reduced glutathione (GSH). Similarly, Uchendu et al. (2010a) showed increased regrowth of blackberry shoot tips with the addition of tocopherol up to 15 mM or ascorbic acid (AsA) at concentrations up to 0.58 mM, while the application of GSH improved *Rubus* recovery (Uchendu et al. 2010b).

However, the continued lack of success with post-cryogenic regeneration with *L. cinerea* required further investigation. Preliminary results suggested an extended preculture period for *L. cinerea* may improve its cryo-capability. In this study, multiple preculture periods (2, 3, 5 & 7 days) were tested to determine their effect on post-cryogenic regeneration. A single concentration of AsA (0.5 mM) was also investigated to assess whether or not the presence of AsA in the preculture medium leads to any observable differences in post cryopreservation survival of *L. cinerea*. In addition to assessing the effects of extended preculture medium treatments, the formation of LPO as a marker of oxidative stress was also assessed by quantifying MDA in shoot tips after cryopreservation. Oxidative stress was determined using the C11-BODIPY$^{581/591}$ fluorophore using the ratio of red to green fluorescent intensity, while fluorescein diacetate (FDA) staining was used as an indicator of viability in the shoot tips after cryopreservation.

Materials and methods

Plant material and propagation medium

In vitro shoots were excised from *L. cinerea* (clone XH804) obtained from Alcoa of Australia's Marrinup Nursery. The *in vitro* shoots were maintained on a basal medium (BM), consisting of half strength Murashige and Skoog medium (Murashige 1962) modified to include 100 µM NaFeEDTA, 1 µM thiamine hydrochloride, 2.5 µM pyridoxine, 4 µM nicotinic acid, 500 µM myo-inositol and 500 µM 4-morpholineethanesulfonic acid. *In vitro* shoots were maintained on multiplication medium consisting of BM, 60 mM sucrose, 0.2 µM benzylaminopurine (BA) and 8 g L^{-1} agar with pH set at 6.0. Three weeks prior to the cryopreservation experiment, the shoots were transferred onto fresh multiplication medium and cultured at 5°C with a

12 h light/dark photoperiod for preconditioning. Preculture medium (PM) consisted of BM with 0.2 M sucrose, and 8 g L^{-1} agar with pH set at 6.0, and recovery medium (RM) consisted of BM with 60 mM sucrose, 2 mM choline chloride, 1 µM gibberellic acid, 1 µM zeatin, and 8 g L^{-1} agar with pH set at 6.0.

Cryopreservation solutions

Loading solution (LS) consisted of 2 M glycerol and 0.4 M sucrose. Plant Vitrification Solution 2 (PVS2) consisted of 0.4 M sucrose, 30% w/v glycerol, 15% w/v ethylene glycol and DMSO at 15% w/v (Sakai et al. 1990). Washing solution (WS) consisted of 0.75 M sucrose.

Cryopreservation protocol

Loxocarya cinerea shoot tips were cryopreserved using the vacuum infiltration vitrification cryopreservation method (Funnekotter et al. 2015). Shoot tips were isolated onto PM for 2, 3, 5 and 7 days at 25°C without light, then shoot tips were incubated in LS for 5 min with 80 kPa vacuum before being transferred to PVS2 for 10 min incubation with 80 kPa vacuum. LS and PVS2 incubations were done at room temperature. After the PVS2 incubation, shoot tips were placed into 1 µl droplets of PVS2 on aluminium foils and transferred to a cryovial which was plunged in to liquid nitrogen (LN) for 1 h. Cryovials were re-warmed by plunging them into a 40°C water-bath for 30 s. The foil strips containing the shoot tips were then incubated in WS for 20 min at room temperature. Shoot tips were then placed on RM at 25°C with no light and regeneration of shoot tips was analysed after 4 weeks. Shoot tips were classified as regenerating if they resumed normal shoot growth following cryopreservation.

Addition of exogenous ascorbic acid

Ascorbic acid stock was set to a pH 5.8 then filter-sterilised and added into the PM after autoclaving for a final concentration of 0.50 mM (88 mg L^{-1}). Shoot tips of *L. cinerea* were isolated onto the PM containing AsA for 2, 3, 5 and 7 days and cryopreserved as described above.

Lipid peroxidation analysis

MDA was analysed using the commercially available BioVision assay kit, which measures the formation of thiobarbituric acid reactive substances (TBARS) (Heath and Packer 1968). Four weeks post-cryopreservation on RM, shoot tips of *L. cinerea* were pooled (~20 mg) and homogenised in 300 µl lysis buffer (supplied with assay kit). 200 µl of sample after centrifugation (13000 *g* x 10 min at 4°C) was incubated

with 600 µl thiobarbituric acid (TBA) reagent at 95°C for 1 h and then cooled on ice for 10 min. 200 µl of sample was then placed in a 96 well plate and absorbance of the MDA-TBA adduct was measured at 532 nm. The formation of the MDA-TBA adduct was calculated from external standards over a linear absorbance range ($R^2 = 0.998$).

Fluorescent probes

Shoot tips of *L. cinerea* freshly isolated or on RM 4 weeks post-cryopreservation were incubated in 50 µM C11-BODIPY$^{581/591}$, obtained from the commercially available Image-iT lipid peroxidation kit (C10445, Life Technologies, USA), for 30 min at room temperature prior to observation using a Nikon Eclipse 80i microscope with a Nikon C-SHG1 Super High Pressure 100 W mercury lamp. A Nikon Triple Band Excitation DAPI-FITC-TRITC filter combination was used and images were captured using a Nikon DS-Fi1 camera with a Nikon DS-L2 stand-alone control unit.

Analysis of the red and green intensities was performed using ImageJ software (version 1.48v). Images were split into their respective RGB channels, with the integrated density (product of area and mean grey value) of three 200 µm^2 boxes measured from the red and green channels and corrected by subtracting the background integrated density. The relative proportion of green fluorescence was then calculated per shoot tip (green fluorescence intensity / total (red+green) fluorescence intensity). Merged images were made using ImageJ by overlaying the respective colour channels into one picture.

Fluorescein diacetate (FDA) staining

Shoot tips of *L. cinerea* freshly isolated or on RM 4 weeks post-cryopreservation were stained with FDA (5 mg/ml acetone) for 30 seconds. Shoot tips were then counter-stained for 30 seconds in DAPI (4'-6'-diamidino-2-phenylindol, 0.1 mg/ml) at room temperature. Shoot tips were observed using the same microscope and capture equipment as described above.

Statistical analysis

Cryopreservation results for each treatment were replicated four times with 20 non-cryopreserved shoot tips and 20 cryopreserved shoot tips per replicate. MDA analysis consisted of three replicates per treatment. Fluorescent staining of the shoot tips with BODIPY and FDA was done on 10 shoot tips per treatment. Statistical differences were calculated with one-way ANOVA using SigmaPlot (Systat Software Inc. version

12.0, 2011). Where required, *post hoc* tests were completed using Tukey's test to determine specific treatment differences ($P < 0.05$).

Results

Cryopreservation

Shoot tips of *L. cinerea* were cryopreserved to compare extended preculture times of 2, 3, 5 and 7 days, with or without 50 mM AsA in the preculture medium (Fig 6.1). The control non-cryopreserved (-LN) shoot tips showed a significant increase in regeneration from the 2 day to the 3 and 5 day preculture treatments (63%, 90% and 90%, respectively), with regeneration then decreasing for the 7 day precultured shoot tips (67%). The addition of AsA to the preculture medium showed no significant differences between the various preculture periods with regeneration of the non-cryopreserved shoot tips above 86%; however, the application of AsA resulted in greater regeneration compared to the control, and the 2 day AsA

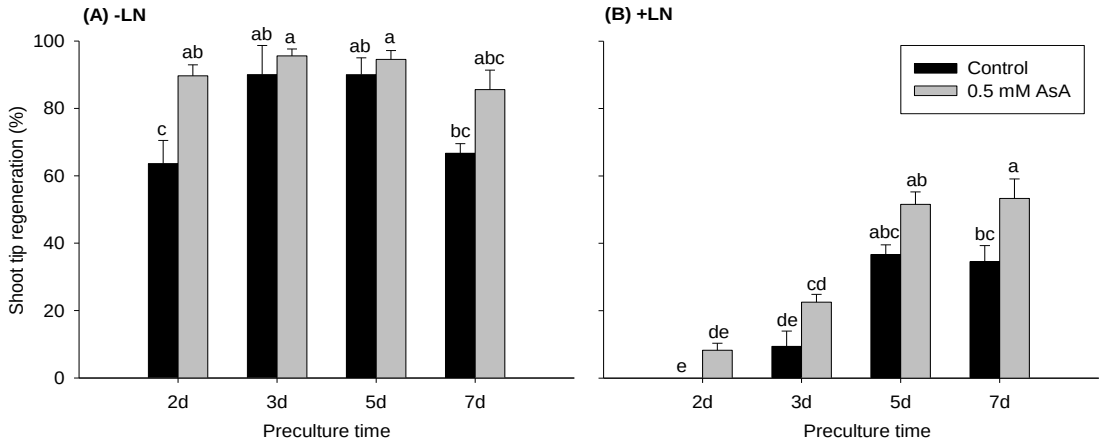

Figure 6.1. Regeneration of *Loxocarya cinerea* shoot tips 4 weeks post-cryopreservation. Comparison of regeneration of non-cryopreserved (-LN, A) and cryopreserved (+LN, B) shoot tips precultured for 2, 3, 5 and 7 days. Black bars show regeneration of control shoot tips with no ascorbic acid exposure while grey bars show regeneration of shoot tips exposed to 0.5 mM ascorbic acid during the preculture period. Different letters denote a statistically significant difference between all treatments ($P < 0.05$) in -LN or +LN.

precultured shoot tips also exhibited significantly higher regeneration compared to the 2 day control shoot tips (Fig 6.1A). Extending the preculture time resulted in progressively greater post-cryogenic (+LN) regeneration for *L. cinerea*, with the 5 and 7 day precultured shoot tips showing significantly higher regeneration compared to the 2 and 3 day precultured shoot tips for both the control and AsA treatment (Fig 6.1B). The greatest post-cryogenic regeneration of *L. cinerea* shoot tips was observed in the 7 day precultured shoot tips exposed to 50 mM AsA (Fig 6.1B, 53%), with the application of AsA resulting in significantly higher post-cryogenic regeneration of shoot tips in the 7 day preculture treatment compared to shoot tips that were not exposed to AsA (35%), and generally higher post-cryogenic regeneration for the other preculture times (2, 3 and 5 days), although these increases were not significant.

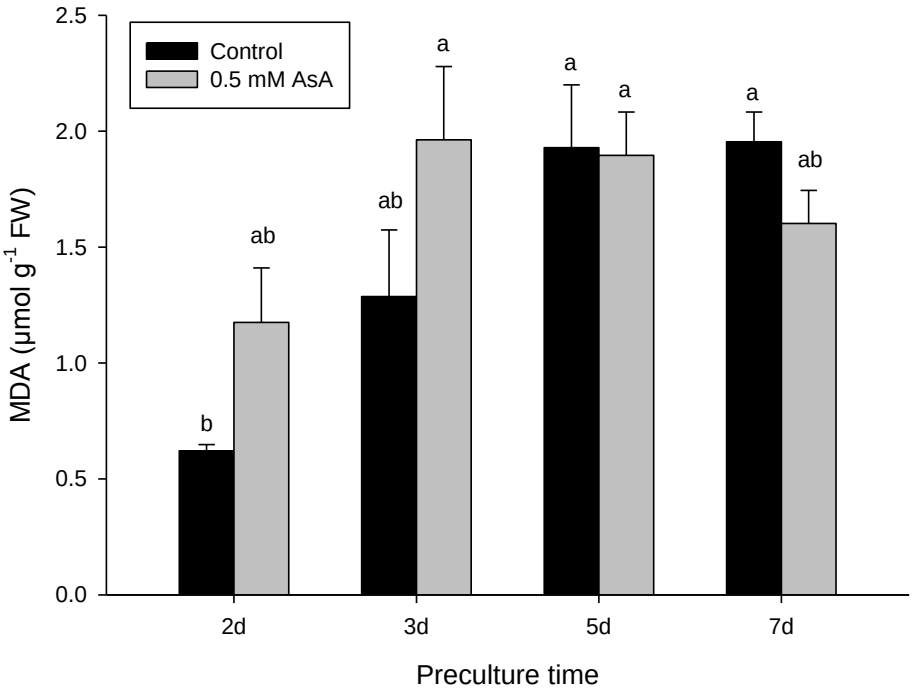

Figure 6.2. MDA concentration in shoot tips 4 weeks post-cryopreservation for various preculture times. Black bars show MDA concentration of control shoot tips with no ascorbic acid exposure while grey bars show MDA concentration of shoot tips exposed to 0.5 mM ascorbic acid during the preculture period. Different letters denote a statistically significant difference between all treatments ($P < 0.05$).

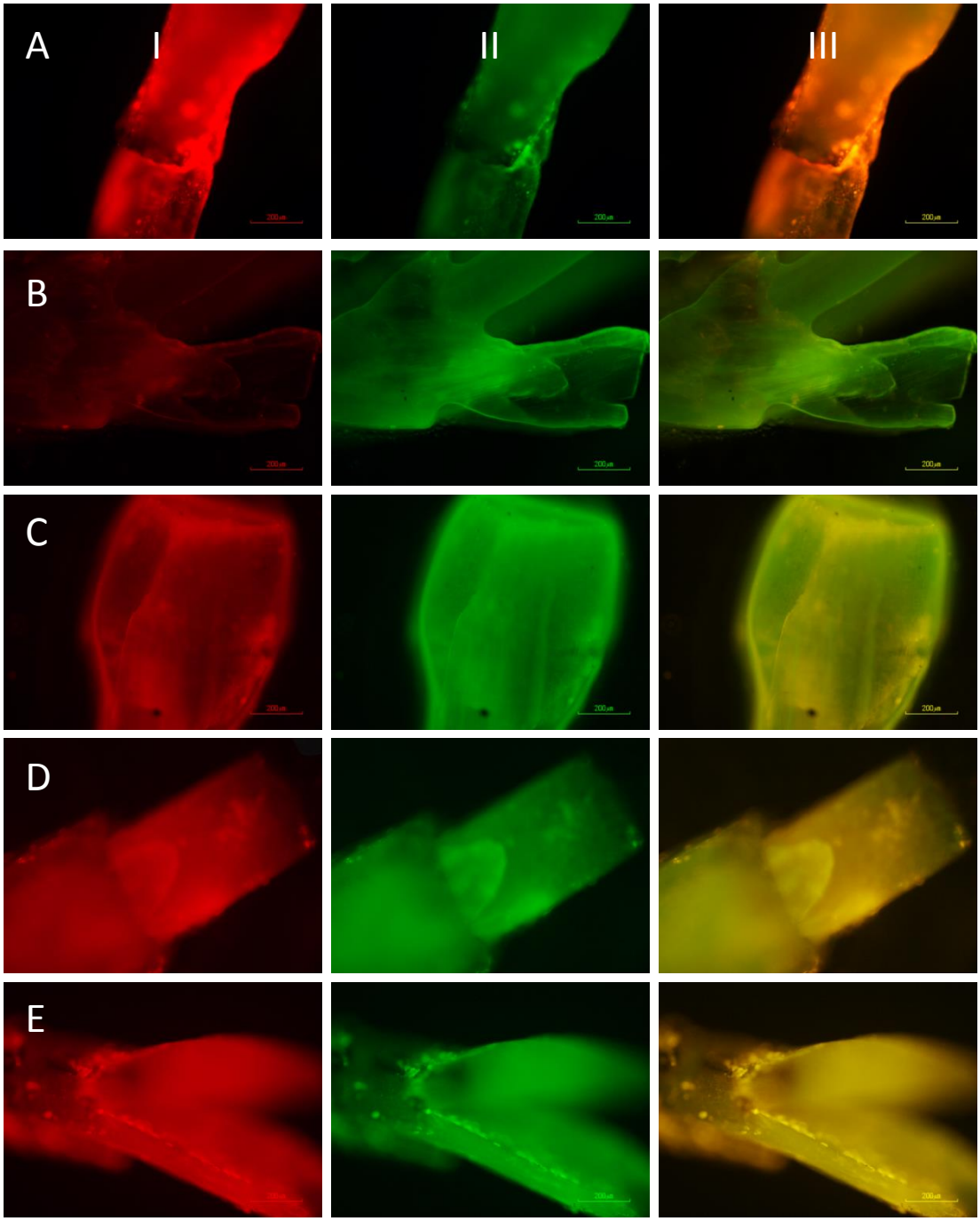

Figure 6.3. Representative shoot tips stained with C11-BODIPY$^{581/591}$. (A) Freshly isolated shoot tip, 2 (B), 3 (C), 5 (D) and 7 (E) day precultured shoot tips 4 weeks post-cryopreservation. (I) red, (II) green and (III) merged red and green fluorescence images.

Lipid peroxidation results MDA

The 2 d control precultured shoot tips had the lowest concentration of MDA detected which increased in the 3, 5 and 7 d precultured shoot tips (0.6, 1.3, 1.9 and 2.0 µmol g^{-1} FW respectively). The AsA precultured shoot tips was lowest in the 2 d shoot tips that increased in the 3 d shoot tips before decreasing in the 5 then 7 d shoot tips (1.2, 2.0, 1.9, 1.6 µmol g^{-1} FW respectively). The MDA concentration of 2 d control precultured shoot tips was significantly lower than the 5 d and 7 d control precultured shoot tips, as well as the 3 d and 5 d AsA precultured shoot tips (1.9, 2.0, 2.0 and 1.9 µmol g^{-1} FW respectively, Fig 6.2).

C11-BODIPY$^{581/591}$ fluorophore staining

As seen in Fig 6.3, the red (Fig 6.3I) and green (Fig 6.3II) fluorescence intensities of the C11-BODIPY$^{581/591}$ stained post-cryogenic shoot tips differed depending on the preculture time, with this difference most noticeable when comparing with the fresh shoot tips, which showed predominantly red fluorescence (Fig 6.3A), while the 2 d control precultured shoot tips showed predominantly green fluorescence, indicating large amounts of oxidative stress in this treatment (Fig 6.3B).

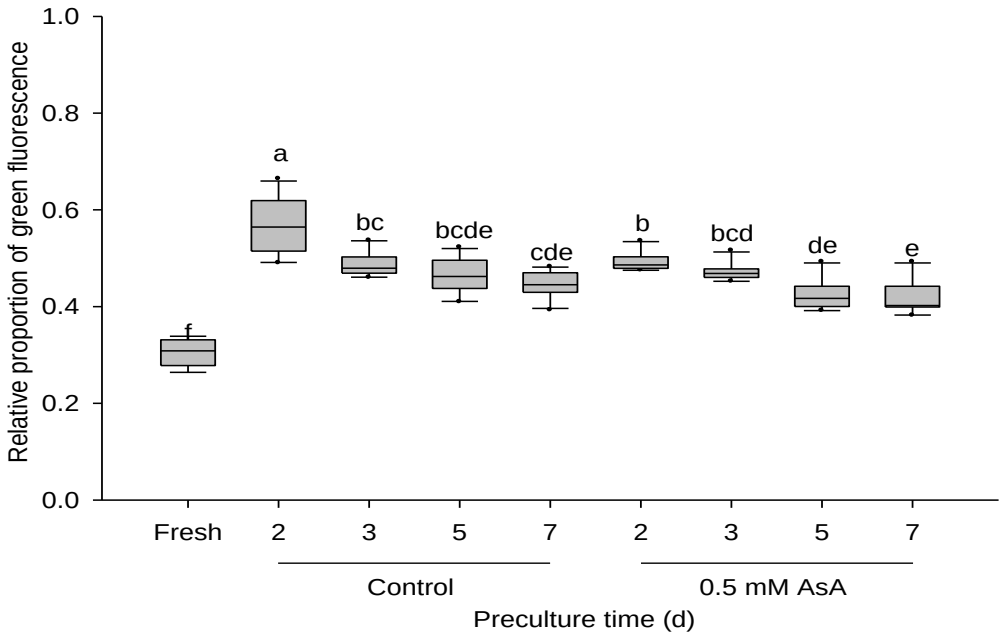

Figure 6.4. Analysis of C11-BODIPY$^{581/591}$ relative proportion of green fluorescence (green intensity/red+green intensity) in freshly isolated shoot tips or shoot tips 4 weeks post-cryopreservation. Comparison of 2, 3, 5 & 7 d precultured shoot tips with AsA (0.5 mM) and without AsA (control). Different letters denote a statistically significant difference between the average green fluorescence conditions ($P < 0.05$).

The analysed images for red and green intensity were used to calculate the relative proportion of green fluorescence (Fig 6.4), with freshly isolated shoot tips showing the lowest green fluorescence, being significantly lower than all the post-cryogenic shoot tips tested. The 2 d control precultured shoot tips showed the highest relative proportion of green fluorescence of all tested treatments, which decreased with the extended 3, 5 and 7 d preculture period (0.57, 0.49, 0.46 and 0.45 respectively). The application of AsA to the preculture medium further decreased the observed relative proportion of green fluorescence at each preculture time, which was statistically lower in the 2 d precultured shoot tips (Fig 6.4).

FDA / DAPI fluorophore staining

FDA fluorescence staining was used to identify viable shoot tips, as upon hydrolysis, it begins fluorescing (emission at 530 nm), indicating the presence of living tissue. As seen in Fig 6.5, FDA staining of the post-cryogenic shoot tips showed green fluorescence, indicating 100% survival across all treatments (data not shown). Shoot tips were then counter-stained with DAPI as a control background stain to normalise the fluorescence intensity. The merged images better represent the difference between a freshly isolated shoot tip and the dead, surviving and regenerating shoot tips after cryopreservation (Fig 6.5).

Discussion

Previous attempts to cryopreserve *L. cinerea* shoot tips either showed no post-cryogenic regeneration (Funnekotter et al. 2013), or very low regeneration (<10%) (Funnekotter et al. 2015; Kaczmarczyk et al. 2013). Kaczmarczyk et al. (2013) also cryopreserved callus tissue of *L. cinerea* with much greater success (>90%), yet the recovering callus tissue showed very slow regrowth and may be more susceptible to genetic alterations (Kaczmarczyk et al. 2012; Harding 2004).The application of a low temperature preconditioning regime prior to cryopreservation has shown benefits for a wide variety of plant species. Low temperature preconditioning in *L. cinerea* has shown to alter phospholipid and sterol compositions in plant membranes (Funnekotter et al. 2013), and increase the cellular concentrations of glutathione and ascorbic acid (Chapter 4). While preconditioning did not increase post-cryogenic survival in those studies, these changes are thought to be beneficial for increasing tolerance to the stresses of cryopreservation, and this was the reason why a 3 week 5°C preconditioning regime was used in this study. On examination of prior results, modifications to the preculture temperature and duration were deemed necessary to

assist with recovery from excision stress to obtain healthy shoot tips for cryopreservation.

Extending the preculture period for *L. cinerea* shoot tips significantly improved post-cryogenic regrowth from zero shoot tip regeneration for a 2 day preculture to 37% with the 5 day preculture. Control (-LN) regrowth showed improved tolerance to the CPAs

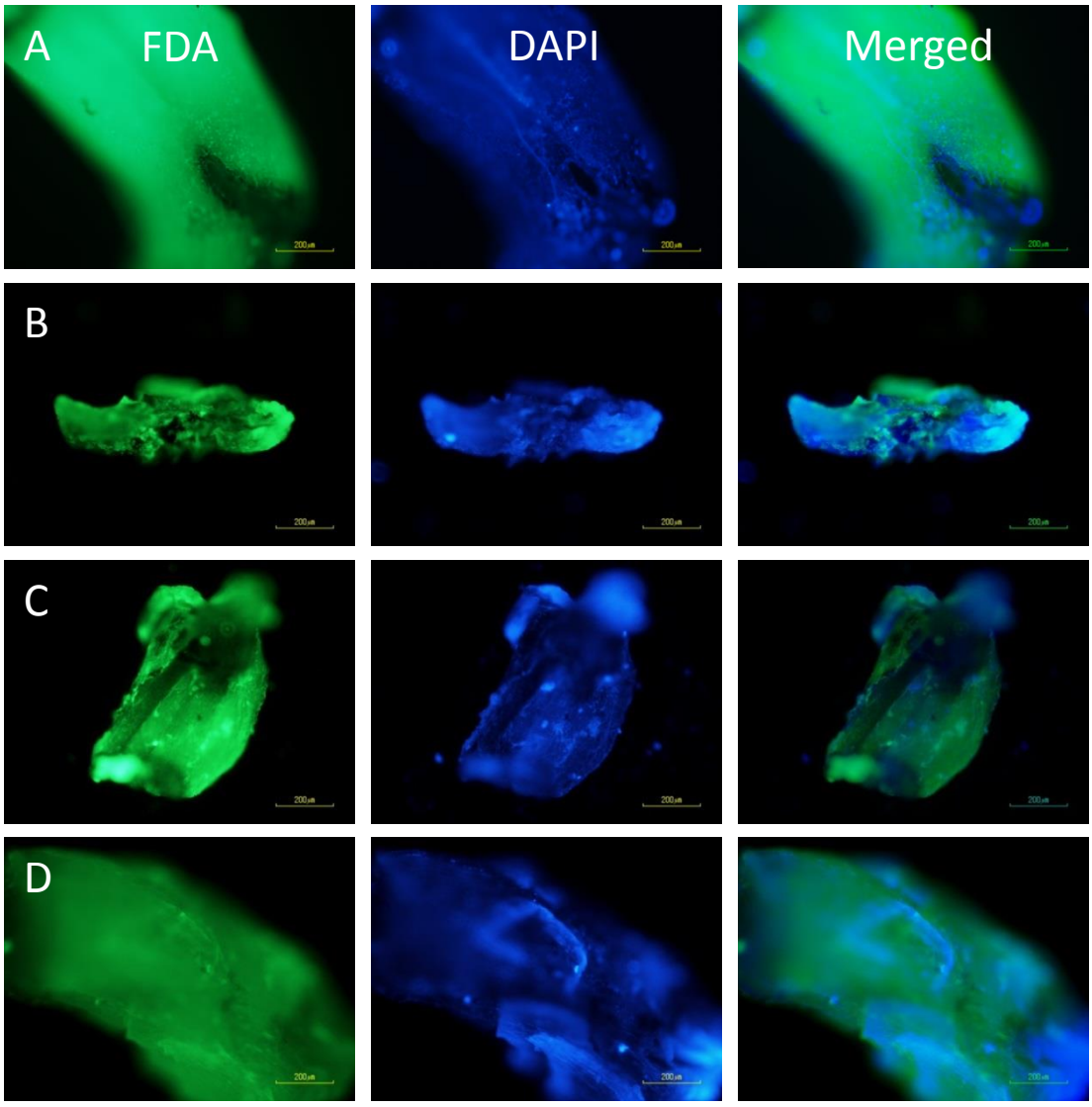

Figure 6.5. Representative shoot tips stained with FDA and DAPI. (A) Freshly isolated shoot tip, (B) dead shoot tip, (C) surviving shoot tip, (D) regenerating shoot tip 4 weeks post-cryopreservation. Scale bar represents 200 µm.

with the extended 3 and 5 day preculture duration. The additional time on the preculture medium may provide the shoot tips with additional time to recover from excision damage, as previous work has shown significant oxidative burst formation during excision of embryonic axis of chestnuts (Roach et al. 2008). The application of AsA to the preculture medium resulted in greater regeneration of the shoot tips across all treatments in comparison to the control precultured shoot tips, which is consistent with previous work. For example, Uchendu et al. (2010a) showed a large uptake of AsA in shoot tips when treated with exogenous AsA in the preculture medium and observed corresponding increases in regrowth of the treated shoot tips after cryopreservation. AsA and iron can participate in the Fenton reaction to produce hydroxyl radicals (Halliwell and Gutteridge 2007), and the addition of exogenous AsA with iron present in the media, especially in the recovery media, has been shown to be detrimental for regrowth after cryopreservation (Uchendu et al. 2010a; Canepa et al. 2011). The LS and PVS2 solutions in this study incorporates no MS salts (which contains 100 µM iron in the form of NaFeEDTA) to avoid the possible deleterious effects of iron, which otherwise may have resulted in increased oxidative stress during CPA incubation. For this reason the addition of AsA was not tested in the recovery medium.

Lipid peroxidation can be comprised of many different reactions, resulting in a range of oxidation products. MDA is just one of the possible products of LPO and commonly measured using TBA. The stresses experienced by shoot tips during cryopreservation have been shown to increase MDA formation (Uchendu et al. 2010a; Verleysen et al. 2004; Zhang et al. 2010; Fretz and Lörz 1995). In this study, an increase in MDA was observed in the recovered shoot tips that showed the greatest post-cryogenic survival (Fig 6.2), contrary to what was expected, i.e. that MDA content should be decrease with treatments that increase regrowth after cryopreservation. For example, Muckenschnabel et al. (2002) observed a decrease in MDA in rotting tissue of *Arabidopsis thaliana* infected with *Botrytis cinerea*. Other studies have also observed decreasing MDA with extended aging in *Pisum sativum* seeds and soybean axes (Colville et al. 2012; Stewart and Bewley 1980). Thus the higher levels of MDA observed in this study may be attributed to the decomposition of the lipid fractions of surviving cells during the heating stage of the extraction process, as well as the interactions of TBA with other compounds (Halliwell and Gutteridge 2007). The detected concentration of MDA in this study was similar to that reported by Benson et al. (1992), and Fretz and Lörz (1995); however, the use of HPLC for the detection of

MDA has shown greater sensitivity and specificity for MDA (Uchendu et al. 2010a; Davey et al. 2005; Zhao et al. 2011).

The use of the C11-BODIPY$^{581/591}$ probe has been used as a measure of lipid peroxidation in spermatozoa freeze-thawing and cryopreservation with increased relative proportion of green fluorescence linked to spermatozoan damage (Ferrusola et al. 2009; Domínguez-Rebolledo et al. 2010; Partyka et al. 2011). Analysis of fresh shoot tips stained with the C11-BODIPY$^{581/591}$ probe (Fig 6.3) showed the lowest green fluorescence intensity, indicating the presence of low levels of oxidative stress, and this was used for comparison with the cryopreserved shoot tips from the different preculture treatments (Fig 6.4). The relative proportion of green fluorescence was highest in the 2 day control precultured shoot tips and decreased as the preculture period increased, indicating decreasing oxidative stress in the shoot tips with an extended preculture period. This trend agrees with the rates of post-cryogenic regeneration, as extended preculture shoot tips showed greater regrowth (Fig 6.4). The addition of AsA to the preculture appears to have further decreased the relative proportion of green fluorescence, however, the addition of exogenous antioxidants may interfere with the ratiometric fluorescence of this probe (MacDonald et al. 2007). As the C11-BODIPY$^{581/591}$ probe is more sensitive to oxidation than PUFA (MacDonald et al. 2007), future work on the addition of the probe prior to cryopreservation may be beneficial, as this will also allow observation of oxidation of the probe through cryopreservation, and the probe may provide a small amount of antioxidant activity.

FDA has been used as a marker of survival after cryopreservation in many previous studies (Sakai et al. 1991; Yamada et al. 1991). In this study FDA was used to confirm the presence of surviving shoot tips after cryopreservation (Fig 6.5); however, all shoot tips showed FDA fluorescence, indicating the presence of viable tissue. DAPI was used to counter-stain and normalise the fluorescence intensity to try and separate dead shoot tips from shoot tips that survived cryopreservation. Merged images showed little DAPI stain in the fresh shoot tips and regenerating shoot tips, whilst the surviving and dead post-cryogenic shoot tips showed large areas of low FDA fluorescence, indicating the presence of dead tissue with smaller sections of live tissue. FDA-DAPI staining showed no discernible point to differentiate between dead and surviving shoot tips.

Conclusions

This study shows that extending the preculture time increased regrowth of *L. cinerea* shoot tips after cryopreservation, and this was a significant improvement over previous work. The addition of 0.5 µM AsA further increased regrowth, with 53% of shoot tips regenerating after cryopreservation. MDA formation during recovery of the shoot tips was not as expected, with greater MDA detected in the shoot tips that showed greater post-cryogenic regrowth; however, this may have been an artefact of the extraction method and requires further investigation. However C11-BODIPY$^{581/591}$ fluorescence showed increased oxidative stress occurring in shoot tips with low regeneration after cryopreservation, consistent with previous known studies. Future work using this probe may be applicable to other problematic cryogenically recalcitrant species for analysing fluorescence throughout the cryopreservation protocol, and may be useful for identifying the particular stages where oxidative stress occurs in the shoot tip. This information may guide improvements to the cryoprotective stages of cryopreservation, including the preculture step and the more precise use of appropriate antioxidants.

References

Benson, E.E. 2008. "Cryopreservation Theory." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 15-32. New York: Springer.

Benson, E.E., P.T. Lynch, and J. Jones. 1992. "The Detection of Lipid Peroxidation Products in Cryoprotected and Frozen Rice Cells: Consequences for Post-Thaw Survival." *Plant Science* 85 (1): 107-114

Brouwers, J.F., P.F. Silva, and B.M. Gadella. 2005. "New Assays for Detection and Localization of Endogenous Lipid Peroxidation Products in Living Boar Sperm after Bts Dilution or after Freeze–Thawing." *Theriogenology* 63 (2): 458-469

Canepa, P., A. Panta, and D. Tay. 2011. "The Effect of Antioxidants on the Cryopreservation Recovery of Two Potato Cultivars Following Post-Thawing Culture." In *International Symposium on Cryopreservation in Horticultural Species*, 101-105. Acta Hort. (ISHS).

Castellano, C.-A., I. Audet, J. Bailey, J.-P. Laforest, and J. Matte. 2010. "Dietary Omega-3 Fatty Acids (Fish Oils) Have Limited Effects on Boar Semen Stored at 17°C or Cryopreserved." *Theriogenology* 74 (8): 1482-1490

Colville, L., E.L. Bradley, A.S. Lloyd, H.W. Pritchard, L. Castle, and I. Kranner. 2012. "Volatile Fingerprints of Seeds of Four Species Indicate the Involvement of Alcoholic Fermentation, Lipid Peroxidation, and Maillard Reactions in Seed Deterioration During Ageing and Desiccation Stress." *Journal of Experimental Botany* 63 (18): 6519-6530

Davey, M., E. Stals, B. Panis, J. Keulemans, and R. Swennen. 2005. "High-Throughput Determination of Malondialdehyde in Plant Tissues." *Analytical Biochemistry* 347 (2): 201-207

Domínguez‐Rebolledo, Á., F. Martínez‐Pastor, M. Fernández‐Santos, E. Del Olmo, A. Bisbal, J. Ros‐Santaella, and J. Garde. 2010. "Comparison of the Tbars Assay and Bodipy C11 Probes for Assessing Lipid Peroxidation in Red Deer Spermatozoa." *Reproduction in Domestic Animals* 45 (6): e360-e368

Drummen, G.P., B.M. Gadella, J.A. Post, and J.F. Brouwers. 2004. "Mass Spectrometric Characterization of the Oxidation of the Fluorescent Lipid Peroxidation Reporter Molecule C11-Bodipy$^{581/591}$." *Free Radical Biology and Medicine* 36 (12): 1635-1644

Esterbauer, H., R.J. Schaur, and H. Zollner. 1991. "Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde and Related Aldehydes." *Free Radical Biology and Medicine* 11 (1): 81-128

Ferrusola, C.O., L.G. Fernández, J. Morrell, C.S. Sandoval, B.M. García, H. Rodríguez-Martinez, J. Tapia, and F. Peña. 2009. "Lipid Peroxidation, Assessed with Bodipy-C11, Increases after Cryopreservation of Stallion Spermatozoa, Is Stallion-Dependent and Is Related to Apoptotic-Like Changes." *Reproduction* 138 (1): 55-63

Fretz, A., and H. Lörz. 1995. "Cryopreservation of *in Vitro* Cultures of Barley *(Hordeum Vulgare L*. And *H. Murinum L*.) and Transgenic Cells of Wheat (*Triticum Aestivum L*.)." *Journal of Plant Physiology* 146 (4): 489-496

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Funnekotter, B., S.E. Whiteley, S.R. Turner, E. Bunn, and R.L. Mancera. 2015. "Evaluation of the New Vacuum Infiltration Vitrification (Viv) Cryopreservation Tehnique for Native Australian Plant Shoot Tips." *CryoLetters* In Press: 1-11

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Hammerstedt, R.H., J.K. Graham, and J.P. Nolan. 1990. "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive." *Journal of Andrology* 11 (1): 73-88

Harding, K. 2004. "Genetic Integrity of Cryopreserved Plant Cells: A Review." *CryoLetters* 25 (1): 3-22

Heath, R.L., and L. Packer. 1968. "Photoperoxidation in Isolated Chloroplasts: I. Kinetics and Stoichiometry of Fatty Acid Peroxidation." *Archives of Biochemistry and Biophysics* 125 (1): 189-198

Kaczmarczyk, A., B. Funnekotter, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., B. Funnekotter, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for *Loxocarya Cinerea*-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519

Koch, J.M., and G.P. Samsa. 2007. "Restoring Jarrah Forest Trees after Bauxite Mining in Western Australia." *Restoration Ecology* 15: S17-S25

MacDonald, M.L., I.V. Murray, and P.H. Axelsen. 2007. "Mass Spectrometric Analysis Demonstrates That Bodipy 581/591 C11 Overestimates and Inhibits Oxidative Lipid Damage." *Free Radical Biology and Medicine* 42 (9): 1392-1397

Marnett, L.J. 1999b. "Lipid Peroxidation—DNA Damage by Malondialdehyde." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 424 (1): 83-95

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Muckenschnabel, I., B. Goodman, B. Williamson, G. Lyon, and N. Deighton. 2002. "Infection of Leaves of *Arabidopsis Thaliana* by *Botrytis Cinerea*: Changes in Ascorbic Acid, Free Radicals and Lipid Peroxidation Products." *Journal of Experimental Botany* 53 (367): 207-214

Murashige, T. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473

Pap, E., G. Drummen, V. Winter, T. Kooij, P. Rijken, K. Wirtz, J. Op den Kamp, W. Hage, and J. Post. 1999. "Ratio-Fluorescence Microscopy of Lipid Oxidation in Living Cells Using C11-Bodipy$^{581/591}$." *FEBS Letters* 453 (3): 278-282

Partyka, A., E. Łukaszewicz, W. Niżański, and J. Twardoń. 2011. "Detection of Lipid Peroxidation in Frozen-Thawed Avian Spermatozoa Using C $_{11}$-Bodipy$^{581/591}$." *Theriogenology* 75 (9): 1623-1629

Roach, T., M. Ivanova, R.P. Beckett, F.V. Minibayeva, I. Green, H.W. Pritchard, and I. Kranner. 2008. "An Oxidative Burst of Superoxide in Embryonic Axes of Recalcitrant Sweet Chestnut Seeds as Induced by Excision and Desiccation." *Physiologia Plantarum* 133 (2): 131-139

Sakai, A., S. Kobayashi, and I. Oiyama. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb. Var. Brasiliensis Tanaka) by Vitrification." *Plant Cell Reports* 9 (1): 30-33

Sakai, A., S. Kobayashi, and I. Oiyama. 1991. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb.) by a Simple Freezing Method." *Plant Science* 74 (2): 243-248

Steponkus, P.L. 1984. "Role of the Plasma Membrane in Freezing Injury and Cold Acclimation." *Annual Review of Plant Physiology* 35 (1): 543-584

Stewart, R.R., and J.D. Bewley. 1980. "Lipid Peroxidation Associated with Accelerated Aging of Soybean Axes." *Plant Physiology* 65 (2): 245-248

Uchendu, E.E., S.W. Leonard, M.G. Traber, and B.M. Reed. 2010a. "Vitamins C and E Improve Regrowth and Reduce Lipid Peroxidation of Blackberry Shoot Tips Following Cryopreservation." *Plant Cell Reports* 29 (1): 25-35

Uchendu, E.E., M. Muminova, S. Gupta, and B.M. Reed. 2010b. "Antioxidant and Anti-Stress Compounds Improve Regrowth of Cryopreserved *Rubus* Shoot Tips." *In Vitro Cellular & Developmental Biology-Plant*: 1-8

Verleysen, H., G. Samyn, E. Van Bockstaele, and P. Debergh. 2004. "Evaluation of Analytical Techniques to Predict Viability after Cryopreservation." *Plant Cell, Tissue and Organ Culture* 77 (1): 11-21

Wang, Z.C., and X.X. Deng. 2004. "Cryopreservation of Shoot-Tips of Citrus Using Vitrification: Effect of Reduced Form of Glutathione." *CryoLetters* 25 (1): 43-50

Willyams, D. 2005. "Tissue Culture of Geophytic Rush and Sedge Species for Revegetation of Bauxite Mine Sites in the Northern Jarrah Forest of Western Australia." In *Contributing to a sustainable future. Proceedings of the Australian Branch of the International Association for Plant Tissue Culture & Biotechnology. IAPTC&B, Perth, Western Australia*, 226–241.

Yamada, T., A. Sakai, T. Matsumura, and S. Higuchi. 1991. "Cryopreservation of Apical Meristems of White Clover (*Trifolium Repens* L.) by Vitrification." *Plant Science* 78 (1): 81-87

Zhang, C., Z. Ding, X. Xu, Q. Wang, G. Qin, and S. Tian. 2010. "Crucial Roles of Membrane Stability and Its Related Proteins in the Tolerance of Peach Fruit to Chilling Injury." *Amino Acids* 39 (1): 181-194

Zhao, Y., L.W. Qi, W.M. Wang, P.K. Saxena, and C.Z. Liu. 2011. "Melatonin Improves the Survival of Cryopreserved Callus of *Rhodiola Crenulata*." *Journal of Pineal Research* 50 (1): 83-88

Chapter 7 – Conclusions and Future Directions

Introduction

The research reported in this thesis provides an in-depth analysis of oxidative stress and the antioxidants that neutralise these stresses during the cryopreservation process of a number of native Australian plant species. The main aims of this were to try to determine why some species show good or average cryo-capability (such as *Anigozanthos viridis* and *Lomandra sonderi*), while other species show a very limited capacity to regenerate after cryopreservation for no obvious reasons (such as *Loxocarya cinerea*). Understanding where and when the damage occurs during the process of cryopreservation is essential for the improvement of current cryopreservation protocols. The following is a summary of the most significant findings reported in the previous chapters and concludes with recommendations for future research and areas where new and insightful findings may be uncovered.

Developing a successful cryopreservation protocol for *Loxocarya cinerea*

Loxocarya cinerea has been labelled cryogenically recalcitrant due to very low or no post-cryogenic regeneration for this species. Extensive work has been done to understand why this species does not survive the stresses imposed by cryopreservation using thermal analysis (Kaczmarczyk et al. 2013), analysis of phospholipids, sterols and sugars (Funnekotter et al. 2013) as well as analysing antioxidants (Chapter 4). Improvements to the previous cryopreservation protocol was first achieved in Chapter 5, the application of a vacuum during the CPA incubations increased penetration of the CPAs. Resulting in a reduced incubation time in the toxic CPAs, reducing stress on the shoot tips. Low levels of post-cryogenic regeneration was achieved for *L. cinerea*. Further improvements to the VIV protocol was completed in Chapter 6 with the addition of an extended preculture with exogenous AsA included in the medium. Utilising this modified cryopreservation protocol resulted in over 50% regeneration for *L. cinerea*.

Oxidative stress during cryopreservation

Achieving regeneration of any plant species after cryopreservation relies on a wide range of factors: from the health of the *in vitro* shoots to the ability of shoot tips to resist ice crystallisation damage, osmotic stress, the toxicity of cryoprotective agents (CPAs) and oxidative stress during the cryopreservation process and finally the provision of the appropriate conditions to nurture and support the recovery, growth and regeneration of competent cells and tissues into healthy new plants.

In vitro shoot preconditioning for stress abatement

All the species investigated in this study are found exclusively in the south-west Western Australian Floristic region, and there have been many problems with obtaining healthy *in vitro* cultures for highly propagation-recalcitrant species in this region. Slow *in vitro* establishment, latent contamination, general poor growth, low multiplication, hyperhydric transformation (of shoots), callusing of stems and/or leaves or abnormal growth (e.g. fasciation, stunting, leaf curling), blackening or necrosis of various tissues and various levels of sometimes damaging phenolic leakage into the medium have previously been reported for species found in this region (Bunn 2005; Bunn et al. 2005; Bunn et al. 2007; Rossetto et al. 1992; Bunn et al. 2011; Bunn and Tan 2002).

Preconditioning regimes prior to cryopreservation are used to stimulate the stress metabolism responses of plants, commonly resulting in increased sugar production, altered membranes that exhibit greater stability, and increased antioxidant content. These alterations can help shoot tips resist the stresses associated with cryopreservation, resulting in greater post-cryogenic survival. However, little is known about the response of Australian species in *in vitro* cultures to the stresses of preconditioning. The application of a preconditioning regime (be it low or high temperature, low or high light or increased osmotic stress) resulted in no observable improvements in the cryopreservation of *L. sonderi*, *A. viridis* and *L. cinerea* (Chapter 3 & 4). It is likely that these preconditioning regimes applied excessive stress to the *in vitro* shoots, as discussed in Chapter 3. High temperature, high light, and high sucrose preconditioning stresses resulted in stunted growth, low multiplication rate and increased number of dead leaves in the cultures. This damage to *in vitro* shoots is a likely cause for the reduced post-cryogenic survival seen with *L. sonderi* and the reduced antioxidant activity observed in the preconditioned shoot tips. However, the application of a low temperature preconditioning regime did increase the glutathione content in shoot tips of *L. cinerea* and *L. sonderi* but not *A. viridis* (Chapter 4).

Therefore, it appears that the successful application of preconditioning regime is reliant on the health of the *in vitro* shoots (i.e. increased growth, production of new shoots, green leaves) after preconditioning and the ability of shoots to acclimate to the stress without experiencing excessive damage, leading to increased resilience to the stresses of cryopreservation.

Preculture

The preculture period is defined as the period after excision of the shoot tips (containing actively growing meristematic tissues) from the in vitro shoots, but before the shoot tips are exposed to CPAs such as loading solutions and PVS1, PVS2 and PVS3. Shoot tips are usually placed onto a desiccation medium for 1 – 7 days, depending on the species. This period allows shoot tips to recover from any excision damage that may have occurred as well as beginning the desiccation process as water is removed from tissues due to negative osmotic pressure. Indeed, extending the preculture period for L. cinerea from 2 days to 5 days significantly improved post-cryogenic regeneration. The extended preculture is likely beneficial as it allows the shoot tips to recover to a greater extent due to the slow growth response seen in L. cinerea (results not shown). Analysis of glutathione and ascorbic acid (AsA) in the 2 day precultured shoot tips of L. cinerea showed a large reduction in antioxidants compared to the preconditioned shoot tips, whereas A. viridis shoot tips precultured at 25°C showed an increase in glutathione and AsA (Chapter 4). Interestingly, L. sonderi showed similar reductions of glutathione and AsA in its precultured shoot tips and may also benefit from an extended preculture period.

Cryoprotection

The addition of CPAs is critical for achieving the vitrification of water within plant tissues and is integral to the whole cryogenic process. Without adequate cryoprotection by CPAs, ice crystallisation will occur within the shoot tips with disastrous consequences. Nevertheless, excessive exposure to CPAs can be just as deleterious as many CPAs are cytotoxic and can also lead to excessive dehydration causing significant tissue damage. Chapter 5 reports the testing of the vacuum infiltration vitrification (VIV) cryopreservation technique aimed at reducing exposure times to CPAs without compromising vitrification. The application of a vacuum during the incubation of shoot tips in CPAs showed significant benefits for reducing incubation times, which was confirmed by determining the extent of ice formation in the shoot tips using differential scanning calorimetry (DSC) measurements. As some CPAs are highly toxic, any process that avoids excessive CPA exposure is likely to

be beneficial for all species to a lesser or greater degree and may be particularly applicable to those that appear to be unusually sensitive to CPA exposure. Interestingly, post-cryogenic regeneration was obtained from all four species tested with the VIV technique utilising the same incubation times (5 min loading solution + 10 min PVS2), suggesting that this technique provides more consistent CPA penetration into the shoot tips. However as this is the first report the use of VIV to cryopreserve shoot tips from any Australian species, additional work on a greater variety of plant species is recommended to better understand the potential advantages and/or disadvantages of utilising and adopting this approach.

Post-cryogenic recovery

Post-cryogenic recovery was lowest when the greatest amount of oxidative stress to the shoot tips was observed (Chapters 3, 4 & 6). Measurements of the half-cell reduction potential and C11-BODIPY$^{581/591}$ fluorescence, as indicators of oxidative stress in the shoot tips, showed strong correlations between increased oxidative stress and reduced post-cryogenic regeneration. *Loxocarya cinerea* showed the greatest sensitivity to oxidative stress of all the three species tested (Chapter 4), with a large increase in oxidised glutathione disulphide (GSSG) detected in the washing and recovery stages of cryopreservation, which was confirmed with C11-BODIPY$^{581/591}$ fluorescence experiments (Chapter 6).

The change in redox potential seen in the three species may be attributed to the large reduction in antioxidant activity of the antioxidant enzyme glutathione reductase (GR) following cryopreservation (Chapter 3). This may account for the increased formation of GSSG detected after cryopreservation, as the reduction of GSSG back to GSH will occur at a much slower rate if GR titre and synthesis is severely reduced or disrupted. Thus, the degradation of GR may be a major cause of oxidative stress in shoot tips of the study species after cryopreservation.

Future work

This study focused on the activity of antioxidants during the cryopreservation process and investigated lipid peroxidation damage through the formation of malondialdehyde (MDA). However, reactive oxygen species (ROS) can still interact with and damage other cellular components, such as DNA and proteins. Damage to vital proteins involved in metabolism will severely inhibit the recovery and regeneration of shoot tips after cryopreservation, but similarly damage to DNA can result in deleterious genetic

mutations. Future studies that explore these areas will be essential for providing an overall picture on the role of oxidative stress in cryopreservation.

Analysis of MDA in the post-cryogenic shoot tips of *L. cinerea* led to interesting findings, where greater levels of MDA were observed in shoot tips with higher post-cryogenic regeneration rates (Chapter 6). It was hypothesised that shoot tips with low post-cryogenic regeneration would have an initial increase in MDA that is degraded over time as the shoot tip dies. Further testing of MDA at multiple stages in the recovery period will be needed to confirm these observations. Lipid peroxidation can also result in the formation of 4-hydroxynonenal (HNE), which has shown to exhibit greater toxicity to cells as it can damage mitochondria, inhibit the synthesis of DNA and proteins, and interfere with the action of repair proteins such as chaperones (Halliwell and Gutteridge 2007; Nishikawa et al. 1992). Therefore combined analysis of MDA and HNE may give a more comprehensive representation of lipid peroxidation in post-cryogenic shoot tips.

Future work using the C11-BODIPY$^{581/591}$ fluorophore probe to observe oxidative stress of individual shoot tips throughout the various stages of cryopreservation may be extremely useful to identify where oxidative stress occurs. This knowledge will guide improvements to specific cryoprotective stages with, for example, the addition of exogenous antioxidants.

While cold temperatures are commonly used to precondition plants prior to cryopreservation, preconditioning appeared to have little effect on post-cryogenic regeneration (*L. cinerea* and *L. sonderi*) or was even detrimental (*A. viridis)* (Chapter 4). Likewise, abiotic preconditioning stresses (light, osmotic and high temperatures) showed no benefit for *L. sonderi*. Artificially inducing a stress response with plant growth regulators, such as acetyl salicylic acid and salicylic acid, has shown to increase drought, heat and chilling stress tolerance (Senaratna et al. 2000), and may prove more beneficial in Australian plant species.

Conclusions

The findings reported in this thesis show that oxidative stress plays a significant role in cryogenic survival influencing tissue survival and recovery in several different ways. Reduced antioxidant enzyme activity and sacrificial antioxidant content, a lesser reducing redox environment and increased oxidation of C11-BODIPY$^{581/591}$ were all found to correlate with decreased cryogenic survival. Understanding the complex interactions of the antioxidant enzymes and the many redundancies plants have in their antioxidant systems means that it is essentially impossible to determine whether a single antioxidant enzyme (or lack thereof) is responsible for the lack of post-cryogenic survival for any given plant species as the complex interactions and interplay between different factors is likely to be just as important.

Cryopreservation, as a long-term conservation technique, provides one of the best storage solutions for valuable and conservation dependant plant species. The development of the cryopreservation protocol for *L. cinerea* has been a valuable learning experience on the recalcitrance of some species to the stresses of cryopreservation. Furthering our understanding of the physiology and biochemistry of Australian plants is instrumental in advancing and improving current conservation programs, and may have benefits for many other species around the world.

References

Bunn, E. 2005. "Development of in Vitro Methods for *Ex Situ* Conservation of *Eucalyptus Impensa,* an Endangered Mallee from Southwest Western Australia." *Plant Cell, Tissue and Organ Culture* 83 (1): 97-102

Bunn, E., T. Senaratna, K. Sivasithamparam, and K.W. Dixon. 2005. "*In Vitro* Propagation of *Eucalyptus Phylacis* L. Johnson and K. Hill., a Critically Endangered Relict from Western Australia." *In Vitro Cellular & Developmental Biology-Plant* 41 (6): 812-815

Bunn, E., and B. Tan. 2002. "Microbial Contaminants in Plant Tissue Culture Propagation." In *Microorganisms in Plant Conservation and Biodiversity*, eds K Sivasithamparam, K Dixon and R Barratt, 307-335. Netherlands: Kluwer Academic Press.

Bunn, E., S. Turner, M. Panaia, and K.W. Dixon. 2007. "The Contribution of in Vitro Technology and Cryogenic Storage to Conservation of Indigenous Plants." *Australian Journal of Botany* 55 (3): 345-355

Bunn, E., S.R. Turner, and K.W. Dixon. 2011. "Biotechnology for Saving Rare and Threatened Flora in a Biodiversity Hotspot." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 188-200

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Kaczmarczyk, A., B. Funnekotter, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for *Loxocarya Cinerea*-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519

Nishikawa, A., R. Sodum, and F.L. Chung. 1992. "Acute Toxicity of Trans-5-Hydroxy-2-Nonenal in Fisher 344 Rats." *Lipids* 27 (1): 54-58

Rossetto, M., K.W. Dixon, and E. Bunn. 1992. "Aeration: A Simple Method to Control Vitrification and Improve in Vitro Culture of Rare Australian Plants." *In Vitro–Plant* 28 (4): 192-196

Senaratna, T., D. Touchell, E. Bunn, and K. Dixon. 2000. "Acetyl Salicylic Acid (Aspirin) and Salicylic Acid Induce Multiple Stress Tolerance in Bean and Tomato Plants." *Plant Growth Regulation* 30 (2): 157-161

References

Agarwal, S. 2007. "Increased Antioxidant Activity in *Cassia* Seedlings under Uv-B Radiation." *Biologia Plantarum* 51 (1): 157-160

Airaki, M., M. Leterrier, R.M. Mateos, R. Valderrama, M. Chaki, J.B. Barroso, L.A. Del Rio, J.M. Palma, and F.J. Corpas. 2012. "Metabolism of Reactive Oxygen Species and Reactive Nitrogen Species in Pepper (*Capsicum Annuum* L.) Plants under Low Temperature Stress." *Plant, Cell & Environment* 35 (2): 281-295

Anchordoguy, T.J., A.S. Rudolph, J.F. Carpenter, and J.H. Crowe. 1987. "Modes of Interaction of Cryoprotectants with Membrane Phospholipids During Freezing." *Cryobiology* 24 (4): 324-331

Antony, J.J.J., C.L. Keng, M. Mahmood, and S. Subramaniam. 2013. "Effects of Ascorbic Acid on Pvs2 Cryopreservation of *Dendrobium* Bobby Messina's Plbs Supported with Sem Analysis." *Applied Biochemistry and Biotechnology* 171 (2): 315-329

Apel, K., and H. Hirt. 2004. "Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction." *Annual Review of Plant Biology* 55: 373-399

Arora, R., M.E. Wisniewski, and R. Scorza. 1992. "Cold Acclimation in Genetically Related (Sibling) Deciduous and Evergreen Peach (*Prunus Persica* [L.] Batsch) I. Seasonal Changes in Cold Hardiness and Polypeptides of Bark and Xylem Tissues." *Plant Physiology* 99 (4): 1562-1568

Asada, K. 2006. "Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions." *Plant Physiology* 141 (2): 391-396

Ashmore, S.E., K.N. Hamilton, and C.A. Offord. 2011. "Conservation Technologies for Safeguarding and Restoring Threatened Flora: Case Studies from Eastern Australia." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 99-109

Avery, S. 2014. "Oxidative Stress and Cell Function." In *Systems Biology of Free Radicals and Antioxidants*, ed. Ismail Laher, 89-112. Springer Berlin Heidelberg.

Baek, K.H., and D.Z. Skinner. 2003. "Alteration of Antioxidant Enzyme Gene Expression During Cold Acclimation of near-Isogenic Wheat Lines." *Plant Science* 165 (6): 1221-1227

Bajaj, Y.P.S. 1995. "Cryopreservation of Plant Cell, Tissue, and Organ Culture for the Conservation of Germplasm and Biodiversity." In *Biotechnology in Agriculture and Forestry 32*, ed. Y.P.S. Bajaj, 1-18. New York: Springer.

Bechtold, N., and G. Pelletier. 1998. "In Planta Agrobacterium Mediated Transformation of Adult *Arabidopsis Thaliana* Plants by Vacuum Infiltration." In *Arabidopsis Protocols*, eds José Martinez-Zapater and Julio Salinas, 259-266. Humana Press.

Benson, E.E. 1990. *Free Radical Damage in Stored Plant Germplasm*. Rome: International Board for Plant Genetic Resources.

Benson, E.E. 1995. "Cryopreservation of Shoot-Tips and Meristems." In *Methods in Molecular Biology Volume 38: Cryopreservation and Freeze-Drying Protocols*, eds J.G. Day and M.R. McLellan, 121-132. Totwa, NJ: Humana Press Inc.

Benson, E.E. 2008. "Cryopreservation Theory." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 15-32. New York: Springer.

Benson, E.E., and D. Bremner. 2004. "Oxidative Stress in the Frozen Plant: A Free Radical Point of View." In *Life in the Frozen State*, eds B.J. Fuller, N. Lane and E.E. Benson, 206–241. Boca Raton, Florida: CRC Press.

Benson, E.E., P.T. Lynch, and J. Jones. 1992. "The Detection of Lipid Peroxidation Products in Cryoprotected and Frozen Rice Cells: Consequences for Post-Thaw Survival." *Plant Science* 85 (1): 107-114

Benson, E.E., and L.A. Withers. 1987. "Gas Chromatographic Analysis of Volatile Hydrocarbon Production by Cryopreserved Plant Tissue Cultures: A Non-Destructive Method for Assessing Stability." *CryoLetters* 8: 35-46

Berjak, P., B. Varghese, and N. Pammenter. 2011. "Cathodic Amelioration of the Adverse Effects of Oxidative Stress Accompanying Procedures Necessary for Cryopreservation of Embryonic Axes of Recalcitrant-Seeded Species." *Seed Science Research* 21 (03): 187-203

Birtic, S., L. Colville, H.W. Pritchard, S.R. Pearce, and I. Kranner. 2011. "Mathematically Combined Half-Cell Reduction Potentials of Low-Molecular-Weight Thiols as Markers of Seed Ageing." *Free Radical Research* 45 (9): 1093-1102

Bowler, C., M. van Montagu, and D. Inze. 1992. "Superoxide Dismutase and Stress Tolerance." *Annual Review of Plant Biology* 43 (1): 83-116

Brouwers, J.F., P.F. Silva, and B.M. Gadella. 2005. "New Assays for Detection and Localization of Endogenous Lipid Peroxidation Products in Living Boar Sperm after Bts Dilution or after Freeze–Thawing." *Theriogenology* 63 (2): 458-469

Bunn, E. 2005. "Development of in Vitro Methods for *Ex Situ* Conservation of *Eucalyptus Impensa,* an Endangered Mallee from Southwest Western Australia." *Plant Cell, Tissue and Organ Culture* 83 (1): 97-102

Bunn, E., T. Senaratna, K. Sivasithamparam, and K.W. Dixon. 2005. "*In Vitro* Propagation of *Eucalyptus Phylacis* L. Johnson and K. Hill., a Critically Endangered Relict from Western Australia." *In Vitro Cellular & Developmental Biology-Plant* 41 (6): 812-815

Bunn, E., and B. Tan. 2002. "Microbial Contaminants in Plant Tissue Culture Propagation." In *Microorganisms in Plant Conservation and Biodiversity*, eds K Sivasithamparam, K Dixon and R Barratt, 307-335. Netherlands: Kluwer Academic Press.

Bunn, E., S. Turner, M. Panaia, and K.W. Dixon. 2007. "The Contribution of in Vitro Technology and Cryogenic Storage to Conservation of Indigenous Plants." *Australian Journal of Botany* 55 (3): 345-355

Bunn, E., S.R. Turner, and K.W. Dixon. 2011. "Biotechnology for Saving Rare and Threatened Flora in a Biodiversity Hotspot." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 188-200

Cakmak, I., and H. Marschner. 1992. "Magnesium Deficiency and High Light Intensity Enhance Activities of Superoxide Dismutase, Ascorbate Peroxidase, and Glutathione Reductase in Bean Leaves." *Plant Physiology* 98 (4): 1222-1227

Canepa, P., A. Panta, and D. Tay. 2011. "The Effect of Antioxidants on the Cryopreservation Recovery of Two Potato Cultivars Following Post-Thawing Culture." In *International Symposium on Cryopreservation in Horticultural Species*, 101-105. Acta Hort. (ISHS).

Cao, W.-L., Y.-X. Wang, Z.-Q. Xiang, and Z. Li. 2003. "Cryopreservation-Induced Decrease in Heat-Shock Protein 90 in Human Spermatozoa and Its Mechanism." *Asian journal of andrology* 5 (1): 43-46

Castellano, C.-A., I. Audet, J. Bailey, J.-P. Laforest, and J. Matte. 2010. "Dietary Omega-3 Fatty Acids (Fish Oils) Have Limited Effects on Boar Semen Stored at 17°C or Cryopreserved." *Theriogenology* 74 (8): 1482-1490

Chaitanya, K., D. Sundar, S. Masilamani, and A.R. Reddy. 2002. "Variation in Heat Stress-Induced Antioxidant Enzyme Activities among Three Mulberry Cultivars." *Plant growth regulation* 36 (2): 175-180

Chang, Y., R.E. Barker, and B.M. Reed. 2000. "Cold Acclimation Improves Recovery of Cryopreserved Grass *(Zoysia* and *Lolium* Sp.)." *CryoLetters* 21 (2): 107-116

Chen, H., D. Osuna, L. Colville, O. Lorenzo, K. Graeber, H. Küster, G. Leubner-Metzger, and I. Kranner. 2013. "Transcriptome-Wide Mapping of Pea Seed Ageing Reveals a Pivotal Role for Genes Related to Oxidative Stress and Programmed Cell Death." *PloS One* 8 (10): e78471

Cho, E.G., Y.L. Hor, H.H. Kim, V.R. Rao, and F. Engelmann. 2002. "Cryopreservation of *Citrus Madurensis* Embryonic Axes by Vitrification: Importance of Loading and Treatment with Vitrification Solution." *CryoLetters* 23 (5): 317-324

Chua, S., and M. Normah. 2011. "Effect of Preculture, Pvs2 and Vitamin C on Survival of Recalcitrant *Nephelium Ramboutan*-Ake Shoot Tips after Cryopreservation by Vitrification." *CryoLetters* 32 (6): 506-515

Colville, L., E.L. Bradley, A.S. Lloyd, H.W. Pritchard, L. Castle, and I. Kranner. 2012. "Volatile Fingerprints of Seeds of Four Species Indicate the Involvement of Alcoholic Fermentation, Lipid Peroxidation, and Maillard Reactions in Seed Deterioration During Ageing and Desiccation Stress." *Journal of Experimental Botany* 63 (18): 6519-6530

Colville, L., and I. Kranner. 2010. "Desiccation Tolerant Plants as Model Systems to Study Redox Regulation of Protein Thiols." *Plant Growth Regulation* 62 (3): 241-255

Creissen, G., J. Firmin, M. Fryer, B. Kular, N. Leyland, H. Reynolds, G. Pastori, F. Wellburn, N. Baker, and A. Wellburn. 1999. "Elevated Glutathione Biosynthetic Capacity in the Chloroplasts of Transgenic Tobacco Plants Paradoxically Causes Increased Oxidative Stress." *The Plant Cell Online* 11 (7): 1277-1291

Crowe, J.H., L.M. Crowe, J.F. Carpenter, A. Rudolph, C.A. Wistrom, B. Spargo, and T. Anchordoguy. 1988. "Interactions of Sugars with Membranes." *Biochimica et Biophysica Acta* 947 (2): 367-384

Davey, M., E. Stals, B. Panis, J. Keulemans, and R. Swennen. 2005. "High-Throughput Determination of Malondialdehyde in Plant Tissues." *Analytical Biochemistry* 347 (2): 201-207

Day, J.G., K.C. Harding, J. Nadarajan, and E.E. Benson. 2008. "Cryopreservation: Conservation of Bioresources at Ultra Low Tempratures." In *Molecular Biomethods Handbook*, eds J Walker and R Rapley, 917-947. Hertfordshire UK: Humana Press.

Dedon, P.C., J.P. Plastaras, C.A. Rouzer, and L.J. Marnett. 1998. "Indirect Mutagenesis by Oxidative DNA Damage: Formation of the Pyrimidopurinone Adduct of Deoxyguanosine by Base Propenal." *Proceedings of the National Academy of Sciences* 95 (19): 11113-11116

Department of Parks and Wildlife. 2014. Wildelife Conservation (Rare Flora) Notice. Accessed 20 Jan 2015, http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/threatened-plants.

Dereuddre, J., J. Fabre, and Basaglia. 1988. "Resistance of Alginate-Coated Somatic Embryos of Carrot (*Daucus Carota* L.) to Desiccation and Freezing in Liquid Nitrogen." *Plant Cell Reports* 7: 170-173

Dixon, D.P., M. Skipsey, N.M. Grundy, and R. Edwards. 2005. "Stress-Induced Protein S-Glutathionylation in *Arabidopsis*." *Plant Physiology* 138 (4): 2233-2244

Domínguez‐Rebolledo, Á., F. Martínez‐Pastor, M. Fernández‐Santos, E. Del Olmo, A. Bisbal, J. Ros‐Santaella, and J. Garde. 2010. "Comparison of the Tbars Assay and Bodipy C11 Probes for Assessing Lipid Peroxidation in Red Deer Spermatozoa." *Reproduction in Domestic Animals* 45 (6): e360-e368

Dowgert, M.F., and P.L. Steponkus. 1984. "Behavior of the Plasma Membrane of Isolated Protoplasts During a Freeze-Thaw Cycle." *Plant Physiology* 75 (4): 1139-1151

Drummen, G.P., B.M. Gadella, J.A. Post, and J.F. Brouwers. 2004. "Mass Spectrometric Characterization of the Oxidation of the Fluorescent Lipid Peroxidation Reporter Molecule C11-Bodipy$^{581/591}$." *Free Radical Biology and Medicine* 36 (12): 1635-1644

Dumet, D., W. Block, R. Worland, B.M. Reed, and E.E. Benson. 2000. "Profiling Cryopreservation Protocols for *Ribes Ciliatum* Using Differential Scanning Calorimetry." *CryoLetters* 21 (6): 367-378

Ďuračková, Z. 2014. "Free Radicals and Antioxidants for Non-Experts." In *Systems Biology of Free Radicals and Antioxidants*, ed. Ismail Laher, 3-38.

Dussert, S., N. Chabrillange, J.L. Montillet, J.P. Agnel, F. Engelmann, and M. Noirot. 2003. "Basis of Coffee Seed Sensitivity to Liquid Nitrogen Exposure: Oxidative Stress or Imbibitional Damage?" *Physiologia Plantarum* 119 (4): 534-543

Esterbauer, H., R.J. Schaur, and H. Zollner. 1991. "Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde and Related Aldehydes." *Free Radical Biology and Medicine* 11 (1): 81-128

Fabre, J., and J. Dereuddre. 1990. "Encapsulation Dehydration: A New Approach to Cryopreservation of *Solanum* Shoot-Tips." *CryoLetters* 11: 413-426

Fadzillah, N.M., V. Gill, R.P. Finch, and R.H. Burdon. 1996. "Chilling, Oxidative Stress and Antioxidant Responses in Shoot Cultures of Rice." *Planta* 199 (4): 552-556

Fahy, G.M., D.R. Mac Farlane, C.A. Angell, and H.T. Meryman. 1984. "Vitrification as an Approach to Cryopreservation." *Cryobiology* 21 (4): 407-426

Fang, J.Y., A. Wetten, and J. Johnston. 2008. "Headspace Volatile Markers for Sensitivity of Cocoa (*Theobroma Cacao* L.) Somatic Embryos to Cryopreservation." *Plant Cell Reports* 27 (3): 453-461

Farrugia, G., and R. Balzan. 2012. "Oxidative Stress and Programmed Cell Death in Yeast." *Molecular and Cellular Oncology* 2 (64): 1-21

Ferrusola, C.O., L.G. Fernández, J. Morrell, C.S. Sandoval, B.M. García, H. Rodríguez-Martinez, J. Tapia, and F. Peña. 2009. "Lipid Peroxidation, Assessed with Bodipy-C11, Increases after Cryopreservation of Stallion Spermatozoa, Is Stallion-Dependent and Is Related to Apoptotic-Like Changes." *Reproduction* 138 (1): 55-63

Fki, L., N. Bouaziz, O. Chkir, R. Benjemaa-Masmoudi, A. Rival, R. Swennen, N. Drira, and B. Panis. 2013. "Cold Hardening and Sucrose Treatment Improve Cryopreservation of Date Palm Meristems." *Biologia Plantarum* 57 (2): 375-379

Fleck, R.A., E.E. Benson, D.H. Bremner, and J.G. Day. 2000. "Studies of Free Radical-Mediated Cryoinjury in the Unicellular Green Alga *Euglena Gracilis* Using a Non-Destructive Hydroxyl Radical Assay: A Novel Approach for Developing Protistan Cryopreservation Strategies." *Free Radical Research* 32 (2): 157-70

Foyer, C.H., L.D. Gomez, and D.R. van Heerden. 2008. "Glutathione." In *Antioxidants and Reactive Oxygen Species in Plants*, ed. N. Smirnoff, 1-24. Wiley.

Foyer, C.H., M. Lelandais, and K.J. Kunert. 1994. "Photooxidative Stress in Plants." *Physiologia Plantarum* 92 (4): 696-717

Foyer, C.H., and G. Noctor. 2009. "Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications." *Antioxidants & Redox Signaling* 11 (4): 861-905

Foyer, C.H., and G. Noctor. 2011. "Ascorbate and Glutathione: The Heart of the Redox Hub." *Plant Physiology* 155 (1): 2-18

Fretz, A., and H. Lörz. 1995. "Cryopreservation of *in Vitro* Cultures of Barley *(Hordeum Vulgare L*. And *H. Murinum L*.) and Transgenic Cells of Wheat (*Triticum Aestivum L*.)." *Journal of Plant Physiology* 146 (4): 489-496

Fryer, M.J., K. Oxborough, P.M. Mullineaux, and N.R. Baker. 2002. "Imaging of Photo‐Oxidative Stress Responses in Leaves." *Journal of Experimental Botany* 53 (372): 1249-1254

Fujikawa, S., Y. Jitsuyama, and K. Kuroda. 1999. "Determination of the Role of Cold Acclimation-Induced Diverse Changes in Plant Cells from the Viewpoint of Avoidance of Freezing Injury." *Journal of Plant Research* 112 (2): 237-244

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture* 114 (1): 83-96

Funnekotter, B., S.E. Whiteley, S.R. Turner, E. Bunn, and R.L. Mancera. 2015. "Evaluation of the New Vacuum Infiltration Vitrification (Viv) Cryopreservation Tehnique for Native Australian Plant Shoot Tips." *CryoLetters* In Press: 1-11

George, E.F., and P.D. Sherrington. 1993. *Plant Propagation by Tissue Culture, Part 1. The Technology*. Edington UK: Exegentics.

Gill, S.S., and N. Tuteja. 2010. "Reactive Oxygen Species and Antioxidant Machinery in Abiotic Stress Tolerance in Crop Plants." *Plant Physiology and Biochemistry* 48 (12): 909-930

Gonzalez-Arnao, M.T., C. Urra, F. Engelmann, R. Ortiz, and C. de la Fe. 1999. "Cryopreservation of Encapsulated Sugarcane Apices: Effect of Storage Temperature and Storage Duration." *CryoLetters* 20 (6): 347-352

Gordon-Kamm, W.J., and P.L. Steponkus. 1984. "Lamellar-to-Hexagonalii Phase Transitions in the Plasma Membrane of Isolated Protoplasts after Freeze-Induced Dehydration." *Proceedings of the National Academy of Sciences of the United States of America* 81 (20): 6373-7

Gratão, P.L., A. Polle, P.J. Lea, and R.A. Azevedo. 2005. "Making the Life of Heavy Metal-Stressed Plants a Little Easier." *Functional Plant Biology* 32 (6): 481-494

Gupta, A.S., J.L. Heinen, A.S. Holaday, J.J. Burke, and R.D. Allen. 1993. "Increased Resistance to Oxidative Stress in Transgenic Plants That Overexpress Chloroplastic Cu/Zn Superoxide Dismutase." *Proceedings of the National Academy of Sciences* 90 (4): 1629-1633

Gupta, S., and B.M. Reed. 2006. "Cryopreservation of Shoot Tips of Blackberry and Raspberry by Encapsulation-Dehydration and Vitrification." *CryoLetters* 27 (1): 29-42

Halliwell, B., and J.M. Gutteridge. 2007. *Free Radicals in Biology and Medicine*. Edited by Barry Halliwell and John MC Gutteridge. 4 ed. Vol. 3. New York: Oxford University Press Inc.

Hamilton, K.N., S.R. Turner, and S.E. Ashmore. 2009. "Cryopreservation." In *Plant Germplasm Conservation in Australia*, eds C.A. Offord and P.F. Meagher, 129-148. Sydney: Australian Network for Plant Conservation Inc.

Hammerstedt, R.H., J.K. Graham, and J.P. Nolan. 1990. "Cryopreservation of Mammalian Sperm: What We Ask Them to Survive." *Journal of Andrology* 11 (1): 73-88

Hao, Y.J., C.X. You, and X.X. Deng. 2002. "Analysis of Ploidy and the Patterns of Amplified Fragment Length Polymorphism and Methylation Sensitive Amplified Polymorphism in Strawberry Plants Recovered from Cryopreservation." *CryoLetters* 23 (1): 37-46

Harding, K. 2004. "Genetic Integrity of Cryopreserved Plant Cells: A Review." *CryoLetters* 25 (1): 3-22

Harding, K., J.W. Johnston, and E.E. Benson. 2009. "Exploring the Physiological Basis of Cryopreservation Success and Failure in Clonally Propagated in Vitro Crop Plant Germplasm." *Agricultural and Food Science* 18 (2): 103-116

Hatanaka, T., T. Yasuda, T. Yamaguchi, and A. Sakai. 1994. "Direct Regrowth of Encapsulated Somatic Embryos of Coffee (*Coffea Canephora*) after Cooling in Liquid Nitrogen." *CryoLetters* 15: 47-52

Heath, R.L., and L. Packer. 1968. "Photoperoxidation in Isolated Chloroplasts: I. Kinetics and Stoichiometry of Fatty Acid Peroxidation." *Archives of Biochemistry and Biophysics* 125 (1): 189-198

Hincha, D., and E. Zuther. 2014. "Introduction: Plant Cold Acclimation and Freezing Tolerance." In *Plant Cold Acclimation*, eds Dirk K. Hincha and Ellen Zuther, 1-6. Springer New York.

Hipkiss, A.R., J.E. Preston, D. Himswoth, V.C. Worthington, and N.J. Abbot. 1997. "Protective Effects of Carnosine against Malondialdehyde-Induced Toxicity Towards Cultured Rat Brain Endothelial Cells." *Neuroscience Letters* 238 (3): 135-138

Hirai, D., and A. Sakai. 1999. "Cryopreservation of in Vitro-Grown Axillary Shoot-Tip Meristems of Mint *(Mentha Spicata* L.) by Encapsulation Vitrification." *Plant Cell Reports* 19 (2): 150-155

Hong, T.D., and R.H. Ellis. 1996. *A Protocol to Determine Seed Storage Behaviour*. Edited by IPGRI Technical Bulletin No. 1. Rome, Italy: International Plant Genetic Resources Institute.

Hong, T.D., S. Linington, and R.H. Ellis. 1998. *Compendium of Information on Seed Storage Behaviour* UK: Kew Publishing.

Hopper, S.D., and P. Gioia. 2004. "The Southwest Australian Floristic Region: Evolution and Conservation of a Global Hot Spot of Biodiversity." *Annual Review of Ecology, Evolution, and Systematics*: 623-650

Hughes, Z.E., and R.L. Mancera. 2013. "Molecular Dynamics Simulations of Mixed Dopc−B-Sitosterol Bilayers and Their Interactions with Dmso." *Soft Matter* 9: 2920-2935

Hughes, Z.E., and R.L. Mancera. 2014. "Molecular Mechanism of the Synergistic Effects of Vitrification Solutions on the Stability of Phospholipid Bilayers." *Biophysical Journal* 106 (12): 2617-2624

Hughes, Z.E., A.E. Mark, and R.L. Mancera. 2012. "Molecular Dynamics Simulations of the Interactions of Dmso with Dppc and Dopc Phospholipid Membranes." *The Journal of Physical Chemistry B* 116 (39): 11911-11923

Huner, N., G. Öquist, and F. Sarhan. 1998. "Energy Balance and Acclimation to Light and Cold." *Trends in Plant Science* 3 (6): 224-230

Johnston, J.W., K. Harding, and E.E. Benson. 2007. "Antioxidant Status and Genotypic Tolerance of *Ribes in Vitro* Cultures to Cryopreservation." *Plant Science* 172 (3): 524-534

Kaczmarczyk, A., B. Funnekotter, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., B. Funnekotter, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for *Loxocarya Cinerea*-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519

Kaczmarczyk, A., A. Houben, E. Keller, and M.F. Mette. 2010. "Influence of Cryopreservation on the Cytosine Methylation State of Potato Genomic DNA." *CryoLetters* 31 (5): 380-391

Kaczmarczyk, A., V.-M. Rokka, and E.J. Keller. 2011a. "Potato Shoot Tip Cryopreservation. A Review." *Potato Research* 54 (1): 45-79

Kaczmarczyk, A., N. Shvachko, Y. Lupysheva, M.R. Hajirezaei, and E.R. Keller. 2008. "Influence of Alternating Temperature Preculture on Cryopreservation Results for Potato Shoot Tips." *Plant Cell Reports* 27 (9): 1551-8

Kaczmarczyk, A., S.R. Turner, E. Bunn, R.L. Mancera, and K.W. Dixon. 2011b. "Cryopreservation of Threatened Native Australian Species—What Have We Learned and Where to from Here?" *In Vitro Cellular & Developmental Biology - Plant* 47 (1): 17-25

Kaity, A., R.A. Drew, and S. Ashmore. 2013. "Genetic and Epigenetic Integrity Assessment of Acclimatised Papaya Plants Regenerated Directly from Shoot-Tips Following Short-and Long-Term Cryopreservation." *Plant Cell, Tissue and Organ Culture* 112 (1): 75-86

Kamata, T., and M. Uemura. 2004. "Solute Accumulation in Wheat Seedlings During Cold Acclimation: Contribution to Increased Freezing Tolerance." *CryoLetters* 25 (5): 311-322

Kartha, K., N. Leung, and L. Mroginski. 1982. "*In Vitro* Growth Responses and Plant Regeneration from Cryopreserved Meristems of Cassava (*Manihot Esculenta* Crantz)." *Zeitschrift für Pflanzenphysiologie* 107 (2): 133-140

Kauzmann, W. 1948. "The Nature of the Glassy State and the Behavior of Liquids at Low Temperatures." *Chemical Reviews* 43 (2): 219-256

Kim, H.H., Y.G. Lee, S.U. Park, S.C. Lee, H.J. Baek, E.G. Cho, and F. Engelmann. 2009a. "Development of Alternative Loading Solutions in Droplet-Vitrification Procedures." *CryoLetters* 30 (4): 291-299

Kim, H.H., Y.G. Lee, D.J. Shin, H.C. Ko, J.G. Gwag, E.G. Cho, and F. Engelmann. 2009b. "Development of Alternative Plant Vitrification Solutions in Droplet-Vitrification Procedures." *CryoLetters* 30 (5): 320-34

Kim, Y.J., M.G. Jang, H.Y. Noh, H.J. Lee, J. Sukweenadhi, J.H. Kim, S.Y. Kim, W.S. Kwon, and D.C. Yang. 2014. "Molecular Characterization of Two Glutathione Peroxidase Genes of *Panax Ginseng* and Their Expression Analysis against Environmental Stresses." *Gene* 535 (1): 33-41

Koch, J.M., and G.P. Samsa. 2007. "Restoring Jarrah Forest Trees after Bauxite Mining in Western Australia." *Restoration Ecology* 15: S17-S25

Kocsy, G., G. Galiba, and C. Brunold. 2001. "Role of Glutathione in Adaptation and Signalling During Chilling and Cold Acclimation in Plants." *Physiologia Plantarum* 113 (2): 158-164

Kocsy, G., G. Szalai, A. Vágújfalvi, L. Stéhli, G. Orosz, and G. Galiba. 2000. "Genetic Study of Glutathione Accumulation During Cold Hardening in Wheat." *Planta* 210 (2): 295-301

Kranner, I. 1998. "Determination of Glutathione, Glutathione Disulphide and Two Related Enzymes, Glutathione Reductase and Glucose-6-Phosphate Dehydrogenase, in Fungal and Plant Cells." In *Mycorrhiza Manual*, ed. Ajit Varma, 227-241. Springer Berlin Heidelberg.

Kranner, I., and S. Birtić. 2005. "A Modulating Role for Antioxidants in Desiccation Tolerance." *Integrative and Comparative Biology* 45 (5): 734-740

Kranner, I., S. Birtic, K.M. Anderson, and H.W. Pritchard. 2006. "Glutathione Half-Cell Reduction Potential: A Universal Stress Marker and Modulator of Programmed Cell Death?" *Free Radical Biology and Medicine* 40 (12): 2155-2165

Kulus, D., and M. Zalewska. 2014. "Cryopreservation as a Tool Used in Long-Term Storage of Ornamental Species–a Review." *Scientia Horticulturae* 168: 88-107

Kuranuki, Y., and A. Sakai. 1995. "Cryopreservation of in Vitro-Grown Shoot Tips of Tea (*Camellia Sinensis*) by Vitrification." *CryoLetters* 16 (6): 345-352

Lambers, H. 2014. *Plant Life on the Sandplains in Southwest Australia: A Global Biodiversity Hotspot*: University of Western Australia Press.

Leifert, C., C.E. Morris, and W.M. Waites. 1994. "Ecology of Microbial Saprophytes and Pathogens in Tissue-Culture and Field-Grown Plants - Reasons for Contamination Problems in Vitro." *Critical Reviews in Plant Sciences* 13 (2): 139-183

Leifert, C., J. Ritchie, and W. Waites. 1991. "Contaminants of Plant-Tissue and Cell Cultures." *World Journal of Microbiology and Biotechnology* 7 (4): 452-469

Leunufna, S., and E.R. Keller. 2005. "Cryopreservation of Yams Using Vitrification Modified by Including Droplet Method: Effects of Cold Acclimation and Sucrose." *CryoLetters* 26 (2): 93-102

Lovelock, J.E. 1953. "Het Mechanism of the Protective Action of Glycerol against Haemolysis by Freezing and Thawing." *Biochimica et Biophysica Acta* 11: 28-36

Lowe, F. 2014. "Biomarkers of Oxidative Stress." In *Systems Biology of Free Radicals and Antioxidants*, ed. Ismail Laher, 65-87. Springer Berlin Heidelberg.

Luyet, B.J. 1937. "The Vitrification of Organic Colloids and of Protoplasm." *Biodynamica* 1 (29): 1-14

Lynch, D.V., and P.L. Steponkus. 1987. "Plasma Membrane Lipid Alterations Associated with Cold Acclimation of Winter Rye Seedlings (*Secale Cereale* L. Cv Puma)." *Plant Physiology* 83 (4): 761-767

Lynch, P.T., A. Siddika, J.W. Johnston, S.M. Trigwell, A. Mehra, C. Benelli, M. Lambardi, and E.E. Benson. 2011. "Effects of Osmotic Pretreatments on Oxidative Stress, Antioxidant Profiles and Cryopreservation of Olive Somatic Embryos." *Plant Science* 181: 47-56

MacDonald, M.L., I.V. Murray, and P.H. Axelsen. 2007. "Mass Spectrometric Analysis Demonstrates That Bodipy 581/591 C11 Overestimates and Inhibits Oxidative Lipid Damage." *Free Radical Biology and Medicine* 42 (9): 1392-1397

Malan, C., M.M. Greyling, and J. Gressel. 1990. "Correlation between Cuzn Superoxide Dismutase and Glutathione Reductase, and Environmental and Xenobiotic Stress Tolerance in Maize Inbreds." *Plant Science* 69 (2): 157-166

Mandumpal, J.B., C.A. Kreck, and R.L. Mancera. 2011. "A Molecular Mechanism of Solvent Cryoprotection in Aqueous Dmso Solutions." *Physical Chemistry Chemical Physics* 13 (9): 3839-3842

Marnett, L.J. 1999a. "Chemistry and Biology of DNA Damage by Malondialdehyde." *IARC Scientific Publications* (150): 17-27

Marnett, L.J. 1999b. "Lipid Peroxidation—DNA Damage by Malondialdehyde." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 424 (1): 83-95

Martinez-Montero, M., N. Mora, J. Quinones, M. Gonzalez-Arnao, F. Engelmann, and J. Lorenzo. 2002. "Effect of Cryopreservation on the Structural and Functional Integrity of Cell Membranes of Sugarcane (*Saccharum* Sp.) Embryogenic Calluses." *CryoLetters* 23 (4): 237-244

Matsumoto, T., A. Sakai, and K. Yamada. 1994. "Cryopreservation of in Vitro-Grown Apical Meristems of Wasabi (*Wasabia Japonica*) by Vitrification and Subsequent High Plant Regeneration." *Plant Cell Reports* 13 (8): 442-446

Mazur, P. 2004. "Priciples of Cryobiology." In *Life in the Frozen State*, eds B.J. Fuller, N. Lane and E. E. Benson, 3-65. Boca Raton, Florida: CRC Press.

McCord, J.M., and I. Fridovich. 1968. "The Reduction of Cytochrome C by Milk Xanthine Oxidase." *Journal of Biological Chemistry* 243 (21): 5753-5760

McKersie, B.D., Y. Chen, M. de Beus, S.R. Bowley, C. Bowler, D. Inzé, K. D'Halluin, and J. Botterman. 1993. "Superoxide Dismutase Enhances Tolerance of Freezing Stress in Transgenic Alfalfa (*Medicago Sativa* L.)." *Plant Physiology* 103 (4): 1155-1163

Meloni, D.A., M.A. Oliva, C.A. Martinez, and J. Cambraia. 2003. "Photosynthesis and Activity of Superoxide Dismutase, Peroxidase and Glutathione Reductase in Cotton under Salt Stress." *Environmental and Experimental Botany* 49 (1): 69-76

Mengutay, M., Y. Ceylan, U.B. Kutman, and I. Cakmak. 2013. "Adequate Magnesium Nutrition Mitigates Adverse Effects of Heat Stress on Maize and Wheat." *Plant and Soil* 368 (1-2): 57-72

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270

Menon, A., B. Funnekotter, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species *Lomandra Sonderi* (Asparagaceae)." *Plant Cell, Tissue and Organ Culture* 119 (2): 347-358

Mishima, O., and H.E. Stanley. 1998. "The Relationship between Liquid, Supercooled and Glassy Water." *Nature* 396 (6709): 329-335

Mishra, N.P., R.K. Mishra, and G.S. Singhal. 1993. "Changes in the Activities of Anti-Oxidant Enzymes During Exposure of Intact Wheat Leaves to Strong Visible Light at Different Temperatures in the Presence of Protein Synthesis Inhibitors." *Plant Physiology* 102 (3): 903-910

Møller, I.M., P.E. Jensen, and A. Hansson. 2007. "Oxidative Modifications to Cellular Components in Plants." *Annual Review of Plant Biology* 58: 459-481

Muckenschnabel, I., B. Goodman, B. Williamson, G. Lyon, and N. Deighton. 2002. "Infection of Leaves of *Arabidopsis Thaliana* by *Botrytis Cinerea*: Changes in Ascorbic Acid, Free Radicals and Lipid Peroxidation Products." *Journal of Experimental Botany* 53 (367): 207-214

Munne-Bosch, S. 2005. "The Role of *A*-Tocopherol in Plant Stress Tolerance." *Journal of Plant Physiology* 162 (7): 743-748

Murashige, T. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473

Murashige, T., and F. Skoog. 1962. "A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures." *Physiologia Plantarum* 15 (3): 473-497

Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. da Fonseca, and J. Kent. 2000. "Biodiversity Hotspots for Conservation Priorities." *Nature* 403 (6772): 853-858

Nadarajan, J., and H.W. Pritchard. 2014. "Biophysical Characteristics of Successful Oilseed Embryo Cryoprotection and Cryopreservation Using Vacuum Infiltration Vitrification: An Innovation in Plant Cell Preservation." *PloS One* 9 (5): e96169

Nagel, M., I. Kranner, K. Neumann, H. Rolletschek, C.E. Seal, L. Colville, B. Fernández-Marín, and A. Börner. 2014. "Genome-Wide Association Mapping and Biochemical Markers Reveal That Seed Ageing and Longevity Are Intricately Affected by Genetic Background and Developmental and Environmental Conditions in Barley." *Plant, Cell & Environment*: doi: 10.1111/pce.12474

Naraikina, N., M. Sin'kevich, I. Demin, A. Selivanov, I. Moshkov, and T. Trunova. 2014. "Changes in the Activity of Superoxide Dismutase Isoforms in the Course of Low-Temperature Adaptation in Potato Plants of Wild Type and Transformed with Δ12-Acyl-Lipid Desaturase Gene." *Russian Journal of Plant Physiology* 61 (3): 332-338

Navrot, N., V. Collin, J. Gualberto, E. Gelhaye, M. Hirasawa, P. Rey, D.B. Knaff, E. Issakidis, J.-P. Jacquot, and N. Rouhier. 2006. "Plant Glutathione Peroxidases Are Functional Peroxiredoxins Distributed in Several Subcellular Compartments and Regulated During Biotic and Abiotic Stresses." *Plant Physiology* 142 (4): 1364-1379

Nishikawa, A., R. Sodum, and F.L. Chung. 1992. "Acute Toxicity of Trans-5-Hydroxy-2-Nonenal in Fisher 344 Rats." *Lipids* 27 (1): 54-58

Noctor, G., and C.H. Foyer. 1998. "Ascorbate and Glutathione: Keeping Active Oxygen under Control." *Annual Review of Plant Biology* 49 (1): 249-279

Noctor, G., A. Mhamdi, S. Chaouch, Y. Han, J. Neukermans, B. Marquez‐Garcia, G. Queval, and C.H. Foyer. 2012. "Glutathione in Plants: An Integrated Overview." *Plant, Cell & Environment* 35 (2): 454-484

Noctor, G., G. Queval, A. Mhamdi, S. Chaouch, and C.H. Foyer. 2011. "Glutathione." *The Arabidopsis Book / American Society of Plant Biologists* 9: e0142. doi:10.1199/tab.0142

O'Kane, D., V. Gill, P. Boyd, and R. Burdon. 1996. "Chilling, Oxidative Stress and Antioxidant Responses in *Arabidopsis Thaliana* Callus." *Planta* 198 (3): 371-377

Obert, B., E.E. Benson, S. Millam, A. Pretová, and D.H. Bremner. 2005. "Moderation of Morphogenetic and Oxidative Stress Responses in Flax in Vitro Cultures by Hydroxynonenal and Desferrioxamine." *Journal of Plant Physiology* 162 (5): 537-547

Offord, C.A., E. Bunn, S.R. Turner, K.D. Sommerville, J. Seimon, and S.E. Ashmore. 2009. "Tissue Culture." In *Plant Germplasm Conservation in Australia*, eds C.A. Offord and P.F. Meagher, 109-128. Sydney: Australian Network for Plant Conservation Inc.

Offord, C.A., and P.F. Meagher. 2009. *Plant Germplasm Conservation in Australia*. Edited by C.A. Offord and P.F. Meagher. Sydney: Australian Network for Plant Conservation Inc.

Paczkowska, G. 1998. *Lomandra Sonderi*. Western Australian Herbarium (FloraBase). Accessed 13 March, http://florabase.dec.wa.gov.au/browse/profile/1244.

Panis, B., and M. Lambardi. 2006. "Status of Cryopreservation Technologies in Plants (Crops and Forest Trees)." In *The Role of Biotechnology in Exploring and Protecting Agricultural Genetic Resources*, eds J. Ruane and A. Sonnino, 61-78. Rome, Italy: United Nations Food and Agriculture Organization (FAO).

Pap, E., G. Drummen, V. Winter, T. Kooij, P. Rijken, K. Wirtz, J. Op den Kamp, W. Hage, and J. Post. 1999. "Ratio-Fluorescence Microscopy of Lipid Oxidation in Living Cells Using C11-Bodipy$^{581/591}$." *FEBS Letters* 453 (3): 278-282

Paques, M., V. Monod, M. Poissonnier, and J. Dereuddre. 2002. "Cryopreservation of *Eucalyptus* Sp Shoot Tips by the Encapsulation-Dehydration Procedure." In *Cryopreservation of Plant Germplasm Ii*, eds L. E. Towill and YPS Bajaj, 234-245. Berlin: Springer-Verlag Berlin.

Partyka, A., E. Łukaszewicz, W. Niżański, and J. Twardoń. 2011. "Detection of Lipid Peroxidation in Frozen-Thawed Avian Spermatozoa Using C $_{11}$-Bodipy$^{581/591}$." *Theriogenology* 75 (9): 1623-1629

Plummer, J., A. Crawford, and S. Taylor. 1995. "Germination of *Lomandra Sonderi* (Dasypogonaceae) Promoted by Pericarp Removal and Chemical-Stimulation of the Embryo." *Australian Journal of Botany* 43 (2): 223-230

Polge, C., A.U. Smith, and A.S. Parkes. 1949. "Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures." *Nature* 164 (4172): 666-666

Ponappa, T., J.C. Scheerens, and A.R. Miller. 1993. "Vacuum Infiltration of Polyamines Increases Firmness of Strawberry Slices under Various Storage Conditions." *Journal of Food Science* 58 (2): 361-364

Prasad, T.K. 1996. "Mechanisms of Chilling‐Induced Oxidative Stress Injury and Tolerance in Developing Maize Seedlings: Changes in Antioxidant System, Oxidation of Proteins and Lipids, and Protease Activities." *The Plant Journal* 10 (6): 1017-1026

Pritchard, H., B. Grout, and K. Short. 1986. "Osmotic Stress as a Pregrowth Procedure for Cryopreservation: 1. Growth and Ultrastructure of Sycamore and Soybean Cell Suspensions." *Annals of Botany* 57 (1): 41-48

Pritchard, H.W. 2004. "Classification of Seed Storage 'Types' for Ex Situ Conservation in Relation to Temperature and Moisture." In *Ex Situ Plant Conservation: Supporting Species Survival in the Wild*, eds E. O. Guerrant, K. Havens and M. Maunder, 139-161. Washington, DC: Island Press.

Rao, A.C., and A.R. Reddy. 2008. "Glutathione Reductase: A Putative Redox Regulatory System in Plant Cells." In *Sulfur Assimilation and Abiotic Stress in Plants*, 111-147. Springer.

Reed, B. 2011. "Choosing and Applying Cryopreservation Protocols to New Plant Species or Tissues." *Acta Horticulturae* 908: 363-372

Reed, B.M. 2008. "Cryopreservation - Practical Considerations." In *Plant Cryopreservation. A Practical Guide*, ed. B.M. Reed, 3-13. New York: Springer.

Reed, B.M., V. Sarasan, M. Kane, E. Bunn, and V.C. Pence. 2011. "Biodiversity Conservation and Conservation Biotechnology Tools." *In Vitro Cellular & Developmental Biology-Plant* 47 (1): 1-4

Reinhoud, P.J., E.W.M. Schrijnemakers, F. Iren, and J.W. Kijne. 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 (3): 261-267

Roach, T., M. Ivanova, R.P. Beckett, F.V. Minibayeva, I. Green, H.W. Pritchard, and I. Kranner. 2008. "An Oxidative Burst of Superoxide in Embryonic Axes of Recalcitrant Sweet Chestnut Seeds as Induced by Excision and Desiccation." *Physiologia Plantarum* 133 (2): 131-139

Romero-Puertas, M.C., F.J. Corpas, L.M. Sandalio, M. Leterrier, M. Rodríguez-Serrano, L.A. Del Río, and J.M. Palma. 2006. "Glutathione Reductase from Pea Leaves: Response to Abiotic Stress and Characterization of the Peroxisomal Isozyme." *New Phytologist* 170 (1): 43-52

Rossetto, M., K.W. Dixon, and E. Bunn. 1992. "Aeration: A Simple Method to Control Vitrification and Improve in Vitro Culture of Rare Australian Plants." *In Vitro–Plant* 28 (4): 192-196

Sakai, A. 1960. "Survival of the Twig of Woody Plants." *Nature, London* 185 (4710): 393-4

Sakai, A. 2004. "Plant Cryopreservation." In *Life in the Frozen State*, eds B.J. Fuller, N Lane and E. E. Benson, 329-345. Boca Raton, Florida: CRC Press.

Sakai, A., and F. Engelmann. 2007. "Vitrification, Encapsulation-Vitrification and Droplet-Vitrification: A Review." *CryoLetters* 28 (3): 151-72

Sakai, A., D. Hirai, and T. Niino. 2008. "Development of Pvs-Based Vitrification and Encapsulation–Vitrification Protocols." In *Plant Cryopreservation: A Practical Guide*, ed. B.M. Reed, 33-57. New York: Springer.

Sakai, A., S. Kobayashi, and I. Oiyama. 1990. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb. Var. Brasiliensis Tanaka) by Vitrification." *Plant Cell Reports* 9 (1): 30-33

Sakai, A., S. Kobayashi, and I. Oiyama. 1991. "Cryopreservation of Nucellar Cells of Navel Orange (*Citrus Sinensis* Osb.) by a Simple Freezing Method." *Plant Science* 74 (2): 243-248

Sakai, A., and S. Yoshida. 1967. "Survival of Plant Tissue at Super-Low Temperature Vi. Effects of Cooling and Rewarming Rates on Survival." *Plant Physiology* 42 (12): 1695-1701

Sánchez-Fernández, R., M. Fricker, L.B. Corben, N.S. White, N. Sheard, C.J. Leaver, M. Van Montagu, D. Inzé, and M.J. May. 1997. "Cell Proliferation and Hair Tip Growth in the *Arabidopsis* Root Are under Mechanistically Different Forms of Redox Control." *Proceedings of the National Academy of Sciences* 94 (6): 2745-2750

Sasaki, H., K. Ichimura, and M. Oda. 1996. "Changes in Sugar Content During Cold Acclimation and Deacclimation of Cabbage Seedlings." *Annals of Botany* 78 (3): 365-369

Schafer, F.Q., and G.R. Buettner. 2001. "Redox Environment of the Cell as Viewed through the Redox State of the Glutathione Disulfide/Glutathione Couple." *Free Radical Biology and Medicine* 30 (11): 1191-1212

Senaratna, T., D. Touchell, E. Bunn, and K. Dixon. 2000. "Acetyl Salicylic Acid (Aspirin) and Salicylic Acid Induce Multiple Stress Tolerance in Bean and Tomato Plants." *Plant Growth Regulation* 30 (2): 157-161

Shao, H.-B., L.-Y. Chu, G. Wu, J.-H. Zhang, Z.-H. Lu, and Y.-C. Hu. 2007. "Changes of Some Anti-Oxidative Physiological Indices under Soil Water Deficits among 10 Wheat (*Triticum Aestivum* L.) Genotypes at Tillering Stage." *Colloids and Surfaces B: Biointerfaces* 54 (2): 143-149

Shu, D.-F., L.-Y. Wang, M. Duan, Y.-S. Deng, and Q.-W. Meng. 2011. "Antisense-Mediated Depletion of Tomato Chloroplast Glutathione Reductase Enhances Susceptibility to Chilling Stress." *Plant Physiology and Biochemistry* 49 (10): 1228-1237

Skyba, M., L. Petijová, J. Košuth, D.P. Koleva, T.G. Ganeva, V.M. Kapchina-Toteva, and E. Čellárová. 2012. "Oxidative Stress and Antioxidant Response in *Hypericum Perforatum* L. Plants Subjected to Low Temperature Treatment." *Journal of Plant Physiology* 169 (10): 955-964

Skyba, M., M. Urbanová, V. Kapchina-Toteva, J. Košuth, K. Harding, and E. Čellárová. 2010. "Physiological, Biochemical and Molecular Characteristics of Cryopreserved *Hypericum Perforatum* L. Shoot Tips." *CryoLetters* 31 (3): 249-260

Smallwood, M., and D.J. Bowles. 2002. "Plants in a Cold Climate." *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 357 (1423): 831-847

Smirnoff, N., and G.L. Wheeler. 2000. "Ascorbic Acid in Plants: Biosynthesis and Function." *Critical Reviews in Biochemistry and Molecular Biology* 35 (4): 291-314

Smulders, M., and G. De Klerk. 2011. "Epigenetics in Plant Tissue Culture." *Plant Growth Regulation* 63 (2): 137-146

Soejima, K., K. Shimoda, M. Takeuchi, T. Yamaki, T. Kono, H. Sakurai, and H. Nakazawa. 2012. "Preliminary Report of Novel Technique for Cryopreservation—Vacuum-Assisted Cryoprotectant Infiltration." *Cryobiology* 65 (1): 21-26

Srivastava, A.K., P. Bhargava, and L.C. Rai. 2005. "Salinity and Copper-Induced Oxidative Damage and Changes in the Antioxidative Defence Systems of

Anabaena Doliolum." *World Journal of Microbiology and Biotechnology* 21 (6-7): 1291-1298

Steponkus, P.L. 1984. "Role of the Plasma Membrane in Freezing Injury and Cold Acclimation." *Annual Review of Plant Physiology* 35 (1): 543-584

Stewart, R.R., and J.D. Bewley. 1980. "Lipid Peroxidation Associated with Accelerated Aging of Soybean Axes." *Plant Physiology* 65 (2): 245-248

Sumithra, K., P. Jutur, B.D. Carmel, and A.R. Reddy. 2006. "Salinity-Induced Changes in Two Cultivars of *Vigna Radiata*: Responses of Antioxidative and Proline Metabolism." *Plant Growth Regulation* 50 (1): 11-22

Suzuki, N., and R. Mittler. 2006. "Reactive Oxygen Species and Temperature Stresses: A Delicate Balance between Signaling and Destruction." *Physiologia Plantarum* 126 (1): 45-51

Tausz, M., H. Šircelj, and D. Grill. 2004. "The Glutathione System as a Stress Marker in Plant Ecophysiology: Is a Stress-Response Concept Valid?" *Journal of Experimental Botany* 55 (404): 1955-1962

Thierry, C., B. Florin, and V. Pétiard. 1999. "Changes in Protein Metabolism During the Acquisition of Tolerance to Cryopreservation of Carrot Somatic Embryos." *Plant Physiology and Biochemistry* 37 (2): 145-154

Thomashow, M.F. 1998. "Role of Cold-Responsive Genes in Plant Freezing Tolerance." *Plant Physiology* 118 (1): 1-7

Thomashow, M.F., E.J. Stockinger, K.R. Jaglo-Ottosen, S.J. Gilmour, and D.G. Zarka. 1997. "Function and Regulation of *Arabidopsis Thaliana* Cor (Cold-Regulated) Genes." *Acta Physiologiae Plantarum* 19 (4): 497-504

Touchell, D.H., S.R. Turner, E. Bunn, and K.W. Dixon. 2002. "Cryostorage of Stomatic Tissues of Endangered Australian Species." In *Biotechnology in Agriculture and Forestry 50: Cryopreservation of Plant Germplasm Ii*, eds LE Towill and YPS Bajaj. New York: Springer.

Tsang, E., C. Bowler, D. Hérouart, W. Van Camp, R. Villarroel, C. Genetello, M. Van Montagu, and D. Inzé. 1991. "Differential Regulation of Superoxide Dismutases in Plants Exposed to Environmental Stress." *The Plant Cell Online* 3 (8): 783-792

Turner, S., D. Touchell, K. Dixon, and B. Tan. 2000. "Cryopreservation of *Anigozanthos Viridis* Ssp. *Viridis* and Related Taxa from the South-West of Western Australia." *Australian Journal of Botany* 48 (6): 739-744

Uchendu, E.E., S.W. Leonard, M.G. Traber, and B.M. Reed. 2010a. "Vitamins C and E Improve Regrowth and Reduce Lipid Peroxidation of Blackberry Shoot Tips Following Cryopreservation." *Plant Cell Reports* 29 (1): 25-35

Uchendu, E.E., M. Muminova, S. Gupta, and B.M. Reed. 2010b. "Antioxidant and Anti-Stress Compounds Improve Regrowth of Cryopreserved *Rubus* Shoot Tips." *In Vitro Cellular & Developmental Biology-Plant*: 1-8

Uchendu, E.E., M.R. Shukla, B.M. Reed, and P.K. Saxena. 2013. "Melatonin Enhances the Recovery of Cryopreserved Shoot Tips of American Elm (*Ulmus Americana* L.)." *Journal of Pineal Research* 55 (4): 435-442

Uemura, M., R.A. Joseph, and P.L. Steponkus. 1995. "Cold Acclimation of Arabidopsis Thaliana (Effect on Plasma Membrane Lipid Composition and Freeze-Induced Lesions)." *Plant Physiology* 109 (1): 15-30

Uemura, M., Y. Tominaga, C. Nakagawara, S. Shigematsu, A. Minami, and Y. Kawamura. 2006. "Responses of the Plasma Membrane to Low Temperatures." *Physiologia Plantarum* 126 (1): 81-89

van Camp, W., K. Capiau, M. Van Montagu, D. Inze, and L. Slooten. 1996. "Enhancement of Oxidative Stress Tolerance in Transgenic Tobacco Plants Overproducing Fe-Superoxide Dismutase in Chloroplasts." *Plant Physiology* 112 (4): 1703-1714

Vandenbussche, B., S. Leurdian, V. Verdoodt, M. Gysemberg, and M. De Proft. 1999. "Changes in Sugar Content and Fatty Acid Composition of in Vitro Sugar Beet Shoots after Cold Acclimation: Influence on Survival after Cryopreservation." *Plant Growth Regulation* 28 (3): 157-163

Verleysen, H., G. Samyn, E. Van Bockstaele, and P. Debergh. 2004. "Evaluation of Analytical Techniques to Predict Viability after Cryopreservation." *Plant Cell, Tissue and Organ Culture* 77 (1): 11-21

Volk, G.M., J.L. Harris, and K.E. Rotindo. 2006. "Survival of Mint Shoot Tips after Exposure to Cryoprotectant Solution Components." *Cryobiology* 52 (2): 305-308

Volk, G.M., and C. Walters. 2006. "Plant Vitrification Solution 2 Lowers Water Content and Alters Freezing Behavior in Shoot Tips During Cryoprotection." *Cryobiology* 52 (1): 48-61

Voulgaridou, G.-P., I. Anestopoulos, R. Franco, M.I. Panayiotidis, and A. Pappa. 2011. "DNA Damage Induced by Endogenous Aldehydes: Current State of Knowledge." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 711 (1): 13-27

Wang, Z.C., and X.X. Deng. 2004. "Cryopreservation of Shoot-Tips of Citrus Using Vitrification: Effect of Reduced Form of Glutathione." *CryoLetters* 25 (1): 43-50

Wen, B., R. Wang, H. Cheng, and S. Song. 2010. "Cytological and Physiological Changes in Orthodox Maize Embryos During Cryopreservation." *Protoplasma* 239 (1-4): 57-67

Whitaker, C., R.P. Beckett, F.V. Minibayeva, and I. Kranner. 2010. "Production of Reactive Oxygen Species in Excised, Desiccated and Cryopreserved Explants of *Trichilia Dregeana* Sond." *South African Journal of Botany* 76 (1): 112-118

Willyams, D. 2005. "Tissue Culture of Geophytic Rush and Sedge Species for Revegetation of Bauxite Mine Sites in the Northern Jarrah Forest of Western Australia." In *Contributing to a sustainable future. Proceedings of the Australian Branch of the International Association for Plant Tissue Culture & Biotechnology. IAPTC&B, Perth, Western Australia*, 226–241.

Xu, J., J. Yang, X. Duan, Y. Jiang, and P. Zhang. 2014. "Increased Expression of Native Cytosolic Cu/Zn Superoxide Dismutase and Ascorbate Peroxidase Improves Tolerance to Oxidative and Chilling Stresses in Cassava (*Manihot Esculenta* Crantz)." *BMC Plant Biology* 14 (1): 208

Yamada, T., A. Sakai, T. Matsumura, and S. Higuchi. 1991. "Cryopreservation of Apical Meristems of White Clover (*Trifolium Repens* L.) by Vitrification." *Plant Science* 78 (1): 81-87

Yamamoto, S., T. Rafique, K. Fukui, K. Sekizawa, and T. Niino. 2012. "V-Cryo-Plate Procedure as an Effective Protocol for Cryobanks: Case Study of Mint Cryopreservation." *CryoLetters* 33 (1): 12-23

Yang, Y., C. Han, Q. Liu, B. Lin, and J. Wang. 2008. "Effect of Drought and Low Light on Growth and Enzymatic Antioxidant System of *Picea Asperata* Seedlings." *Acta Physiologiae Plantarum* 30 (4): 433-440

Young, I., and J. McEneny. 2001. "Lipoprotein Oxidation and Atherosclerosis." *Biochemical Society Transactions* 29: 358-362

Zarghami, R., M. Pirseyedi, S. Hasrak, and B.P. Sardrood. 2008. "Evaluation of Genetic Stability in Cryopreserved *Solanum Tuberosum*." *African Journal of Biotechnology* 7 (16): 2798-2802

Zgallaï, H., K. Steppe, and R. Lemeur. 2006. "Effects of Different Levels of Water Stress on Leaf Water Potential, Stomatal Resistance, Protein and Chlorophyll Content and Certain Anti - Oxidative Enzymes in Tomato Plants." *Journal of Integrative Plant Biology* 48 (6): 679-685

Zhai, Z., Y. Wu, F. Engelmann, R. Chen, and Y. Zhao. 2003. "Genetic Stability Assessments of Plantlets Regenerated from Cryopreserved in Vitro Cultured Grape and Kiwi Shoot-Tips Using Rapd." *CryoLetters* 24 (5): 315-322

Zhang, C., Z. Ding, X. Xu, Q. Wang, G. Qin, and S. Tian. 2010. "Crucial Roles of Membrane Stability and Its Related Proteins in the Tolerance of Peach Fruit to Chilling Injury." *Amino Acids* 39 (1): 181-194

Zhang, J., and M. Kirkham. 1994. "Drought-Stress-Induced Changes in Activities of Superoxide Dismutase, Catalase, and Peroxidase in Wheat Species." *Plant and Cell Physiology* 35 (5): 785-791

Zhao, Y., L.W. Qi, W.M. Wang, P.K. Saxena, and C.Z. Liu. 2011. "Melatonin Improves the Survival of Cryopreserved Callus of *Rhodiola Crenulata*." *Journal of Pineal Research* 50 (1): 83-88

Zhao, Y., and J. Xie. 2004. "Practical Applications of Vacuum Impregnation in Fruit and Vegetable Processing." *Trends in Food Science & Technology* 15 (9): 434-451

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

Appendix

Additional Publications

Funnekotter, B., A. Kaczmarczyk, S.R. Turner, E. Bunn, W. Zhou, S. Smith, G. Flematti, and R.L. Mancera. 2013. "Acclimation-Induced Changes in Cell Membrane Composition and Influence on Cryotolerance of in Vitro Shoots of Native Plant Species." *Plant Cell, Tissue and Organ Culture (PCTOC)* 114 (1): 83-96.

Kaczmarczyk, A., **B. Funnekotter**, A. Menon, P.Y. Phang, A. Al-Hanbali, E. Bunn, and R.L. Mancera. 2012. "Current Issues in Plant Cryopreservation." In *Current Frontiers in Cryobiology*, ed. Igor I Katkov, 417-438. InTech, Available from: http://www.intechopen.com/books/currentfrontiers-in-cryobiology/current-issues-in-plant-cryopreservation.

Kaczmarczyk, A., **B. Funnekotter**, S.R. Turner, E. Bunn, G. Bryant, T.E. Hunt, and R.L. Mancera. 2013. "Development of Cryopreservation for Loxocarya Cinerea-an Endemic Australian Plant Species Important for Post-Mining Restoration." *CryoLetters* 34 (5): 508-519.

Menon, A., **B. Funnekotter**, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2012. "Cryopreservation of *Lomandra Sonderi* (Asparagaceae) Shoot Tips Using Droplet-Vitrification." *CryoLetters* 33 (4): 259-270.

Menon, A., **B. Funnekotter**, A. Kaczmarczyk, E. Bunn, S. Turner, and R.L. Mancera. 2014. "Cold-Induced Changes Affect Survival after Exposure to Vitrification Solution During Cryopreservation in the South-West Australian Mediterranean Climate Species Lomandra Sonderi (Asparagaceae)." *Plant Cell, Tissue and Organ Culture (PCTOC)* 119 (2): 347-358.

Reviewer 1	
Comment	**Author's response**
Vacuum infiltration seems to be an alternative approach in cryoprotection of larger and/or hardy (semipermeable) materials, rather than the small and tender samples, like in vitro shoot tips. Also, the effect can be significant only if the samples are not seriously sensitive to the cytotoxicity of cryoprotection with vitrification solutions. Therefore, I should say that the approach/rationale of this study is inappropriate, since the material is small and tender as well as sensitive to cytotoxicity of cryoprotection, though the author did not provide the size of the shoot tips.	While the application of the VIV technique may give a greater difference in larger hardy samples, we feel that this method still provides some benefits for *in vitro* shoots. We have inserted into the methods an indication of the average shoot tip size for each of the three species listed in the 'Conventional droplet-vitrification (CV) cryopreservation technique' methods section (page 2 line 149 & 153).
The authors tried to select the optimum cryoprotection duration using DSC and then did cryopreservation experiments under the conditions defined through DSC. But, as described in INTRODUCTION section, the cryopreservation protocols for these species were already developed. Thus, more direct parallel comparison of both regrowth of –LN, +LN and DSC data is recommended with a narrow range of cryoprotection duration.	Due to time constraints we did not measure specific growth rates of recovering shoots from the different treatments assessed so cannot add this extra information. We have however added some photographs to illustrate regenerating shoot tips as the other reviewer suggested (Figure 1 page 3).
Another issue need to address is the interpretation of the results. For example, the post-cryo regeneration of *L. sonderi*, ~40% of post-cryo regeneration is likely the outcome of the less sensitivity to cytotoxicity during cryoprotection (no crystallization + less toxicity with ~60% rege at 10 min); *L. cinerea*, crystallization + low regeneration of –LN (40%) at 10 min resulted nil post-cryo rege.; *A. perlaria*, high rege of >90% with no crystallization at 10 min produced >70% post-cryo rege	We have added several points in the discussion to reflect this comment (page 9 line 561-579). "This study shows the large variation in cryo-capability commonly seen when applying the same cryopreservation protocol to multiple plant species, comparison of the 10 min PVS2 incubation between the species showed the greatest post-cryogenic survival in *A. perlaria* then *L. sonderi*, both of which also showed good –LN regeneration. Whilst *A. viridis* had the lowest –LN regeneration with the 10 min PVS2 incubation, these three species all showed very little to no ice formation with 10 min PVS2 incubation and thus the post-cryogenic regeneration rates are most likely linked to the plants sensitivity to the CPAs. *Loxocarya cinerea* however showed a similar sensitivity to the CPAs as *A. viridis* and the largest amount of ice

	formation in the shoot tips, thus the low levels of post-cryogenic regeneration for this species is likely due to a combined effect of CPA phyototoxicity and crystallisation damage.”
The half of the four species seems very sensitive to cryoprotection with PVS2 and thus optimized preconditioning of the samples is a critical factor rather than vacuum infiltration.	We generally agree with this comment. Previous work (Menon et al. 2012, 2014) has shown the benefits of preconditioning (with low temperatures) for *L. sonderi* and indeed several of the study species seem to be very sensitive to the cryoprotection chemicals. However, in our study we have demonstrated that similar levels of survival can be attained when using much shorter VIV approaches thus significantly reducing exposure to deleterious cryogenic chemicals.
Fig. 1 seems to be redundant. The DSC data are presented in Table 1. Hence, Fig. 1 can be deleted; otherwise a model diagram of fresh, preculture, osmoprotected, cryoprotected with a range of duration might be enough.	As suggested by both reviewers Figure 1 has been removed.
The manuscript is well written and easy to follow.	We thank the reviewer for this positive feedback.

Reviewer 2

Comment	**Author's response**
This paper describes optimization of cryopreservation protocols using vacuum infiltration for shoot tips of four native species in Western Australia. The use of vacuum is useful for the materials, which have microtopographical features with easy making air bubbles, difficult to penetrate cryoprotectant solutions or/and dehydrate. So the contribution here lies in the demonstration that exposure time to cryoprotective solutions can be shortened by the use of the vacuum with infiltration check by thermal analysis.	We agree with the reviewer’s view of our manuscript that the main contribution of our paper to the cryogenic literature is demonstrating that through adopting VIV approaches exposure times to cryogenic chemicals can be significantly shortened.
It is recommended to authors to add evidences of getting normal shoots (photos).	As suggested by the reviewer we have added several photographs to highlight the healthy growth of recovering shoot tips for each species (Figure 1 page 3).
In the text any repetition should be avoided.	Where identified repeated text has been deleted.

Table 1 and Figure 1 have almost same results, it is better to remove Figure 1. As well as, long sentences should be changed to short, especially using semicolon.	As suggested by both reviewers Figure 1 has been removed.
As well as, long sentences should be changed to short, especially using semicolon	Where possible we have changed long sentences to short ones and removed some semicolons.
Introduction	
Lines 28-57: Paragraph should be transferred to discussion section (at top of discussion). “Cryoprotective agents (CAPs) have two main ----------plant tissues following cryopreservation [5;------23].”	As suggested by the reviewer this paragraph has been moved to the start of the discussion (page 6 line 327 - 356).
Lines 87-96: Add red words “Four native species including two threatened, used to test the new VIV cryopreservation technique, Androcalva perlaria (Malvaceae), Anigozanthos viridis ssp. terrapectans (Haemodoraceae), Lomandra sonderi (Asparagaceae) and Loxocarya cinerea (Restionaceae).”	As suggested by the reviewer this has been done. (page 2 line 85-91)
Remove this sentence. “Cryopreservation protocols exist for these species, two of which are threatened A. viridis ssp. terrapectans and A. perlaria); with post LN survival ranging from 10-80% [7; 17; 28].” If needed, put others.	As suggested by the reviewer this has been done.
Materials and Methods *Plant material and* in vitro *conditions*	
Lines 4-5: Replace “L. sonderi (clone LSOP) and A. perlaria (clone 96)” with “Lomandra sonderi (clone LSOP) and Androcalva perlaria (clone 96)”.	As suggested by the reviewer this has been done. (page 2 line 106)
Lines 20-21: Replace “Androcalva perlaria in vitro shoots” with “In vitro shoots of A. perlaria”.	As suggested by the reviewer this has been done. (page 2 line 122)
CV cryopreservation techniques	
Lines 3-4: “without no exposure to light” Is this usually in the 3 species shoot tips?. If so, indicate Ref.	As suggested by the reviewer references have been added to the section. (page 2 line 152)

Line 12: “transferred to a cryo-vial and plunged into LN.” Is this mean the foil was directly immersed into LN or not? Make clear.	This has been modified to improve clarity as follows: “transferred to a cryo-vial and cryo-vial was plunged into LN” (page 2 line 162)
Lines 13-15: Also, “Cryo-vials were rewarmed after 1 h in a 40°C water-bath for 10 s then incubated in WS for 20 min at room temperature.” Is this mean cryo-vial with the foil was rewarmed in a 40C water bath for 10 s and then the foils were taken out from cryo-vial and moved into WS? Make clear and rewrite in detail.	This has been modified to improve clarity as follows: “Cryo-vials were rewarmed after 1 h in a 40°C water-bath for 10 s then shoot tips on foils were removed from the cryo-vials and incubated in WS for 20 min at room temperature.” (page 3 line 163)
Survival and regeneration assessment post-cryopreservation	
Line 2: “for 4 weeks at 25°C with no light.” Is this incubation normal? Some references?	As suggested by the reviewer several references have been added to the section (page 3 line 185)
Line 5-6: Change “ μ mol m-2 s-1” to “ μ mol m-2s-1”	As suggested by the reviewer this has been corrected. (page 3 line 188)

Results	
Table 1 and Figure 1 have same results. I think Figure 1 is not necessary in this paper. If delate Figure 1, and change other figure numbers.	As suggested by the reviewer Figure 1 has been removed.
Thermal analysis	
Line 5 and elsewhere: Change “Anigozanthos viridis” to “The shoot tips of A. viridis”	As suggested by the reviewer this has been done. (page 3 line 233)
Lines 7-8 and elsewhere: Change “ice peak was detected (Fig 1A); by comparison, no ice peak” to “ice peak was detected (Table 1). By comparison, no ice peak”.	As suggested by the reviewer this has been done. (page 3 line 235)
Lines 10-28: Remove this part and change to “Other three species also showed similar ice peak reductions with increasing incubation time of PVS2 (Table 1).”	This has been partly addressed. We do not wholly agree with the reviewers suggested change but have modified to something similar. (page 3 line 241-252)
Cryopreservation	
Lines 6-10: The sentence “Anigozanthos viridis, -- as previously described.” Should be removed because previously described	As suggested by the reviewer this has been removed.
Lines 19-22 and elsewhere: Change “Anigozanthos viridis showed significantly ---- cryopreservation; however ---.” to “The	We have left this as it is as we see no problem in using semi colons in this context.

shoot tips of A. viridis showed significantly -- -- cryopreservation. However ---."	
Line 30 and elsewhere: Change "Fig 2" to "Fig 1".	This has not been changed as Fig 2 is correct

Discussion	
Line 2; "three of species" is four?	As pointed out by the reviewer this has been addressed. (page 7 line 358)
Line 5 and elsewhere: Change "to the CV technique (Fig 2-5), indicating optimal" to "to the CV technique (Fig 1-4?). Optimal"	The figure numbers are correct but split into two sentences as suggested. (page 7 line 361)
Line 25: "for CPA exposure,," delate ","	As suggested by the reviewer this has been removed.
Line 85: Put "(Table 1)" like "formation peak (Table 1), "	As suggested by the reviewer this has been done. (page 8 line 441)
Line 98: Put "(Fig 1)" like "5 min for VIV (Fig 1).	As suggested by the reviewer this has been done. (page 8 line 454)
Line 153: Put "(Fig 3)" like "tissues (Fig 3). "	As suggested by the reviewer this has been done. (page 9 line 510)
Line 173: Put "(Table 1)" like "the CV technique (Table 1)."	As suggested by the reviewer this has been done. (page 10 line 589)
Line 180: Change "Androcalva perlaria showed high" to "This result on A. perlaria (Fig 4) showed high".	As suggested by the reviewer this has been done. (page 9 line 537)

Permissions

Re: your MS 14_73 Copyright permission for doctoral thesis

Florent ENGELMANN [florent.engelmann@ird.fr]
Sent:02 February 2015 15:30
To: Bryn Funnekotter
Cc: Eric Bunn

Dear Bryn,

There is no problem for you to include this MS in your PhD thesis.

Kind regards.

Florent

Florent Engelmann
Associate Editor, CryoLetters
IRD
911 avenue Agropolis
BP 64501
34394 Montpellier cedex 5
France
tel: +33 (0)4 67 41 63 80
fax: +33 (0)4 67 41 62 52
email: florent.engelmann@ird.fr

http://www.cryoletters.org/
CryoLetters publishes original research reports, authoritative reviews, technical developments and commissioned book reviews of studies of the effects produced by low temperatures on a wide variety of scientific and technical processes, or those involving low temperature techniques in the investigation of physical, chemical, biological and ecological problems.

Le 01/02/15 05:24, « Bryn Funnekotter » <Bryn.Funnekotter@bgpa.wa.gov.au> a écrit :

>Dear Florent,
>
>I would like to include this manuscript (Evaluation of the new vacuum
>infiltration vitrification (VIV) cryopreservation technique for native
>Australian plant shoot tips) in my PhD thesis/dissertation I am currently
>undertaking at Curtin University
>
>Thesis title: The Role of Oxidative Stress in Successful Cryopreservation
>of South-west Western Australian Plant Species
>
>Full acknowledgement of the ownership of the copyright and the source of
>the material will be provided with the material. I would be willing to
>use a specific form of acknowledgement that you may require and to
>communicate any conditions relating to its use.
>
>If you are not the copyright owner of the material in question, I would
>be grateful for any information you can provide as to who is likely to
>hold the copyright. I look forward to hearing from you and thank you in
>advance for your consideration of my request.
>
>Yours sincerely
>
>Bryn Funnekotter
>

>
>
>On 15/01/15 3:47 PM, "Florent ENGELMANN" <florent.engelmann@ird.fr> wrote:
>
>>Dear Eric,
>>
>>I am pleased to inform you that your MS is now accepted for publication
>>in
>>CryoLetters. It has been sent to the Editorial Office for processing
>>before publication.
>>
>>We hope to receive additional MSs from your group in a not too distant
>>future.
>>
>>Kind regards.
>>
>>Florent
>>-------
>>Florent Engelmann
>>Associate Editor, CryoLetters
>>IRD
>>911 avenue Agropolis
>>BP 64501
>>34394 Montpellier cedex 5
>>France
>>tel: +33 (0)4 67 41 63 80
>>fax: +33 (0)4 67 41 62 52
>>email: florent.engelmann@ird.fr
>>
>>
>>http://www.cryoletters.org/
>>CryoLetters publishes original research reports, authoritative reviews,
>>technical developments and commissioned book reviews of studies of the
>>effects produced by low temperatures on a wide variety of scientific and
>>technical processes, or those involving low temperature techniques in the
>>investigation of physical, chemical, biological and ecological problems.
>>
>>
>>
>>
>>Le 08/01/15 11:41, « Eric Bunn » <Eric.Bunn@bgpa.wa.gov.au> a écrit :
>>
>>>Dear Florent
>>>
>>>Please find attached the revised manuscript MS 14-73 and our response to
>>>the reviewer's comments.
>>>
>>>I trust that we have satisfactorily amended the manuscript as required
>>>and
>>>this version is acceptable for CryoLetters.
>>>
>>>Regards
>>>Eric Bunn (Corresponding Author)
>>>
>>>Dr Eric Bunn (Snr Research Scientist)
>>>Botanic Gardens and Parks Authority
>>>(Kings Park and Botanic Garden)
>>>West Perth, Western Australia 6005
>>>++61 8 9480 3647;   Fax: ++61 8 9480 3641
>>>Email: ebunn@bgpa.wa.gov.au
>>>
>>>
>>>
>>>
>>>

>>>
>>>On 19/12/14 2:56 PM, "Florent ENGELMANN" <florent.engelmann@ird.fr>
>>>wrote:
>>>
>>>>Dear Eric,
>>>>
>>>>I have now received the referees' reports on your MS 14-73. I am
>>>>pleased
>>>>to inform you that your MS can be accepted for publication after
>>>>revision.
>>>>
>>>>Please kindly send me within one month a revised version incorporating
>>>>the
>>>>referees' comments along with a letter detailing how these
>>>>modifications
>>>>have been incorporated in the revised MS. Many thanks in advance.
>>>>
>>>>I look forward to hearing back from you very soon.
>>>>
>>>>Kind regards.
>>>>
>>>>Florent Engelmann
>>>>-------
>>>>Florent Engelmann
>>>>Associate Editor, CryoLetters
>>>>IRD
>>>>911 avenue Agropolis
>>>>BP 64501
>>>>34394 Montpellier cedex 5
>>>>France
>>>>tel: +33 (0)4 67 41 63 80
>>>>fax: +33 (0)4 67 41 62 52
>>>>email: florent.engelmann@ird.fr
>>>>
>>>>
>>>>http://www.cryoletters.org/
>>>>CryoLetters publishes original research reports, authoritative reviews,
>>>>technical developments and commissioned book reviews of studies of the
>>>>effects produced by low temperatures on a wide variety of scientific
>>>>and
>>>>technical processes, or those involving low temperature techniques in
>>>>the
>>>>investigation of physical, chemical, biological and ecological
>>>>problems.
>>>>
>>>>
>>>>
>>>>
>>>>
>>>>
>>>>
>>>
>>>
>>>
>>>
>>>
>>>
>>>
>>
>>
>>
>
>

Dear Bryn,

Thank you for your request. You have our permission to use the OUP Material you list in your email below in your thesis for submission to Curtin University.

If at some future date your thesis is published it will be necessary to re-clear this permission. Please also note that if the material to be used is acknowledged to any other source, you will need to clear permission with the rights holder.

Kind regards,
- removed -

IF THIS PERMISSION IS INCLUDED IN ANY THESIS/DISSERTATION MADE AVAILABLE ONLINE PLEASE REMOVE MY NAME AND CONTACT INFORMATION. THANK YOU.

From: no.reply@oup.com [mailto:no.reply@oup.com]
Sent: 25 January 2015 10:25
To: Academic Permissions
Subject: Academic Permissions Request Form

0_0_URL	/uk/academic/rights/permissions/request
A_Z_firstname	Bryn
A_Z_lastname	Funnekotter
B_Z_Company	Curtin University
C_Z_Address	Kent St Bentley WA
C_Z_zip	6102
D_Z_PhoneNo	
D_Z_country	Australia
E_Z_FaxNo	
F_Z_Email	bryn.funnekotter@postgrad.curtin.edu.au
F_Z_VATnumber	

G_Z_TheirTitle	The Role of Oxidative Stress in Successful Cryopreservation of South-west Western Australian Plant Species.
H_Z_Author	Bryn Funnekotter
H_Z_Publisher	Curtin University
I_Z_Covers	Unspecified
I_Z_PrintRunHard	1
I_Z_pubDate	February 2015
J_Z_Territory	Australia
K_Z_Language	English
K_Z_Notes	
L_Z_Media1	illustration
M_Z_Author1	Barry Halliwell and John M. C. Gutteridge
M_Z_Title1	Free Radicals in Biology and Medicine (Fourth Edition)
M_Z_editedby1	Barry Halliwell and John M. C. Gutteridge
N_Z_Material1	Table 1.3. Reactive oxygen species Page 23
O_Z_ISBN1	978-0-19-856869-8
O_Z_OUPpubDate1	08 March 2007
P_Z_Media2	Text
Q_Z_Author2	
Q_Z_Title2	
Q_Z_editedby2	
R_Z_Material2	
S_Z_ISBN2	
S_Z_OUPpubDate2	
T_Z_Media3	Text
U_Z_Author3	
U_Z_Title3	
U_Z_editedby3	
V_Z_ISBN3	
V_Z_Material3	
V_Z_OUPpubDate3	
W_Z_Additional	Type Of Use: Thesis/Dissertation

subject	
view	submitform

SPRINGER LICENSE
TERMS AND CONDITIONS

Jan 25, 2015

This is a License Agreement between Bryn Funnekotter ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3555830869891
License date	Jan 25, 2015
Licensed content publisher	Springer
Licensed content publication	Springer eBook
Licensed content title	Free Radicals and Antioxidants for Non-Experts
Licensed content author	Zdeňka Ďuračková
Licensed content date	Jan 1, 2014
Type of Use	Thesis/Dissertation
Portion	Figures
Author of this Springer article	No
Order reference number	None
Original figure numbers	Fig 1.1
Title of your thesis / dissertation	The Role of Oxidative Stress in Successful Cryopreservation of South-west Western Australian Plant Species.
Expected completion date	Feb 2015
Estimated size(pages)	150
Total	0.00 USD
Terms and Conditions	

Introduction
The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License
With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the

university's intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis limited to university-use only. If the thesis is going to be published, permission needs to be re-obtained (selecting "book/textbook" as the type of use).

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, subject to a courtesy information to the author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice:Disclaimer
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: None

Example 1: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Example 2: Springer Science + Business Media makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity
You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License

This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in The Netherlands, in accordance with Dutch law, and to be conducted under the Rules of the 'Netherlands Arbitrage Instituut' (Netherlands Institute of Arbitration).***OR:***

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other terms and conditions:

v1.3

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

Rights and permissions

Rights

For terms and conditions for online usage of journals published by Portland Press Ltd, please visit the following websites:

- Biochemical Journal
- Biochemical Society Transactions
- Clinical Science
- Essays in Biochemistry
- Biochemical Society Symposia
- Bioscience Reports
- Cell Signalling Biology

Licensing enquiries

Permissions

The guidelines below refer to articles published in the following journals, published by Portland Press Limited:

Biochemical Journal	*Cell Signalling Biology*
Biochemical Society Symposia	*Clinical Science*
Biochemical Society Transactions	*Essays in Biochemistry*
Bioscience Reports	*The Biochemist*

Authors of articles published in the above journals

Authors do NOT usually need to contact Portland Press Limited to request permission to reuse their own material, as long as the material is properly credited to the original publication. It is usual to provide the citation of the original publication thus:

"This research was originally published in Journal Name. Author(s), Title. Journal Name. Year; Volume: pp-pp © copyright holder"

The copyright holder for each publication is as follows:

© the Biochemical Society	**© Portland Press Limited**
Biochemical Journal	*Cell Signalling Biology*
Biochemical Society Symposia	
Biochemical Society Transactions	
Bioscience Reports	
Clinical Science	
Essays in Biochemistry	
The Biochemist	

Provided the original publication of the article, or portion of the article, is properly cited, Authors retain the following non-exclusive rights:

1. To reproduce their article in whole or in part in any printed volume (book or thesis) of which they are the Author or Editor
2. To reproduce their article in whole or in part at the Author's current academic institution for teaching purposes
3. To reuse figures, tables, illustrations or photos from the article in commercial or non-commercial works created by them
4. To post a copy of the Immediate Publication (i.e. the Accepted Manuscript*) at the Author's Institutional Repository, 6 months after publication, provided that this is linked to the article on the journal website (e.g. through the DOI).

In addition, authors of Opt2Pay articles, or authors of articles published in our open access journal *Bioscience Reports*, may post the Version of Record** to their institutional repository. Portland Press Limited will deposit the Version of Record in PubMed Central on behalf of the author, where applicable.

Quick search

alerting
RSS Feeds
Table of contents by email
to subscribe
terms and conditions
latest despatch dates
licensing
offprints
how to claim
journal usage statistics
subscriber help/services
library recommendation form
2015 journal prices
Essential Cellular and Molecular Life Sciences Collection 2015

HINARI ACCESS TO RESEARCH

Requests for non-commercial use

Other parties wishing to use reuse an article in whole or in part for educational purposes (e.g. in lectures or tutorials) may do so at no cost providing the original source is attributed, as outlined above.

You may also reproduce an article in whole or in part in your thesis at no cost providing the original source is attributed.

If you wish to copy and distribute an article in whole for teaching (e.g. in a course pack), please contact your librarian who will advise you on the various clearance options available.

STM Permission Guidelines

Portland Press Limited is a signatory of the STM Permission Guidelines, which aims to reduce the administration involved in clearing permissions.

Publishers who are also signatories to the STM Permission Guidelines may re-use work published in Portland Press Limited journals, without contacting us, as long as the work is appropriately used and attributed as stated in the Guidelines.

All other usage requests, including Books

For all other requests please download and complete the permission request form and send by email or fax to:

(e) permissions@portlandpress.com
(f) +44 (0) 20 7685 2469

---

*Accepted Manuscript – (also known as Immediate Publication) - the version of the article that has been accepted for publication and includes the revisions made following Peer Review.

**Version of Record - the version of the article, after processes such as copyediting, proof corrections, layout and typesetting have been applied.

Please see the NISO/ALPSP guidelines for a full description of these terms.

Sales enquiries
Portland Customer Services
Charles Darwin House
12 Roger Street
London
WC1N 2JU
United Kingdom
Tel: +44 (0)20 7685 2444
Fax: +44 (0)20 7685 2468
E-mail: sales@portland-services.com
Map

Editorial enquiries
Portland Press Ltd.
Third floor
Charles Darwin House
12 Roger Street
London WC1N 2JU
Tel: +44(0) 20 7685 2410
Fax: +44(0) 20 7685 2469
E-mail: editorial@portlandpress.com

PNAS

About PNAS (/site/aboutpnas/index.xhtml#PNAS_Online)

Free Content (/site/aboutpnas/index.xhtml#free)

Special Features (/site/aboutpnas/special.xhtml)

Colloquium Papers (/site/aboutpnas/colloquia.xhtml)

Developing Countries With Free Access to PNAS Online (/site/aboutpnas/developingcountries.xhtml)

2015 Marketing Brochure (/site/misc/2015 Brochure.pdf)

RSS Feeds (/site/aboutpnas/rss.xhtml)

About Direct Submission (/site/aboutpnas/submiss.xhtml)

Reprints (/site/aboutpnas/reprints.xhtml)

Rights and Permissions (/site/aboutpnas/rightperm.xhtml)

Author Rights and Permissions Frequently Asked Questions (/site/aboutpnas/rightpermfaq.xhtml)

Frequently Asked Questions (/site/aboutpnas/faq.xhtml)

PNAS Portals (/site/misc/pnas_portals.xhtml)

PNAS Mobile (/site/misc/pnas_mobile.xhtml)

Android App Permissions (/site/misc/androidfaq.xhtml)

PNAS Alerts (/site/misc/PNASalerts.xhtml)

Editorial Board (/site/misc/masthead.xhtml)

News & Multimedia (/multimedia)

Subscriptions (/site/subscriptions/index.xhtml)

Contact (/site/misc/contact.xhtml)

Rights and Permissions

Beginning with articles submitted in Volume 106 (2009) the author(s) retains copyright to individual articles, and the National Academy of Sciences of the United States of America retains an exclusive license to publish these articles and holds copyright to the collective work. Volumes 90–105 (1993–2008) copyright © by the National Academy of Sciences. Volumes 1–89 (1915–1992), the author(s) retains copyright to individual articles, and the National Academy of Sciences holds copyright to the collective work.

The PNAS listing on the Sherpa RoMEO publisher copyright policies & self-archiving detail pages can be found here (http://www.sherpa.ac.uk/romeo/search.php?id=94&la=en&fIDnum=|).

Requests for Permission to Reprint

Requests for permission should be made in writing. For the fastest response time, please send your request via email to

PNASPermissions{at}nas.edu

. If necessary, requests may be faxed to 202-334-2739 or mailed to:

PNAS Permissions Editor
500 Fifth Street, NW
NAS 340
Washington, DC 20001 USA

Anyone may, without requesting permission, use original figures or tables published in PNAS for noncommercial and educational use (i.e., in a review article, in a book that is not for sale) provided that the original source and the applicable copyright notice are cited.

For permission to reprint material in volumes 1–89 (1915–1992), requests should be addressed to the original authors, who hold the copyright. The full journal reference must be cited.

For permission to reprint material in volumes 90–present (1993–present), requests must be sent via email, fax, or mail and include the following information about the original material:

1. Your full name, affiliation, and title
2. Your complete mailing address, phone number, fax number, and email address
3. PNAS volume number, issue number, and issue date
4. PNAS article title
5. PNAS authors' names
6. Page numbers of items to be reprinted
7. Figure/table number or portion of text to be reprinted

Also include the following information about the intended use of the material:

1. Title of work in which PNAS material will appear
2. Authors/editors of work
3. Publisher of work
4. Retail price of work
5. Number of copies of work to be produced
6. Intended audience
7. Whether work is for nonprofit or commercial use

PNAS authors need not obtain permission for the following cases: (1) to use their original figures or tables in their future works; (2) to make copies of their papers for their own personal use, including classroom use, or for the personal use of colleagues, provided those copies are not for sale and are not distributed in a systematic way; (3) to include their papers as part of their dissertations; or

(/content/current)
Current Issue (/content/current)
Email Alerts (/site/misc/PNASalerts.xhtml)
Subscribe (/site/subscriptions/index.xhtml)
RSS (/site/aboutpnas/rss.xhtml)

Don't Miss

Lens view takes advantage of the dynamic nature of the web; use it to explore PNAS articles in a new way. (/site/aboutpnas/index.xhtml#PNAS_Online)

Other PNAS Media

Image Gallery (/site/media/imagegallery.xhtml)
Video Library (/site/media/videolibrary.xhtml)
Follow Us on Twitter (https://twitter.com/PNASNews)
Find Us on Facebook (http://www.facebook.com/pages/PNAS/182E

MOST READ **MOST CITED**

1. News Feature: Animals that self-medicate (/cgi/content/short/111/49/17339?rss=1&ssource=mfr)
2. Experimental evidence of massive-scale emotional contagion through social networks (/cgi/content/short/111/24/8788?rss=1&ssource=mfr)
3. Links that speak: The global language network and its association with global fame (/cgi/content/short/111/52/E5616?rss=1&ssource=mfr)
4. Extraordinarily high spider densities on islands: flow of energy from the marine to terrestrial food webs and the absence of predation. (/cgi/content/short/92/10/4382?rss=1&ssource=mfr)
5. Evening use of light-emitting eReaders negatively affects sleep, circadian timing, and next-morning alertness (/cgi/content/short/1418490112v1?rss=1&ssource=mfr)

50 Most-Read Articles » (/reports/most-read)

1. DNA sequencing with chain-terminating inhibitors (/cgi/content/short/74/12/5463?rss=1&ssource=mfc)

(4) to use all or part of their articles in printed compilations of their own works. Citation of the original source must be included and copies must include the applicable copyright notice of the original report.

Authors whose work will be reused should be notified. PNAS cannot supply original artwork. Use of PNAS material must not imply any endorsement by PNAS or the National Academy of Sciences. The full journal reference must be cited and, for articles published in Volumes 90–105 (1993–2008), "Copyright (copyright year) National Academy of Sciences, USA."

Requests for Permission to Photocopy

For permission to photocopy beyond that permitted by Section 107 or 108 of the US Copyright Law, contact:

Copyright Clearance Center (http://www.copyright.com)
222 Rosewood Drive
Danvers, MA 01923 USA
Phone: 1-978-750-8400
Fax: 1-978-750-4770
Email:

info{at}copyright.com

Authorization to photocopy items for the internal or personal use of specific clients is granted by The National Academy of Sciences provided that the proper fee is paid directly to CCC.

[12/14]

2. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. (/cgi/content/short/76/9/4350?rss=1&ssource=mfc)
3. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products (/cgi/content/short/97/12/6640?rss=1&ssource=mfc)
4. Cluster analysis and display of genome-wide expression patterns (/cgi/content/short/95/25/14863?rss=1&ssource=mfc)
5. Significance analysis of microarrays applied to the ionizing radiation response (/cgi/content/short/98/9/5116?rss=1&ssource=mfc)

50 Most-Cited Articles »
(/reports/most-cited)

Re: Copyright permission to use Figure

Sasa Marcan [permissions@intechopen.com]
Sent:10 December 2014 20:35
To: Bryn Funnekotter

Hi Bryn,

Thank you for your inquiry.

You may freely reuse and modify the specified figure as long as you cite the source chapter and include the following notice in the figure caption:

Adapted from [short citation]; originally published under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/32860

Please feel free to contact me if I can be of any additional assistance regarding this matter.

Kind regards,
Sasa

--
Sasa Marcan
Library and Information Service Coordinator
_____
InTech - open science | open minds
Email: marcan@intechopen.com
Website: http://www.intechopen.com
Phone: +385 51 688 994
Fax: +385 51 686 166

Journal Department
Janeza Trdine 9
51000 Rijeka, Croatia

On 10/12/14 05:42, Bryn Funnekotter wrote:
> Hello,
>
> I am looking to get copyright permission to use a figure from the following book chapter
>
> Anja Kaczmarczyk, Bryn Funnekotter, Akshay Menon, Pui Ye Phang, Arwa Al-Hanbali, Eric Bunn and Ricardo L. Mancera (2012). Current Issues in Plant Cryopreservation, Current Frontiers in Cryobiology, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0191-8, InTech, DOI: 10.5772/32860. Available from: http://www.intechopen.com/books/current-frontiers-in-cryobiology/current-issues-in-plant-cryopreservation
>
> I will be looking to submit a modified version of Figure 1. Cryopreservation procedure for the droplet vitrification method (page 420)
>
> Best Regards,
> Bryn Funnekotter