

**UNDERSTANDING THE SIGNIFICANCE OF PRE-TREATMENT IN
THE POST-CRYOPRESERVATION SURVIVAL OF KIWIFRUIT
SHOOT TIPS THROUGH BIOCHEMICAL AND
ULTRASTRUCTURAL STUDIES**

A thesis submitted for the degree of

MASTER OF SCIENCE

in

PLANT BIOTECHNOLOGY

At University of Otago, Dunedin, New Zealand

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JUNE 2015

ABSTRACT

The number of vegetatively propagated species conserved by cryopreservation is increasing thanks to the development of vitrification-based methods in recent years. Although the most economically important horticultural crop in New Zealand is kiwifruit (*Actinidia* spp.), there is currently no cryopreserved collection of its genetic resources. The field collections of kiwifruit are limited to a few countries and are at a high risk of genetic erosion due to diseases such as *Pseudomonas syringae* pv. *actinidiae*. An alternative method of *ex situ* conservation is through *in vitro* collections but these are prone to contamination and somaclonal variation, and require maintenance through subculture. Cryopreservation is considered the only method available for the long-term preservation of clonally propagated species while maintaining their genetic fidelity. As cryopreservation outcomes can be species and genotype-specific, understanding biochemical and cell ultrastructural changes during cryopreservation is important for the successful development of protocols for the establishment of a kiwifruit germplasm collection in cryo-storage. Among different vitrification-based cryopreservation techniques, droplet vitrification is becoming the preferred method for many species, as it ensures fast freezing and thawing rates that are vital for vitrification of tissue, but it has not been tested in kiwifruit yet. This research aimed to develop a droplet vitrification protocol for kiwifruit using *A. chinensis* ‘Hort16A’ as a model, by optimising different pre-treatments to enhance the survival after cryopreservation and assessing the levels of enzymatic and non-enzymatic oxidative markers as well as quantification of oxidative damage and ultrastructural changes. Cold acclimation of donor plants at 4 °C for 2 weeks followed by daily increasing sucrose preculture (0.25, 0.5, 0.75 and 1.0 M) of 1-2 mm shoot tips and supplementation of ascorbic acid (0.4 mM) in all media throughout the procedure registered 40 % regeneration after cryopreservation against 0 % regeneration in shoot tips without any pre-treatment. One-hour treatment of shoot tips in a modified vitrification solution at 0 °C is another important step in the protocol. All the antioxidant markers showed a significant increase in pretreated shoot tips compared to non-pretreated shoot tips throughout the steps, especially 24 h after recovery from cryopreservation. Oxidative damage markers, lipid peroxides and protein carbonyls were significantly lower in pretreated shoot tips than in non-pretreated shoot tips after cryopreservation, suggesting better protection as a result of pre-treatment and ascorbic acid supplementation. Transmission electron microscopic images of the ultrastructure after cryopreservation showed damage in cell organelles of all cells of non-pretreated shoot tips

whereas, some cells in pretreated shoot tips had structural integrity confirming the results of oxidative marker assays and recovery after cryopreservation. These observations show that pre-treatment is necessary to ensure survival after droplet vitrification of *Actinidia* species. Testing the applicability of this protocol to other *A. chinensis* genotypes and then to other *Actinidia* species, and further research to improve it would pave the way for the establishment of a cryopreserved *Actinidia* collection.

ACKNOWLEDGEMENT

First and foremost I submit myself and my work to the Lord Almighty who is indeed the cornerstone of all my accomplishments. All the glory and honour belongs to Him.

I would like to express my utmost gratitude to my beloved supervisors, Dr David J Burritt and Dr Ranjith Pathirana for their constant support, help, encouragement, patience, dedication and guidance throughout my thesis. You both always motivated me by pointing out the positives in my work, even though I may have had many drawbacks. I take this opportunity to tell that I'm very lucky to get both of you as my supervisors. Dr Andrew McLachlan, I am extremely grateful for your advice and suggestions in statistics.

I would like to thank University of Otago and the Department of Botany to accept my request for this course and gave me the opportunity to be a part of this reputed University. I would like to thank Plant and Food Research and its management for permitting me to undertake this project at this prestigious institute and providing me all the access to its facilities without any limitations.

I would like to express my heartfelt gratitude to the staff members at Palmerston north site of Plant and Food Research especially, Ed Morgan, John Seelye, Keith Funnell, Andrew Mullan, Belinda, Darren, Lei, Zoe and Carol for all your support given for me whenever I asked. Many thanks to all the lab members of plant tissue culture lab for their friendly and positive attitude that had a positive influence on me. Sriya, your friendship, support and encouragement helped me a lot in surpassing the difficulties I faced during this period. I would like to express my sincere thanks to Allan and Gillian, Microscopy Otago for your assistance in transmission electron microscopy that helped a lot in my thesis. I would like to express my thanks to all staff in the Department of Botany, University of Otago for their assistance and support. Special thanks to Sasha for being my good friend and encouraging me. I would also like to thank Shiji and family for their love and support, and for providing me a wonderful homestay during my course.

The strength in me behind all my success is my family. With a deep sense of indebtedness, I thank my husband who sacrificed all his likes to care, support and encourage me. Dad, I know you are blessing me from heaven and make my life smooth always. I express my thanks to my mother, brother and in-laws for their love, support and prayers. No words are sufficient to thank my loving and pretty daughter, your love has been driving me to the success of this project and I dedicate all the credits of my work to you.

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ABBREVIATION

ABA	– abscisic acid
ANOVA	– analysis of variance
APX	– ascorbate peroxidase
ASA	– ascorbic acid
BM	– basal medium
CA	– cold acclimation
CAT	– catalase
CPA	– cryoprotective agents
DHA	– dehydroascorbate
DHAR	– dehydroascorbate reductase
DMSO	– dimethyl sulfoxide
DNPH	– dinitrophenylhydrazine
DV	– droplet vitrification
ED	– encapsulation dehydration
EV	– encapsulation vitrification
GLN	– generalised linear model
GPX	– glutathione peroxidase
GR	– glutathione reductase
GSH	– glutathione (reduced)
GSSG	– glutathione disulphide
LN	– liquid nitrogen
LPs	– lipid peroxides
MDA	– malondialdehyde
MDHAR	– monodehydroascorbate reductase
mPVS2	– modified plant vitrification solution 2
PCs	– protein carbonyls

PEG	– polyethylene glycol
PVS	– plant vitrification solution
RECP	– recovery plate
RECS	– recovery solution
ROS	– reactive oxygen species
SOD	– superoxide dismutase
TCA	– trichoroacetic acid
TEM	– transmission electron microscopy
UA	– uranyl acetate

1 GENERAL INTRODUCTION

1.1 CROP GERMPLASM AND ITS CONSERVATION

Conservation of crop plant genetic resources ensures maintenance of agro-biodiversity (Benito et al. 2004). There are two approaches for conservation of plant genetic resources, namely *in situ* and *ex situ* conservation. *In situ* conservation involves maintaining genetic resources in the natural habitats where they occur. *Ex situ* conservation on the other hand, involves conservation outside the native habitat in gene banks, this is generally used to safeguard populations that are in danger of destruction, replacement or deterioration, and thus many countries have established different types of gene banks for the conservation of plants (Rao 2004).

1.1.1 GENE BANKS

Field gene banks: In field gene banks, the plant genetic resources are maintained as live plants that undergo continuous growth requiring continuous maintenance. Field gene banks are therefore generally expensive to maintain, requiring labour, inputs in the form of agrochemicals, irrigation, labour etc. They occupy valuable field space as well. They also have a higher level of risk from natural disasters and adverse environmental conditions, such as drought, flood and, fire, and from pest and disease outbreaks (CGKB 2014; Höfer 2015; Karun et al. 2014; Panis & Lambardi 2005).

Vegetative gene banks: Vegetative propagules of tuber and root crops can be conserved for several months between one harvest and the next planting season under optimal temperature conditions. This method can be applied only on limited species and in addition, it cannot be used for long-term storage, and the propagules are sensitive to pathogen and pest attack (CGKB 2014; Niino & Arizaga 2015).

Seed banks: It is the preservation of seeds under controlled environmental conditions that maintain seed viability for long periods. Storing genetic diversity in the form of seeds is very effective, most widely used and is the most convenient method of *ex-situ* conservation (Pritchard et al. 2014; Rao 2004). However, it is difficult or impossible to store recalcitrant seeds and seeds that are very small in nature (Niino & Arizaga 2015; Pritchard et al. 2014). It is not applicable to crops that do not produce seed (e.g. bananas) and the plant species that are

propagated vegetatively to preserve the unique genomic constitution of cultivars, such as several timber, fruit and ornamental species (Panis & Lambardi 2005; Wang et al. 1993).

Pollen banks: The ease of pollen storage and shipment internationally without any disease transfer and the potential for its immediate use help researchers in their breeding programs. The primary limitation of pollen storage within gene banks is that only half the genome is conserved in this method, requiring female flowers for conventional propagation. Also, it is difficult to obtain adequate quantities of pollen in many species (Thormann et al. 2006; Wang et al. 2012), and the collection, processing enough pollen for routine storage and distribution are labour intensive. Tri-cellular pollen storage is extremely difficult. Regeneration of aged pollen is another limiting factor; unlike seeds, associated mother plants are necessary to replenish pollen supplies when quantities are depleted or have deteriorated (CGKB 2014; Wang et al. 1993).

DNA banks: DNA, RNA, cDNA and genes are routinely extracted from the nuclei, mitochondria and chloroplasts of plants and are stored in DNA gene banks. However, use of DNA banks in conservation is limited as there is no technique currently available or designed yet to regenerate whole plants from the stored DNA and these banks are now considered as a means of complimentary conservation (Andreas et al. 2006).

In vitro banks: *In vitro* conservation involves maintenance of explants in a sterile, pathogen-free environment, which is ideal for clonal crop germplasm and multiplication of species that produce recalcitrant seeds, or do not produce seeds. It is however labour intensive; regular subculture is needed and also has the high risk of losing of material due to contamination of cultures, human error or somaclonal variation (CGKB 2014; Panis & Lambardi 2005). Genetic variation or mutation can also be induced during prolonged subculture (Niino & Arizaga 2015).

All these approaches have limitations regarding efficiency, costs, labour, space, security and long-term maintenance (Kami 2012).

1.1.2 CRYO-BANKING

Cryo-banking utilises cryopreservation for long-term preservation of structurally intact living cells and tissues (Pegg 2007) at -196 °C in liquid nitrogen (LN). Cryopreservation has been proven to be an ideal method of storing germplasm, especially of vegetatively

propagated and recalcitrant seed producing plants for the long-term, utilizing minimum space, labour and maintenance and also preserves genetic stability (Niino et al. 2000; Sakai & Nishiyama 1978; Taylor et al. 2000; Yamamoto et al. 2011). However, the cryopreservation of whole recalcitrant seed is difficult to perform, hence shoot apices or embryos of plants are preferred explants (Pritchard et al. 2014). There are several cryobanks across the world for preserving plant germplasm; some are listed in table 1.1.

Table 1.1. Cryo-banks in different parts of the world for preserving plant germplasm.

Cryo-bank name	Species (No. of accessions)	Reference
National Agrobiodiversity Centre, Rural Development Administration, Rep. Korea	Seeds/shoot tips of Potato, lily, garlic and Korean-ginseng	Kim et al. (2012) RDA-Genebank (2014), Engelmann et al. (2014) Niino and Arizaga (2015)
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany	Potato (1403), mint (97), <i>Allium</i> (112), yam and medicinal/aromatic plants	IPK (2014), Benson et al. (2011)
Dresden-Pillnitz Institute, Germany	<i>Fragaria</i> (23) and <i>Malus</i> (77)	Hoefer and Reed (2010) Höfer (2015)
Crop Research Institute, Czech Republic	Potato, grape, <i>Humulus</i> , <i>Allium</i> and fruit crops	Frese et al. (2013)
International Potato Centre (CIP), Peru	Potato (>755)	Panta et al. (2014b)
The International Network for the Improvement of Banana and Plantain (INIBAP), Belgium	<i>Musa</i> (882)	CGKB (2014)
Kings Park and Botanic Garden, Perth, Australia	Seeds from more than 40 % of rare and endangered species	Touchell and Dixon (1994)
Cincinnati Zoo and Botanical Garden, USA	Endangered local species	Engelmann et al. (2014)
National Bureau of Plant genetic Recourses (NBPGR), New Delhi, India	>9915 accessions of 729 species such as seeds, embryonic axes, dormant buds or shoot tips of <i>Allium sativum</i> , <i>Citrus</i> , <i>Dioscorea</i> , <i>Musa</i> , <i>Rubus</i> , <i>Prunus</i> , <i>Capparis</i> , <i>Buchanania lanzan</i> , <i>Manilkara hexandra</i> , <i>Piper nigrum</i> , <i>Juglans regia</i> , <i>Jatropha curcas</i> , <i>Pongamia pinnata</i> etc.	NBPGR (2014) Niino and Arizaga (2015)

Center for Research and Higher Learning in Tropical Agriculture (CATIE), Costa Rica	<i>Coffea</i> (80)	CGIAR (2009), Engelmann et al. (2014)
National Center for Genetic Resources Preservation (NCGRP), Fort Collins, Colorado, USA and National Clonal Germplasm Repository (NCGR), Corvallis, USA	Potato, sweet potato, lettuce, mint, garlic, wheat, tobacco, banana, apple, willow, <i>Pyrus</i> , <i>Prunus</i> , <i>Humulus</i> , <i>Fragaria</i> , <i>Rubus</i> and seeds.	Hummer and Postman (2005), USDA-ARS (2014) Niino and Arizaga (2015)
Association Floret Cellulose (AFOCEL), Nangis, France	Elm (>440)	Altman and Hasegawa (2012), Engelmann et al. (2014)
National Institute of Agrobiological Sciences (NIAS), Japan	Mulberry (>1000), <i>Juncus</i> (50)	Engelmann et al. (2014) Niino and Arizaga (2015)
National Institute of Agronomic Research (INRA), Guadeloupe, France	Yam, grape	Gallet et al. (2007), Engelmann et al. (2014)
International Center for Tropical Agriculture (CIAT), Cali, Colombia	<i>Cassava</i> (>600)	CGKB (2014)
Institute of Research for Development (IRD), France	<i>Coffea</i> (>500)	Engelmann et al. (2014) Niino and Arizaga (2015)
Shimane Agriculture Research Centre(SARC), Japan	Wasabi (40)	Niino and Arizaga (2015)

1.2 CRYOPRESERVATION AS THE METHOD OF CHOICE FOR VEGETATIVELY PROPAGATED SPECIES

Cryopreservation is becoming more widely used method of choice for ensuring cost-effective and safe, long-term storage of genetic resources (Benson 2008; Engelmann 2004) especially for the preservation of vegetatively propagated crops (Hirai 2001). Considering the need of maintenance of the original genotype, the maintenance of genetic diversity of vegetatively propagated species is more demanding than seed producing plants (Höfer 2015). The ultra-low temperatures used during cryopreservation virtually stop all the metabolic and biochemical activities of cells and therefore extends their longevity during long-term storage without any genetic variation and metabolically induced deterioration (Engelmann 2004; Sakai et al. 1990). In addition, the material is protected from contamination and very little maintenance is needed. Moreover, the method relies on LN stored in cryo tanks, which is not dependent on refrigeration or a constant electricity supply. It takes limited space and there is no need for regular regeneration of plant material (CGKB 2014).

Considering these advantages, cryopreservation is the only viable procedure for long-term conservation of vegetatively propagated species (Benito et al. 2004; Clavero-Ramirez et al. 2005) like kiwifruit. The development in the cryopreservation methods significantly favours the preservation of the germplasm of vegetatively propagated species than recalcitrant seed producing species (Engelmann 2004).

Cryopreservation of plant cells, meristems and organs has become an important tool and is the only technique currently available to ensure safe and cost efficient long-term preservation of plant germplasm including non-orthodox seed species, vegetatively propagated plants, rare and endangered species and biotechnology products (Engelmann 2011). The successful plant cryopreservation begun in late 1950s with Sakai (1960) publishing the first successful report, when hardy woody twigs of mulberry survived after immersing in LN. Complete survival was observed when cortical tissue sections from winter twigs of mulberry tree were immersed in LN (Sakai 1966; Sakai & Yoshida 1967). Quatrano (1968) reported the first success of cryopreservation with *in vitro* cell suspensions and Nag and Street (1973) regenerated somatic embryos from *Daucus carota* after preserving callus in LN. Since then numerous successful cryopreservation studies have been conducted with a wide variety of plant species, which led to the development of advanced and improved methods.

In cryopreservation, tissue dehydration plays a vital role in preventing freezing injury and in the maintenance of post-thaw viability after storage in LN. There are mainly two different approaches for cryopreservation on the basis of their physical mechanisms: the classical or conventional cryopreservation (mainly slow freezing) in which freezing is performed in the presence of ice, and vitrification based cryopreservation in which freezing takes place without ice formation, i.e transition of liquid into an amorphous glassy, non-crystalline state (Gonzalez-Arno et al. 2008). To achieve vitrification, cellular content is concentrated by the use of cryoprotective agents and rapid freezing is achieved by the direct immersion of samples in LN (Panis & Lambardi 2005; Reed 2008; Sakai et al. 2000; Sakai et al. 2008).

1.2.1 SLOW FREEZING

Slow freezing or the controlled rate cooling involves step-wise programmed decrease in temperature. Some of the most successful pioneer cryopreservation experiments have involved a combination of slow and stepwise freezing (Withers 1985). In order to minimize the chance of intracellular ice formation, sufficient amount of water need to be removed from the cell. This is done by the osmotic regulation of cell contents and freeze-induced dehydration (Reed & Uchendu 2008). The slow freezing method involves cryoprotection using one or several cryoprotectants, followed by slow cooling at the rate of 0.5-2.0 °C/min to a determined pre-freezing or holding temperature at around -40 °C (Cruz-Cruz et al. 2013) that results in sufficient concentration of unfrozen fraction of the suspending solution and cytosol to enable vitrification (partial vitrification) when cooled in LN (Sakai et al. 2008).

Usually, dimethyl sulfoxide (DMSO) is the main penetrating colligative cryoprotectant and it may be combined with non-penetrating osmotically active additives such as sugars, polyethylene glycol (PEG), polyvinylpyrrolidone etc. Here, the samples are exposed to a slow cooling usually called a “ramp” during which a step may be incorporated for the operator or the machine to “seed” extracellular ice, and initiate an exothermic reaction (Benson 2008). At the freezing point of the cryoprotectant solution about 9 °C, ice nucleation is initiated and ice forms in the cryoprotectant solution and the intercellular spaces, but the cytoplasm remains unfrozen due to the high solute concentration, and the cell wall protects the cell membrane from damage by the ice crystals. As temperature is further decreased to -35 °C or -40 °C, the extracellular solution will become more icy and the intracellular solutes become highly concentrated. The plant cells lose water to the exterior ice and the cytoplasm

continues to get further concentrated. The intracellular water is thus safely reduced before the samples are plunged into LN (Reed & Uchendu 2008).

If the cells are optimally dehydrated, the cytoplasm vitrifies during instant freezing in LN. However, if the samples are under dehydrated leaving freezable water in the cytoplasm, ice will form damaging the cell membrane. If over dehydrated, the cells may die from desiccation (Reed & Uchendu 2008). The slow rates of cooling may also be achieved using alcohol baths, or self-contained units (containing methanol or isopropanol) placed in a -20 or -80 °C freezer. For example, isopropanol contained in a Mr Frosty® unit provides a 1 °C/min cooling rate. These methods are some of the first techniques developed, and are therefore considered as traditional methods (Benson 2008).

The classical cryopreservation techniques have been successfully applied to different plant species involving cell suspensions and calluses, dormant buds and also to apices of cold-tolerant plants (Chen et al. 1985; Reed & Uchendu 2008; Towill et al. 2004; Vogiatzi et al. 2011). A general observation of many researchers is that, greater the frost hardiness, higher the effective prefreezing temperature. If the explants can withstand freezing at temperatures below -30 °C, they are able to survive freezing at extremely low temperatures when slow freezing method is applied (Sakai 1965). Pre-freezing at -30 °C produced the highest survival in LN for mulberry (Sakai 1965) and apple (Tyler & Stushnoff 1988). Sakai et al. (1991) recorded 90 % survival rate when nucellar cell suspensions of navel orange were frozen at the rate of 2 °C/min and held at -30 °C for 20-30 min; the revived cells resumed growth within 3 days and developed into plantlets via embryogenesis. Vogiatzi et al. (2012) improved the efficiency of cryopreservation of winter dormant apple buds by cooling in air at -4 °C for 4 days and then cooling to -30 °C at 1 °C/h and holding at -30 °C for 24 h before plunging into LN. The slow freezing method has been successfully applied so far in apple (Höfer 2015; Lambardi et al. 2009; Tyler et al. 1988; Vogiatzi et al. 2012; Wu et al. 2001), grape (Zhao et al. 2001), *Actinidia* (Li & Guo 1996; Liu et al. 1998) (Wu et al. 2000), mulberry (Sakai 1965), *Pinus* (Salaj et al. 2007), *Prunus* (Choudhary et al. 2014), *Pyrus* (Reed 1990) and *Rubus* (Reed et al. 2008).

Though germplasm of several species is cryopreserved using classical procedures including pre-treatment with cryoprotectants and controlled freezing, survival rates have been generally low, growth recovery was slow, often including a transitory callusing phase and results were not always reproducible (Arnao et al. 1993). It is also not effective to cryopreserve complex organs like shoot apices and somatic embryos (Benito et al. 2004). In

addition, the techniques are generally operationally complex as they require expensive programmable freezers and also they are not effective for species sensitive to low temperatures (Engelmann 2004; Pennycooke & Towill 2000).

1.2.2 VITRIFICATION

Cryopreservation has been applied to a wider range of species with the introduction of new techniques and processes for which vitrification is central. More and more species have become amenable to cryopreservation through vitrification and its modifications, by which liquids solidify without crystallization (Benson 2008). In this technique, the freeze induced dehydration step, which is the characteristic of classical freezing method is eliminated and the slow freezing protocol is replaced by rapid freezing (Benson 2008; Cruz-Cruz et al. 2013). In vitrification protocols, the cell viscosity is elevated through dehydration by increasing the concentration of osmotically active permeate and non permeate cryoprotective agents (CPA) in the vitrification solutions (Fábián et al. 2008). Hence, vitrification is the only way to freeze the cells by avoiding the formation of lethal ice crystals that penetrate membraneous cell structures causing loss of its semi-permeability and thereby cell damage (Helliot et al. 2003).

Vitrification refers to the physical process of transition of an aqueous solution into an amorphous glassy, non-crystalline state. To achieve this, rapid freezing rate and concentrated cellular solution must be achieved (Panis & Lambardi 2005; Sakai et al. 2000). Rapid freezing rate is achieved by the direct immersion of samples in LN without the freeze-concentration step of slow freezing (Sakai et al. 2008). The cell cytosol can be concentrated through air-drying, or by the application of penetrating and non-penetrating cryoprotectants (Panis & Lambardi 2005). The increased viscosity that results from the concentrated cell solutes inhibits the coming together of water molecules to form ice (Benson 2008). Because of the high viscosity of a glass, all chemical reactions that require molecular diffusion of water are virtually arrested, and biological material in this stable condition may be maintained for a long time (Burke 1986). Vitrification is one of the best ways to prevent ice crystallisation in cells (Brockbank et al. 2003) and thus protecting their cellular membranes during LN storage as cellular water content is extremely reduced during the process (Kaviani 2011). This method has been the major cryopreservation method since Uragami et al. (1989) and Langis et al. (1989) developed it for asparagus and brassica respectively.

1.2.2.1 Cryopreservation Methods based on Vitrification

Several improvements to vitrification-based cryopreservation protocols have been introduced in conserving plant cells and tissues, such as pregrowth, dehydration, pregrowth-dehydration, encapsulation dehydration (ED), encapsulation vitrification(EV), droplet vitrification(DV) and cryo-plate (Cruz-Cruz et al. 2013; Engelmann et al. 1997). In pregrowth, the samples are grown in sucrose enriched media prior to cryoprotectant treatments before immersing in LN to enable them to withstand the ultra-low temperature (Reed 1996). Dehydration or desiccation of tissues is achieved using air current of a laminar airflow cabinet or with silica gel, followed by direct plunging into LN (Benson et al. 2011). Pregrowth-dehydration is the combination of the above two methods (Altman & Hasegawa 2012). ED is based on the artificial seed procedure, in which the explants are encapsulated in calcium alginate beads, which are then dehydrated before immersing in LN (Cruz-Cruz et al. 2013). The EV protocol combines the alginate encapsulation with dehydration by plant vitrification solution (PVS) (Benson et al. 2011). In DV, the samples are placed in a droplet of vitrification solution on an aluminium foil, which is then immersed in LN (Sakai & Engelmann 2007). Yamamoto et al. (2011) developed the very recent cryogenic procedure called cryo plate cryopreservation, in which the shoot tips are mounted with a calcium alginate layer on an aluminium cryo plate, which then follows the steps of droplet vitrification protocol. Among these methods, ED, EV and DV techniques are widely used. The vitrification-based methods are not complicated and are widely applicable to complex structures such as embryos and shoot tips (Benito et al. 2004; Withers & Engelmann 1997).

1.2.2.2 Encapsulation Dehydration

Dereuddre et al. (1990) developed ED technique for cryopreserving pear shoot tips based on the synthetic seed technology developed by (Redenbaugh et al. 1986) using calcium alginate beads. The method has been successfully applied to the cryopreservation of shoot tips and somatic embryos of several plant species (Gonzalez-Arno & Engelmann 2006).

ED technique involves encapsulation, preculture, desiccation, freezing/storage, thawing and regrowth (Gonzalez-Arno & Engelmann 2006). The encapsulated explants are first precultured in high sucrose media followed by desiccation. This extreme desiccation that would highly damage the non-encapsulated explants, removes all the water available for freezing from the cell and there by vitrifying the internal solutes during rapid freezing

(Engelmann et al. 1997). The survival rates are generally high and growth recovery is also rapid and direct (Engelmann 1998). It has been applied in wide range of plants including the recent reports on *Malus* (Li et al. 2014a), *Dendrobium* (Antony et al. 2014), coconut (Bandupriya et al. 2014), *Rosa* (Le Bras et al. 2014) and yam (Barraco et al. 2014).

1.2.2.3 Encapsulation Vitrification

The EV method, which utilises the combined application of calcium alginate encapsulation and PVS2 vitrification was first reported by Matsumoto et al. (1995) for cryopreserving shoot apices of *Wasabia japonica*. It is designed to preserve large number of meristems and eliminates the lengthy desiccation treatment time required for the encapsulation-dehydration method (Benson et al. 2011). In addition, the ability to handle small samples as well as the easy handling of large numbers of samples are the main advantages of this method (Sakai & Engelmann 2007).

In this technique, the samples are usually precultured in sucrose media following alginate encapsulation. The beads are then loaded with glycerol and sucrose mixture for about 30-60 min and then treated with PVS2. These treated beads are then placed in cryo vials containing PVS2, which are then immersed in LN. For retrieval, the cryovials are rapidly rewarmed in water bath set at around 35 °C followed by washing with unloading solution for recovery (Benson et al. 2011). Several plant species have been cryopreserved using this technique, which include the recent reports on *Rubia* (Shin et al. 2014), *Colocassia* (Hong et al. 2014a), *Hypericum*, *Nicotiana* (Uchendu et al. 2014) and *Passiflora* (Shin et al. 2014).

1.2.2.4 Droplet Vitrification

The DV method is the modification of droplet method developed by Kartha et al. (1982) for cryopreserving cassava meristems using slow freezing method. Schafermenuhr et al. (1994) first applied the DV method for the cryopreservation of potato meristems. It combines the application of highly concentrated vitrification solutions with ultra-fast freezing and thawing rates (Panis et al. 2005), which are crucial for its successful results (Benson et al. 2011).

DV technique involves the treatment of samples with cryoprotectants, which mainly includes exposure to the loading solution (usually 2 M glycerol and 0.4M sucrose) followed by dehydrating in highly concentrated vitrifying solutions. The commonly used plant vitrification solutions are PVS2 (Sakai et al. 1990) and PVS3 (Nishizawa et al. 1993). The samples are then placed on an aluminium foil strip mounted with a droplet of vitrifying solution and immersed in LN for rapid cooling. During rewarming the removal of cryoprotectant is also achieved using unloading solution, which has a high molarity achieved with sucrose, usually 1.2 M, and then transferred to recovery and regeneration media (Benelli et al. 2013). It is vital that the samples must be sufficiently dehydrated by the vitrification solution in order to vitrify while rapid cooling in LN. Thus the key for successful DV is to quantify and optimize the dehydration tolerance of samples in vitrifying solution, and this tolerance is achieved by optimizing the preconditioning and loading treatments along with the standardization of concentration, time of exposure and temperature of vitrification solution; in addition, the warming and recovery steps are also crucial for its success (Gonzalez-Arnan et al. 2014; Sakai & Engelmann 2007).

When compared to the other vitrification techniques, DV provides higher cooling and warming rates in LN and recovery medium respectively as the samples are placed on aluminium foil which has very high thermal conductivity (Cruz-Cruz et al. 2013; Kulus & Zalewska 2014). Use of aluminium foil helps the explants to get in closer contact with LN than with other techniques and thus increases the chance of achieving vitrified state during ultra-fast freezing (Benelli et al. 2013; Panis et al. 2005).

The DV is being increasingly applied to a wide range of crop plant species successfully (Benson et al. 2011). The very promising results achieved so far calls for a broader utilization of this technique (Sakai & Engelmann 2007). It has been the most effective technique among cryopreservation methods, viz vitrification, ED or EV, (Coelho et al. 2014; Fki et al. 2013; Kaya et al. 2013; Leunufna & Keller 2003; Ozudogru & Kaya 2012; Panta et

al. 2014a). For example, the highest regeneration rates were obtained by DV when three vitrification protocols were applied to China's eight major potato cultivars (Wang et al. 2013). Cassava shoot tips proven to be recalcitrant to ED could be recovered after DV (Escobar et al. 2014). Some of the species so far successfully cryopreserved by DV are listed in Table 1.2 along with the pre-treatments used.

Table 1.2. Successful droplet vitrification protocols developed for different species.

Species	Pre-treatments	Max. regrowth (%)	References
<i>Byrsonima</i>	(a): 0.3M for 24h	90	Silva et al. (2013)
<i>Calanthe</i>	-	82	Lin et al. (2014a)
<i>Callerya</i>	-	60	Li et al. (2013)
Cassava	-	75(b)	Escobar et al. (2014)
<i>Chrysanthemum</i>	(a): 0.5M for 24h	83	Wang et al. (2014b)
	(a): 0.5M for 24h	67	Wang et al. (2014c)
<i>Colocassia</i>	-	80	Hong et al. (2014b)
<i>Eucalyptus</i>	(a): 0.25M for 24h, 0.625M for 24h	85(b)	Kaya et al. (2013)
<i>Ipomoea</i>	(a): 0.35M for 24h	63	Vollmer et al. (2014)
<i>Lilium</i>	(c): 4 °C for 7days; (a): 0.3M-24h, 0.7M-24h	87.5	Yi et al. (2013)
	(a): 0.5M for 24h	87.5	Yin et al. (2014)
<i>Malus</i>	(a): 0.5M for 24h	70	Halmagyi et al. (2010)
	-	70	Condello et al. (2011a)
<i>Musa</i>	-	80	Agrawal et al. (2014)
<i>Paraisometrum</i>	(c): 4 °C for 7days; (a): 0.3M for 24h	93	Lin et al. (2014b)
<i>Phoenix</i>	(a):0.5M or (c): 4 °C for 48h	60	Fki et al. (2013)
<i>Prunus</i>	(a) (liquid med): 0.3M for 15h, 0.7M for 5h	30	Vujovic et al. (2011)
<i>Rosa (ex-vitro)</i>	-	86	Le Bras et al. (2014)
<i>Rubus</i>	-	18	Condello et al. (2011b)
	-	30	Vujovic et al. (2011)
<i>Solanum</i>	(c): 5 °C for 3w; (a): 0.3/0.45M at 5 °C for 1d	100(b)	Wang et al. (2013)
	-	41	Panta et al. (2014a)
	(c): 5 °C for 3w; (a): 0.3M at 5 °C for 3days	75	Wang et al. (2014a)
<i>Saccharum</i>	(a): 0.3M for 24h	20(b)	Nogueira et al. (2013)
<i>Thymus</i>	-	60(b)	Marco-Medina et al. (2010)
	(c): 4 °C for 2weeks; (a): 0.25M for 48h	80	Ozudogru and Kaya (2012)
	(c): alternating 25/-1 °C or const 10 °C;	67	Coelho et al. (2014)
	(a): 0.3M for 24h		
<i>Vitis</i>	(a): 24h each of 0.25M, 0.5M, 0.75, 1.0M	50	Pathirana et al. (2015)
	(a): 0.1M for 24h	50	Markovic et al. (2013)

(a) Sucrose (b) Recovery (c) Cold acclimation

1.2.2.5 Application of vitrification methods for eradicating plant pathogens from infected plants

Another application of vitrification-based cryopreservation is to eradicate systemic plant pathogens through cryotherapy (CGKB 2014; Cruz-Cruz et al. 2013; Pathirana et al. 2015; Wang & Valkonen 2009a). Cryotherapy is a new method in which shoot tips are cryopreserved to remove the infected cells by injuring them with the cryo-treatment and thus regenerating healthy shoots from pathogen-free meristematic cells (Wang et al. 2009). Since meristematic cells are rapidly dividing and are located away from the vascular tissues, they have lesser chance of pathogen infection. The infected cells that are located more distant from the apical dome are more sensitive to the cryo injury than the less vacuolated cells in the apical dome and thus, a larger portion of regenerants after cryopreservation are likely to be pathogen free than with the traditional meristem culture which is difficult, time consuming and often with very poor regeneration rate (Wang & Valkonen 2009a). Successful attempts have been reported in the elimination of a range of pathogens, which includes viruses, phytoplasma and bacteria using cryotherapy in a wide range of plants as listed in Table 1.3. In addition to the high efficiency of recovery in several species, the low operational costs and rapid recovery of plant material without virus infection has resulted in cryotherapy becoming a promising tool in agriculture and horticulture (Wang & Valkonen 2009a).

Table 1.3. Vitrification-based cryotherapy methods applied to different crop germplasm.

Crops	Pathogen	Reference
Citrus	<i>Candidatus Liberibacter asiaticus</i>	Ding et al. (2008)
Globe artichoke	<i>Artichoke latent virus</i>	Taglienti et al. (2013)
Musa	<i>Cucumber mosaic virus</i> and <i>Banana streak virus</i>	Helliot et al. (2002)
Potato	<i>Potato leafroll virus</i> and <i>Potato virus Y</i>	Wang et al. (2006)
Prunus	<i>Plum-pox poty virus</i>	Brison et al. (1997)
Raspberry	<i>Raspberry bushy dwarf virus</i>	Nukari et al. (2009), Wang and Valkonen (2009b)
Sweet potato	<i>Sweet potato little leaf phytoplasma</i>	Wang and Valkonen (2008), Feng et al. (2011)
Sweet potato	<i>Sweetpotato feathery mottle virus</i> and <i>Sweetpotato chlorotic stunt virus</i>	Feng et al. (2011)
Vitis	<i>Grapevine leafroll associated virus 1, 2 & 3</i> <i>Grapevine virus A</i>	Pathirana et al. (2015) Wang et al. (2003), Bayati et al. (2011)
Yam	<i>Yam mosaic virus</i>	Shin et al. (2013)

1.3 CRYOPROTECTION- METHODS AND ADVANCES

As intracellular water plays the central role in cryopreservation, the cell must be dehydrated and the water content must be optimised to avoid ice crystal formation. The cells cannot survive the ultra-freezing temperature of LN without cryoprotective treatments (Gnanapragasam & Vasil 1992; Volk & Walters 2006). The concept of cryoprotection was established in the late 19th and early 20th century, when Hans Molisch in the 1890's examined freezing in plants by direct microscopy using an early version of a cryomicroscope. H. Muller Thurgau reported the accumulation of sugars in cold hardened plants, but the importance of sugars as CPA was clearly recognized by Maximov in the early 1900's (Fuller 2004). The milestone that could open the vast application and possibilities of cryopreservation is the discovery of glycerol as a protective agent against freezing by Polge et al. (1949). The next two decades meant for the investigation of different solutes possessing CPA activity. Sucrose, 1, 2 propanediol, ethanediol, and DMSO were shown to have high CPA activity. Many other low molecular weight solutes, such as amino acids including alanine, glycine and proline, other sugars including glucose, lactose and ribose, and amides including acetamide and formamide also have CPA activity to some extent (Fuller 2004).

1.3.1 CRYOPROTECTANTS

Cryoprotectants are mainly classified into two categories: the penetrating CPA and non-penetrating CPA. The penetrating CPA are those that pass through the plasma-membrane to equilibrate between extracellular solution and the cell interior and the non-penetrating CPA are those that do not pass through the plasma membrane and accumulate in the extracellular solution (Reed 2008). The penetrating CPA mainly comprise DMSO, glycerol and amino acids, and non-penetrating CPA includes sugars, alcohols and high molecular weight additives like PEG that are osmotically active for dehydration (Panis & Lambardi 2005). Among this group of various cryoprotectants, DMSO and glycerol are the most effective when appropriately applied (Nag & Street 1975). DMSO is preferred because of its rapid penetration into the cells, but considering its toxicity, glycerol and amino acids are often used as alternatives to DMSO (Panis & Lambardi 2005).

Since the discovery that the cryoprotectant solutions help in protecting the cells from cryogenic temperatures, various vitrifying solutions (Kim et al. 2009; Nishizawa et al. 1993; Sakai et al. 1990; Uragami et al. 1993; Uragami et al. 1989) have been developed with

variations in the composition for cryopreserving plant cells. However, the most commonly used plant vitrifying solutions are PVS2 that contains 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol and 15 % (w/v) DMSO in MT medium containing 0.15 M sucrose (Sakai et al. 1990), and PVS3 that constitutes 50 % (w/v) glycerol and 50 % (w/v) sucrose in water (Nishizawa et al. 1993). Markovic et al. (2013) suggested that PVS3 is highly toxic as it resulted in no regrowth after cryopreservation in *Vitis* when compared with PVS2.

Pre-treatment of the explant is a common approach that usually enhances the survival after cryopreservation when applied with vitrification solution. Typical pre-treatments include cold hardening in which the donor plants are exposed to a lower temperature, and preculture in which the shoot tips are incubated in media containing high sugar concentration (usually by daily increasing concentration of sugar) which dehydrate cells prior to freezing (Ozudogru & Kaya 2012).

1.3.2 EFFECT OF COLD ACCLIMATION AND SUCROSE

Cold acclimation (CA) or the cold hardening is the process that triggers the plant's natural resistance mechanisms to cold weather or to survive cold stress conditions (Panis & Lambardi 2005; Reed 1996). To improve the regeneration rates, *in vitro* donor plants are commonly exposed to lower temperature (than their natural growth conditions) before excision of shoot tips for cryopreservation (Coelho et al. 2014). In temperate species and naturally occurring cold hardy plants, CA is very effective in the survival of cells after cryopreservation in LN (Benson 2008), and the genotypes that do not cold acclimate well are difficult to cryopreserve (Reed 1993). This is because the lipid and protein compositions of plasma membrane that dynamically alter during cold acclimation enhance the cryostability of the plasma membrane during ultra cooling (Kaviani 2011). The cold acclimation enhanced the uptake of sugar from the media in sugar beet shoot tips, which safeguards the liquid crystalline state of membrane bilayers (Vandenbussche et al. 1999). The decrease in the temperature increase the unsaturation of membrane lipids (Nishida & Murata 1996), which have a direct correlation in maintaining the fluid state in hydrocarbon chains at low temperature (Raison 1973) that is crucial for the proper functioning and survival of cells (Cossins 1994). Chang et al. (1999) and Kushnarenko et al. (2009) also reported the significance of CA in the survival of grass (*Zoysia* and *Lolium* sp.) and apple shoot tips respectively.

The accepted standardised temperature for CA is about 4 ± 1 °C (Fki et al. 2013; Lin et al. 2014b; Uchendu et al. 2014; Wang et al. 2014a) in the dark, but is effectively applied in a diurnal temperature cycle (Coelho et al. 2014; Funnekotter et al. 2013; Niu et al. 2012; Vandenbussche et al. 1999). Cold hardening of donor plants at 5 °C improved recovery of sour cherry, apple and pear after cryopreservation (Barraco et al. 2012; Niino et al. 1992). Liu et al. (1998) observed high survival in *Actinidia arguta* after cryopreservation when the buds were cold acclimated at 5 °C for 4 weeks followed by 0 °C for 2 weeks. Cold acclimating plants with alternating temperature produced best growth after cryopreservation for *Frageria* (Höfer & Reed 2010), pear (Chang & Reed 2000) and potato (Kaczmarczyk 2008). CA with or without abscisic acid (ABA) significantly improved the survival of *Rubus* (Reed 1993). She found that ABA pre-treatment alone cannot increase the survival of plants grown under warm conditions after cryopreservation, but the survival tripled when CA was combined with ABA pre-treatment (Reed 1993).

Preculturing in high sugar media enhances the acclimation of plants to low temperature and stimulates the osmotic dehydration (Reed & Uchendu 2008). It increases the survival after cryopreservation (Matsumoto et al. 1994). Though mannitol or sorbitol (Gnanapragasam & Vasil 1992; Mikula 2006; Mikula et al. 2005; Pritchard et al. 1986) can be used in preculturing media, and also glucose and fructose can replace sucrose without altering the post-cryo survival (Panis et al. 2002), most researchers prefer to use sucrose. Sucrose was found to be the optimal sugar for preculturing the samples as mannitol or sorbitol did not support any regrowth after cryopreservation of olive somatic embryos (Lynch et al. 2011). Halmagyi et al. (2005) also obtained highest regrowth by preculturing potato shoot tips with sucrose media than with glucose, mannitol or sorbitol.

Sucrose acts as osmolyte, excellent glass former and also stabilises membranes and proteins. It stimulates the production of other sugars, glycerol, proline, glycine betaine and polyamines, which have colligative as well as non-colligative effects (Antony et al. 2013; Hirsh 1987). Of these, sugars (Heber & Santarius 1964), glycerol (Williams et al. 1991), proline (Burritt 2012), glycine betaine (Cleland et al. 2004) have proved their cryoprotectant ability, whereas polyamines are known for their antioxidant properties; hence these compounds play a vital role in protecting the cells during cryopreservation. When plant cells undergo abiotic stress, they demand for high energy for which especially the meristematic cells completely depend on glycolysis and oxidative phosphorylation. Proteome analysis of banana meristems showed that high sucrose maintains an osmoprotective intracellular concentration by enhancing the enzymes of glycolytic pathway, thereby protecting the cells

during dehydration and also maintains the integrity of the cell wall (Carpentier et al. 2007). Sucrose protects the membrane stability by forming hydrogen bonds with the polar head groups of the phospholipid bilayer of the membrane and thereby replaces the water molecules bound to the phospholipids (Crowe et al. 1987). It also maintains the protein stability when the cells are exposed to extreme desiccation at ultra-low temperature (Kendall et al. 1993).

Post-cryo viability of *Brassidium* (Yin et al. 2011) and *Dendrobium* (Yin & Hong 2009) after cryopreservation increased with high sucrose concentration in the pre-treatment medium. Survival of kiwifruit (Bachiri et al. 2001) and grapevine (Wang et al. 2004; Zhao et al. 2001) was enhanced when sucrose concentration of the preculture media was daily increased. The preculture in the elevated level of sucrose also increased the tolerance to the dehydration stress induced by PVS2 and also reduced its injurious effect on banana meristems which are vital for successful cryopreservation (Helliot et al. 2003). In addition to several successful cryopreservation experiments using 0.3M - 0.75 M sucrose for preculture, preculture with 1M sucrose was also found to improve survival rates (Plessis et al. 1993; Wu et al. 2000; Zhao et al. 2001).

The cold hardiness significantly increased with the increase of sucrose concentration in the preculture media and decreased the shoot water content (Chang & Reed 2001). Fki et al. (2013) found that CA and sucrose treatments increase the proline content of date palm shoot tips that in turn enhanced the resistance of plants to osmotic stress and low temperature. Barraco et al. (2012) obtained high recovery in *Prunus* after LN either with the combination of cold and sucrose precultures or by replacing cold hardening with a 7 day preculture in sucrose or glycerol media. Niino et al. (1992) obtained an average shoot formation of 80 % when apple shoot tips were cryopreserved after cold acclimation at 5 °C for 3 weeks, followed by preculture on 0.7 M sucrose for 1 day at 5 °C.

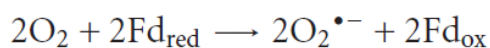
1.3.3 ROLE OF ANTIOXIDANTS IN CRYOPRESERVATION

Antioxidants and anti-stress compounds improve plant regrowth after cryopreservation by preventing or repairing the damage resulting from oxidative stress (Uchendu et al. 2010b). Recent studies have shown that the supplementation of antioxidants in the cryopreservation media reduces the oxidative stress and increases the regrowth of cryopreserved tissues (Reed et al. 2012). Johnston et al. (2007) suggest maintaining an elevated antioxidant protection to improve post-cryo survival and plant regeneration. They observed a higher antioxidant status

in the cold-tolerant *Ribes* genotype, whereas the cold-sensitive genotype, which resulted in poor recovery, had an overall low antioxidant status. There are several reports to support this: inclusion of vitamin C and vitamin E in blackberry (Uchendu et al. 2010a), vitamin C in barley and wheat (Fretz & Lörz 1995), wheat egg cells (Fábián et al. 2008) and mouse embryos (Lane et al. 2002), and desferrioxamine in rice cells (Benson et al. 1995) and algae (Fleck et al. 2000) resulted in enhancing the post-cryo survival. Uchendu et al. (2010b) reported an increase in regeneration percentage with the addition of lipoic acid, glutathione and glycine betaine in different steps of cryopreservation. Absciscic acid known for improving dehydration and freezing tolerance in plant tissues improved survival of gentian axillary buds (Suzuki et al. 2006), kiwifruit shoot tips (Suzuki et al. 1997), and embryos of coconut (Bandupriya et al. 2007) and wheat (Kendall et al. 1993) after cryopreservation.

Ascorbic Acid (ASA) (ascorbate or vitamin C) occurs in all plant tissues, especially higher in photosynthetic cells and meristems. ASA plays key roles in many physiological processes, but is well known for its fundamental role in defence mechanism as an antioxidant; and in association with other components of the antioxidant system, protects plants against oxidative damage resulting from aerobic metabolism, photosynthesis, pollutants and other environmental stresses (Shao et al. 2008; Smirnoff 1996). Thus it helps in the detoxification of reactive oxygen species enzymatically or non-enzymatically (Smirnoff & Wheeler 2000). ASA can directly scavenge singlet oxygen, hydrogen peroxide, superoxide and hydroxyl radical (Shao et al. 2008). It is also well known for the production of collagen, which increases the tolerance of plants to oxidative stress through glutathione cycle (Yazdanpanah et al. 2011). It can terminate radical chain reactions by disproportionation to non-toxic, non-radical products like dehydroascorbate, and in addition, it can donate electrons to various substrates, especially in the antioxidant enzyme pathways (Davey et al. 2000).

The vitrification based cryopreservation produces oxidative stress in cells at various steps (Uchendu et al. 2010a). The oxidative stress cause reduced supply of NADP in the chloroplasts, which results in the transfer of high-energy electrons from reduced ferredoxin of electron transport chain to molecular oxygen instead of NADP, and subsequently reduced to superoxide. This undesirable electron pathway is known as Mehler reaction (Sharma et al. 2012).



The subsequent reduction of this oxygen results in the accumulation of toxic hydrogen peroxides, as chloroplast lacks catalase that detoxifies them. In this state, ASA plays an

essential role by serving as a substrate for ascorbate peroxidase to scavenge the peroxides (Davey et al. 2000). The addition of ASA to the cryopreservation solution decreased the hydrogen peroxide levels in the mouse embryos and thereby enhanced the embryo development after cryopreservation (Lane et al. 2002). ASA increased the survival of barley and wheat by decreasing the lipid peroxidation that occurs during cryopreservation (Fretz & Lörz 1995). The higher ASA content along with the higher APX activity resulted in high tolerance in wheat genotypes to water stress (Sairam et al. 1998). Uchendu et al. (2010a) found that ASA can significantly improve regrowth of shoot tips of blackberry after cryopreservation and hence they recommended adding it to the pre-treatment medium, loading solution and the recovery solution.

1.3.4 ROLE OF VITRIFICATION SOLUTION DURING CRYOPRESERVATION

The success of vitrification-based cryopreservation lies mainly on the careful control of the vitrification solution to prevent the damage of the cells by chemical toxicity or excess osmotic stress during dehydration (Niino et al. 1992). The plant vitrification solution, developed by Sakai et al. (1990) named PVS2 has made a significant contribution to the success of cryopreservation in recent years. This mixture contains high concentrations of penetrating cryoprotectants such as DMSO and glycerol (Si et al. 2004) as well as osmotically active compounds like ethylene glycol and sucrose (Helliott et al. 2003). Volk and Walters (2006) proposed that PVS2 operates through two cryoprotective mechanisms: it replaces cellular water and it also changes the freezing behaviour of water remaining in the cells. The cell penetrating constituents of PVS2 (e.g. DMSO) replace the water during dehydration, thus preventing injurious cell shrinkage caused by dehydration. These penetrating cryoprotectants have a colligative role that moderates the effects of non-penetrating compounds in the solution such as sucrose and glycerol, which osmotically dehydrate the cells (Benson 2008; Volk & Walters 2006).

The PVS2 super cools very fast at temperatures below -100 °C during rapid cooling and solidifies into a meta-stable glass around -115 °C. Using differential scanning calorimetry, it has been shown that while slowly warming the solution from the ultra low temperature, the vitrified PVS2 displays a glass transition state at around -115 °C; when the temperature reaches -75 °C, it crystallises by an exothermic de-vitrification process, and the endothermic melting is recorded at -36 °C (Sakai & Engelmann 2007).

The optimal duration and concentration of vitrifying solution treatment depend up on the size and structure of the shoot tips, and is also highly species specific (Markovic et al. 2013; Niino et al. 1992). The shoot tips larger than 3 mm take longer for dehydration, whereas those smaller than 0.5 mm often appear over-stressed by the toxicity of the PVS2 (Sakai et al. 2008). Deviations from the widely used optimal PVS2-based dehydration periods of 30-50 min at 0 °C (Condello et al. 2011b; Escobar et al. 2014; Fki et al. 2013; Hong et al. 2014b; Marco-Medina et al. 2010; Panis et al. 2005; Panta et al. 2014a) in DV have been recorded in recent times. Thus, for example, maximum regrowth in *Rosa* was achieved when *ex-vitro* shoot tips were dehydrated for only 15 min (Le Bras et al. 2014), whereas the optimal time of 150 min was reported in *Musa* (Agrawal et al. 2014). Optimal dehydration time for *Thymus* ranged from 60 min (Coelho et al. 2014) to 90 min (Ozudogru & Kaya 2012).

Reed and XiaoLing (1995) suggest that treating explants at high concentrations of cryoprotectants may cause toxic effects in the cells and thus affect the survival after cryopreservation. In order to reduce the toxicity of the vitrification solution, a modified vitrification protocol has been developed for *Vitis* by Markovic et al. (2013) in which the samples were first treated with half-strength followed by incubation in full-strength PVS2. They achieved 50 % of regrowth after cryopreservation by this method. Hakozaiki et al. (1996) developed a cryopreservation technique for *Actinidia* in which they treated the samples with 60 % PVS2 prior to dehydration with 100 % PVS2. Kami et al. (2008) experimented diluted PVS2 (50 % and 70 %) to reduce the toxic effect of concentrated PVS2 on shoot tips of cranberry, but resulted in poor regrowth after cryopreservation because of the nucleation caused by the dilution during cooling and /or rewarming.

1.4 ULTRASTRUCTURAL CHANGES IN PLANT CELLS AND TISSUES DURING CRYOPRESERVATION

A comparison of ultrastructure of cryopreserved samples with control samples can help to understand the structural changes in cells at different steps of cryopreservation so that the protocol can be improved (Helliot et al. 2003). Gnanapragasam and Vasil (1992) studied the ultrastructure of suspension-cultured cells of *Panicum maximum* at different stages of cryopreservation. The cells appeared smaller with dense cytoplasm with response to preculture, and the cryoprotectant treatment caused swelling of organelles along with the invagination of tonoplast and vacuolar vesicles. When freeze-fixed at -10 °C, the plasma membrane damage and cell content leakage were seen only in some of the cryoprotected cells, whereas the non-cryoprotected cells showed extensive lethal damages such as disruption of plasma membrane and plasmodesmata. The flaccid, discoloured and water soaked appearance can be considered as the proof for the alteration or disruption of plasma membrane and is the major cause of cryo-injury during the cryopreservation of plant cells or tissues (Steponkus 1985). The cryoprotectants play a significant role in this context by direct interaction with the membrane bilayer throughout the freezing process to safeguard the membrane integrity (Anchordoguy et al. 1987).

The ultrastructural study of the meristems precultured in sucrose medium showed various changes including the fragmentation of large vacuoles into smaller ones, differentiation of proplastids into amyloplasts, swelling of endoplasmic reticulum and undulation of plasma membrane by partial plasmolysis (Ding et al. 2008; Helliot et al. 2003). Ding et al. (2008) observed slight plasmolysis after sucrose preculture of *Citrus* shoot tips, but plasmolysis was clearly visible after treating with loading solution and PVS2 treatment and it reached the peak after immersion in LN and thawing. In banana shoot tips subjected to preculture, the severely plasmolysed cells lost the contact with their neighbouring cells as the intercellular connections through plasmodesmata were broken (Helliot et al. 2003). The osmotically stressed cells resulting from preculturing in high sugar media showed dense cytoplasm, reduced vacuolar cross-sectional area and reduced cell wall thickness (Pritchard et al. 1986). Helliot et al. (2002) investigated the damaged banana cells after cryopreservation and found that the freezing injuries related with water crystallization during vitrification or devitrification were linked with increased vacuolisation. The shoot apices of kiwifruit having increased number of vacuoles suffered severe plasmolysis during pre-culture and PVS2 treatment (Cai et al. 2006; Xu et al. 2006a). Therefore, the more vacuolated the plant cells are,

the more water that needs to be withdrawn in order to prevent the lethal ice crystallisation (Helliot et al. 2003).

The irreversible injury caused by the ice crystallisation during cryopreservation leads to the damage of biological material by physical and biochemical events (Dumet et al. 2000). Techniques that are found effective for cryopreserving cells do not always maintain integrity at tissue level because highly organised multicellular tissues present a special set of problems, mainly the effect of extracellular ice formation that disrupts the tissue architecture (Taylor et al. 2004). Wesley-Smith et al. (1992) observed notable freeze damage in embryonic axes of tea when cryopreserved using slow freezing methods compared to rapid freezing.

Grout and Henshaw (1980) identified many damaged cells in surviving shoot tips after cryopreservation by microscopic examination. The cells did not show any significant changes just after the rewarming treatment of potato shoot tips, but profound damage was observed especially in the meristematic dome area and epidermis such as rupture of plasmalemma, plasmolysis, destruction of cell organelles and strong heterochromatization after two days of rewarming (Kaczmarczyk et al. 2008). Pinker et al. (2009) investigated the ultrastructure of meristematic cells of strawberry shoot tips and found them severely affected by PVS2 and LN treatment. The microscopy revealed the accumulation of starch in meristematic and parenchyma cells of the apical dome after sucrose preculture (Pinker et al. 2009; Vandenbussche et al. 1999).

1.5 OXIDATIVE STRESS AND ANTIOXIDANT DEFENCE DURING CRYOPRESERVATION

There are several approaches that increase the protection to tissue to avoid the lethal ice crystal formation for successful cryopreservation, but yet several other factors including various stresses affect the post-cryo survival. The various stages of cryopreservation impose a series of stresses on the plant material including temperature stress, osmotic stress and water stress that leads to oxidative stress which makes the cells susceptible to induced modifications and thereby cell damage (Benson & Bremner 2004; Engelmann 2004; Kaczmarczyk et al. 2012).

1.5.1 OXIDATIVE STRESS

Oxidative stress is defined as an imbalance between oxidants and the antioxidants, in favour of oxidants potentially leading to cell damage (Bartosz 1997; Sies 1997). Under non-stressful conditions, oxidative stress and antioxidant metabolism are regulated processes, which are maintained in equilibrium (Apel & Hirt 2004). This equilibrium between the two processes thereby stabilizes the normal plant growth, protecting it from cytotoxicity and tissue damage induced by the reactive oxygen species (ROS) resulting from the metabolism of oxygen (Arora et al. 2002). However under stress conditions, when the rate of ROS production in the cells exceeds the natural antioxidant defence levels, it is referred to as oxidative stress (Vaziri et al. 2003).

1.5.2 REACTIVE OXYGEN SPECIES

ROS are a group of oxygen derivatives comprising of free radicals, reactive molecules and ions that play a dual role as both beneficial and deleterious, depending on their concentration in cells. At their normal concentration, ROS act as second messengers in intracellular signalling cascades, whereas, their over-production damages the biomolecules that lead to cell death (Foyer & Noctor 2005; Mittler 2002; Sharma et al. 2012). The ROS that are present in various subcellular loci such as mitochondria, chloroplasts and peroxisomes include singlet oxygen (O_2^1), superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$), which are toxic molecules that are capable of causing oxidative damage to proteins, lipids and DNA (Apel & Hirt 2004; Mittler 2002).

The molecular oxygen (O_2) is totally harmless unless it is activated either by absorbing energy to reverse the spin of one of the unpaired electrons forming O_2^1 or by its stepwise monovalent reduction to form $O_2^{\bullet-}$, H_2O_2 and $\bullet OH$ (Sharma et al. 2012). The O_2^1 is highly destructive and reacts with most biological molecules; the primary source of O_2^1 is the chlorophyll pigments associated with the electron transport system and also produced as a by-product of lipogenase activity. H_2O_2 is produced by the dismutation of superoxide radicals mostly catalysed by superoxide dismutase and is normally scavenged by peroxide reaction or by catalase (Arora et al. 2002). H_2O_2 and $O_2^{\bullet-}$ by themselves are relatively less damaging but can form $\bullet OH$ through Fenton reaction and the Haber-Weiss reaction when the superoxide dismutase and catalase activities are inhibited. $\bullet OH$ can damage essential cell components by lipid peroxidation and by attaching DNA, proteins and other small molecules (Arora et al. 2002; Sharma et al. 2012). The pathway is illustrated in the Figure 1.1.

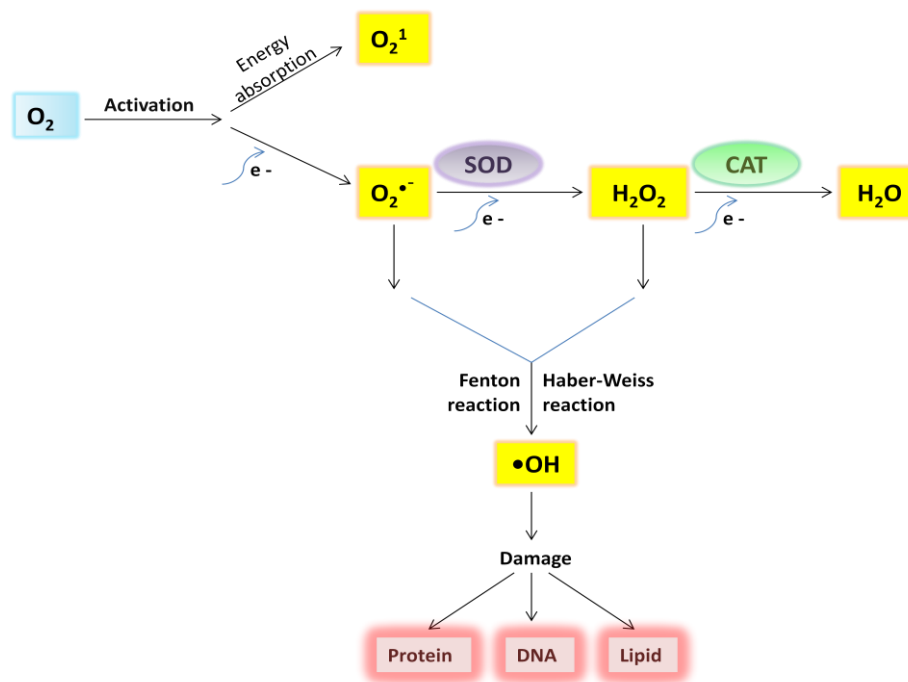
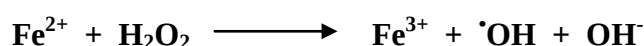
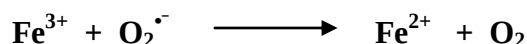


Figure 1.1. Intracellular cascade leading to the formation of reactive oxygen species that cause cell damage.

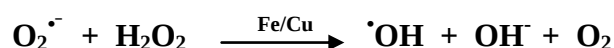
CAT-catalase, e^- -electrons, H_2O_2 -hydrogen peroxide, O_2 -oxygen, $O_2^{\bullet-}$ -superoxide radical, O_2^1 -singlet oxygen, $\bullet OH$ -hydroxyl radical, SOD-superoxide dismutase

The $\cdot\text{OH}$ is one of the most reactive radicals in nature and is considered to be the main causative factor for cryo-injury in plant cells (Benson & Bremner 2004; Johnston et al. 2007). When SOD activity increases in the absence of catalase, the survival decreases due to the formation of $\cdot\text{OH}$ from H_2O_2 by the Fenton and the Haber-Weiss reactions as given below. There is evidence for the role of Fenton and Haber-Weiss chemistry in inducing cryo injury *in vitro* (Fleck et al. 2003).

Fenton reaction:



Haber-Weiss reaction:



1.5.3 OXIDATIVE DAMAGE IN CELLS

The unpaired electrons of the ROS cause damage by extracting the electrons from biological molecules such as lipids, proteins and nucleic acids (Møller et al. 2007; Reed et al. 2012). ROS damage the cells by altering membrane fluidity and ion transport, lipid peroxidation, protein cross-linking and degradation, enzyme inactivation, inhibition of protein synthesis and disruption of DNA (Batkova et al. 2008; Sharma et al. 2012).

Lipids are the main target molecules of ROS in the cell membranes. The lipid peroxidation adversely affects the integrity of the membrane structure (Reed et al. 2012) and also produces lipid-derived radicals, mainly malondialdehyde (MDA) that reacts with and damage proteins and DNA (Uchendu et al. 2010a). The two main sites of phospholipid molecule prone to ROS attack are the unsaturated bond between the carbon atom and the ester linkage between glycerol and the fatty acid, in addition to the polyunsaturated bonds of the fatty acids, which are very sensitive to the ROS attack (Sharma et al. 2012).

The ROS attack the proteins by the modification of amino acids by oxidation, nitrosylation, carbonylation, disulphide bond formation and glutathionylation (Sharma et al. 2012), thereafter these modified proteins are selectively recognised by the proteases and degrade them (Palma et al. 2002). Oxidized proteins are the better substrates for proteolytic

digestion (Sharma et al. 2012). The ROS induced changes in cellular proteases can lead to the accumulation of inactivated cellular proteins (Prasad 1996). Also, the ROS damage the proteins by peptide chain fragmentation, inactivation of enzymes, aggregation of cross-linked reaction products and alteration of electric charges (Sharma et al. 2012).

The damage to DNA can lead to the malfunction or complete inactivation of the encoded protein (Sharma et al. 2012). A relatively large fraction of oxidative damage occurs at the sugar-phosphate backbone leading to single strand breaks, and the bases at these nicks are more accessible to hydroxyl radical attack, whereas the bases undergo deamination by oxidation (Britt 1996). ROS attack on DNA manifests in deoxyribose oxidation, double strand breakage, removal of nucleotides and modification of bases resulting in mutation and DNA-protein cross-links (Sharma et al. 2012). The mitochondrial and cytoplasmic DNA are more vulnerable to oxidative damage compared to nuclear DNA as the former lacks the protective protein, histones and also located close to the ROS production site (Richter 1992).

1.5.4 OXIDATIVE STRESS RESULTING FROM CRYOPRESERVATION

Cryopreservation causes osmotic changes and oxidative damage that have sub-lethal and lethal effects on cells (Li et al. 2010). The steps involved in cryopreservation impose a series of stresses to the plant tissues, which are both osmotic and temperature-related, and can cause oxidative damage. Vitrification-based cryopreservation involves preculture, excision of buds, pre-treatment, loading, treatment with vitrification solution, freezing, rewarming, rinsing and regrowth. All these steps present the risk of oxidative damage caused by increasing the level of ROS in cells (Reed et al. 2012). The formation of ROS, mainly the production of $O_2^{\bullet-}$ was found to be associated with tissue injury caused during excision of the embryonic axes and dehydration of recalcitrant sweet cherry (Roach et al. 2008). The slowing down of enzymatic reactions during cold stress results in a lower demand for ATP which results in the loss of electrons from electron transport chain, leading to increased ROS levels and oxidative stress (Apel & Hirt 2004; Okuda et al. 1992). Oxidative stress that occurs during the different stages of cryopreservation results in reduced viability of the tissues during recovery (Uchendu et al. 2010a). (Thomson et al. 2009) reported a significant rise in DNA fragmentation levels after cryopreservation of human sperm cells and showed its association with the increased oxidative stress.

The exposure of plant tissue to low temperature causes chilling and freezing injury which promotes sub-lethal changes leading to increased ROS production and thereby cell damage (Reed et al. 2012; Uchendu et al. 2010b). Chilling injury results from the transition of liquid state cell membranes to gel state (Ghetler et al. 2005), which cause loss of membrane integrity by changing membrane structure and composition, causing solute leakage, reduced transport through plasmalemma, malfunction of mitochondrial respiration and increase of peroxide and oxidase levels (Prasad et al. 1994; Saltveit & Morris 1990). O'Kane et al. (1996) reported an increased level of lipid peroxidation in *Arabidopsis thaliana* due to chilling injury. This differs from the freezing injury that results from intracellular water crystallisation during freezing and thawing (Ding et al. 2008). Alvarez and Storey (1992) suggested the reduction of phospholipid content along with the loss of phosphatidylcholine and phosphatidylethanolamine during the cryopreservation of human sperm is associated with lipid peroxidation.

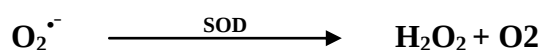
1.5.5 ANTI-OXIDANT METABOLISM IN PLANTS

1.5.5.1 Enzymatic antioxidants

Antioxidants are known to form the first line of defence against ROS damage and are essential for maintaining normal metabolism and the functioning of plant cells (Ali & Alqurainy 2006). The antioxidant defence system of plants comprise a variety of antioxidant molecules, both enzymatic and non-enzymatic, to scavenge the ROS that protect the cells from destructive oxidative reactions (Arora et al. 2002). Plant antioxidants include antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione peroxidase (GPX; EC 1.11.1.9) glutathione reductase (GR; EC 1.6.4.2), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) etc. and also low molecular mass non-enzymatic antioxidants such as ascorbate and glutathione, as well as tocopherols, carotenoids and phenolics (Batkova et al. 2008; Zlatev et al. 2006). Highly complex and integrated physiological pathways are involved in the regulation of antioxidant metabolism (Johnston et al. 2007). The antioxidant enzymes catalyze redox reactions of which most of them depend on electrons supplied by the low molecular weight reductants ascorbate and glutathione (Noctor & Foyer 1998).

Superoxide Dismutase

The SODs, the peroxidation defence enzymes (Lasso et al. 1994) are a family of metallo-enzymes that catalyse the dismutation of $O_2^{\bullet -}$ to H_2O_2 and O_2 , thus it remove superoxide and hence decreases the risk of hydroxyl radical formation via the metal-catalysed Haber-Weiss reaction.



The three isozymes of SOD, differentiated by the metals in their enzyme active site responsible for the catalysis (Michiels et al. 1994), namely FeSOD (in chloroplasts), MnSOD (in mitochondria and peroxisomes) and Cu/Zn-SOD (in cytosol and chloroplasts) have been reported in various plant species (Alscher et al. 2002; Arora et al. 2002). An increase in the activity of SOD indicates the plants' enhanced tolerance against the oxidative stresses (Sharma et al. 2012).

Catalase

CAT is ubiquitous enzyme located in peroxisomes/glyoxisomes (Ali & Alqurainy 2006) and is the first antioxidant enzyme to be discovered and characterized (Sharma et al. 2012). The enzyme catalyses dismutation of two molecules of H_2O_2 into H_2O and oxygen (Michiels et al. 1994; Sharma et al. 2012) as shown below.



CAT activity decreases with the increased water stress and this can lead to the accumulation of H_2O_2 (Ali & Alqurainy 2006). Among the various H_2O_2 degrading enzymes in plants, CAT is unique as it does not depend on reducing equivalents for catalysis and has very fast turnover rates, but has a much lower affinity for H_2O_2 compared to APX (Sharma et al. 2012).

The Ascorbate-Glutathione Cycle

APX, MDHAR, DHAR and GR together form the ascorbate-glutathione cycle that is found in mitochondria, peroxisomes, chloroplasts and the cytosol, which converts H_2O_2 to water and recycles ascorbate and glutathione (Ali & Alqurainy 2006; Batkova et al. 2008; Jimenez et al. 1997). In the first reaction, the APX utilizes ascorbate as the reducing agent to convert H_2O_2 into water, thereby oxidising ascorbate into monodehydroascorbate (MDHA). MDHAR then reduces MDHA into ascorbate with the help of NADPH. DHA is produced spontaneously from MDHA and is reduced to ascorbate by DHA reductase (DHAR) with the help of glutathione (GSH) as the reducing agent, which subsequently gets oxidised to glutathione disulphide (GSSG). GR, which contains FAD at its active site (Michiels et al. 1994), catalyses the reduction of GSSG back to GSH using the reducing agent NADPH (Apel & Hirt 2004; Noctor & Foyer 1998). The cycle is depicted in figure 1.2. Changes in the ratio of ASA to DHA and GSH to GSSG can be used to detect oxidative stress (Sharma et al. 2012).

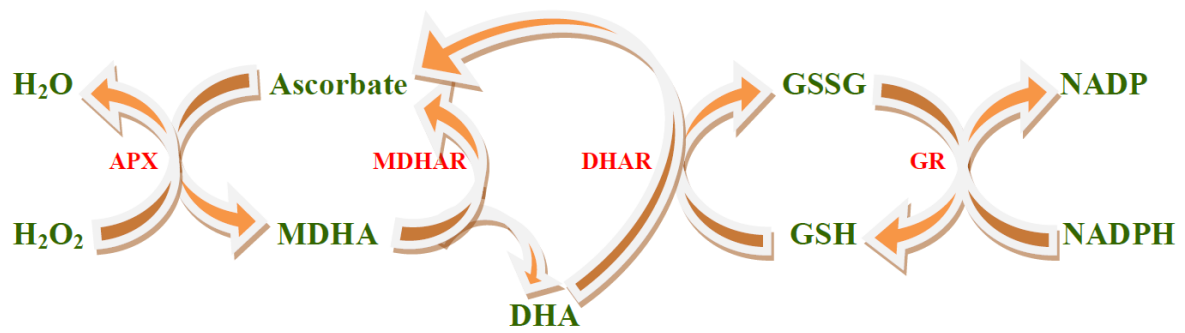


Figure 1.2. The Ascorbate-Glutathione cycle

APX-ascorbate peroxidase, DHA-dehydroascorbate, DHAR-dehydroascorbate reductase, GPX-glutathione peroxidase, GR-glutathione reductase, GSH-glutathione, GSSG-oxidised glutathione, H₂O-water, H₂O₂-hydrogen peroxide, MDHAR-monodehydroascorbate reductase, MDHA-monodehydroascorbate, NADP-nicotinamide adenine dinucleotide phosphate, NADPH-reduced NADP.

Glutathione Peroxidase

Plant GPXs, which are present mainly in chloroplasts, are thiol based enzymes having cysteine in their active site instead of seleno-cysteine, found in animal cells, and reduce H₂O₂ to water, using GSH (x2), which is oxidised to GSSG (Bela et al. 2015; Passaia & Margis-Pinheiro 2015). The oxidised GSSG is converted back to GSH by GR using NADPH as the reducing agent (Apel & Hirt 2004). The GPX cycle is shown in Figure 1.3.



Figure 1.3. Glutathione Peroxidase cycle

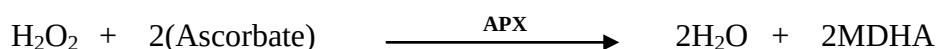
GSSG-oxidised glutathione, GSH-glutathione, GPX-glutathione peroxidase, GR-glutathione reductase, H₂O-water, H₂O₂-hydrogen peroxide, NADP-nicotinamide adenine dinucleotide phosphate, NADPH-reduced NADP.

In addition to its detoxification capability, GPX may also be involved in the maintaining of redox homeostasis in the cell by balancing the ratio of thiol/disulphide or NADPH/NADP⁺ (Bela et al. 2015).

1.5.5.2 Non-enzymatic antioxidants

Ascorbate

Ascorbate is found mainly in chloroplasts and the cytosol, and is a universal reductant and antioxidant in plants. It donates electrons in enzymatic as well as non-enzymatic antioxidant pathways and is the main ROS detoxifying compound in the aqueous phase (Ali & Alqurainy 2006). In addition to the direct scavenging of $O_2^{\cdot 1}$, $O_2^{\cdot -}$ and $\cdot OH$ (Ahmad et al. 2010; Noctor & Foyer 1998), it is considered to be a very important substrate for APX to detoxify H_2O_2 (Mehlhorn et al. 1996). APX utilizes two molecules of ascorbate for the reduction of H_2O_2 into water with the consequent release of two molecules of MDHA (Noctor & Foyer 1998) as given below:



Glutathione

GSH, synthesized in cytosol and chloroplasts (Noctor & Foyer 1998), is a tripeptide bearing a thiol group and reacts with H_2O_2 via GPX to form oxidized glutathione (GSSG), a disulphide molecule. It also can directly reacts with other ROS such as $O_2^{\cdot 1}$, $O_2^{\cdot -}$ and $\cdot OH$ and has a vital role in the regeneration of ascorbate via ascorbate-glutathione cycle (Ali & Alqurainy 2006; Arora et al. 2002).

A high ratio of reduced to oxidized ascorbate and glutathione is essential to scavenge ROS in cells (Apel & Hirt 2004). For example, this ratio was found to decline in cold sensitive tomato genotypes when chilling stress was induced (Walker & Mckersie 1993). Mutants with decreased ascorbate levels or altered GSH content are hypersensitive to stress (Apel & Hirt 2004).

Other non-enzymatic antioxidants include tocopherols, carotenoids and phenolics (Apel & Hirt 2004). Tocopherols (α , β , γ and δ) are comprised of a group of lipophilic antioxidants, which are involved in scavenging oxygen free radicals (Diplock et al. 1989). Among the four types, α -Tocopherol has the highest antioxidant capacity (Kamal-Eldin & Appelqvist 1996). It is a membrane-associated antioxidant that scavenges singlet oxygen and lipid peroxides, whereas carotenoids, which also belong to the group of lipophilic

antioxidants, attack singlet oxygen (Arora et al. 2002). Carotenoids also stabilise excited chlorophylls (Chl* and 3Chl*) to prevent the formation of O_2^1 and thereby protect the photosynthetic apparatus (Sharma et al. 2012). The phenolics are a group of secondary metabolites having antioxidant capacity, consisting of the flavonoids, tannins, hydroxycinnamate esters and the structural polymer lignin (Grace & Logan 2000). They can act as antioxidants by donating electrons or hydrogen atoms and can directly scavenge ROS and inhibit lipid peroxidation, by trapping the lipid alkoxyl radical (Sharma et al. 2012).

1.5.6 THE ROLE OF ANTIOXIDANTS IN CRYOPRESERVATION

The antioxidants can counteract oxidative reactions, which reduce cell viability, and thereby improve cell recovery after cryopreservation (Uchendu et al. 2010a). Several studies have reported that a higher cell antioxidant status confers protection against ROS in plants (Bela et al. 2015; Johnston et al. 2007; Prasad 1996). Dhindsa and Matowe (1981) observed an increase in SOD and catalase activity in drought tolerant moss, whereas enzyme activity was less in drought sensitive moss, when both the mosses are subjected to dehydration. Likewise, cold tolerant tomato species have higher total ascorbate and glutathione levels under chilling stress compared to sensitive genotypes (Walker & Mckersie 1993).

Plant cell alters their antioxidant activity in response to low temperature in order to protect cell membranes from damage (Xu et al. 2008). For example, acclimated maize seedlings have an elevated level of antioxidant enzymes and show chilling tolerance compared to non-acclimated seedlings. The accumulation of higher levels of ROS in the latter induced the oxidation of proteins and lipids and also inhibited protease activity (Prasad 1996). Chilling stress resulting from the exposure of plants to low temperature and increases the activity of SOD (Sala 1998), GPX (Song et al. 2006) and GR (Lee & Lee 2000).

Sucrose preculture enhances the antioxidant status of olive somatic embryos, especially GR activity, and thus enhances antioxidant protection during post-cryo recovery when stressed cells need to preserve membrane stability (Lynch et al. 2011). However, the GR activity was inhibited while cryopreserving the common carp sperm after treating with DMSO for 15 min (Li et al. 2010). During the cryopreservation of oocytes of Greenshell mussel, the cryoprotectants used significantly increased protein carbonyls (PCs) and lipid peroxide (LPs) levels and also enhanced the levels of antioxidant enzymes, but when cooled, GPX activity remained the same whereas the levels of PCs and LPs elevated and CAT and

SOD showed slightly lower activities (Gale et al. 2014). Bilodeau et al. (2000) also reported decreased SOD and GSH levels after cryopreservation of bovine sperm. Alvarez and Storey (1992) observed loss of SOD activity in human sperm cells after cryopreservation. But supplementation of antioxidants enhanced protection of the membrane with increased SOD activity and lower MDA production (Beconi et al. 1993).

1.6 THE OBJECTIVES AND RATIONALE BEHIND THIS THESIS

Kiwifruit (*Actinidia spp*) is one of nature's perfect foods: low in calories and glycemic index, high in energy and is an excellent source of antioxidants. Kiwifruit delivers a world of nutrition benefits including vitamin C, fibre, potassium, antioxidants, magnesium, lutein, folate, zinc and vitamin E (Kiwifruit 2014). Kiwifruit accounts for about 0.22 % of the total major fruit crop production in the world (Wang & Gleave 2012). Cryopreservation is suggested as an excellent means of preserving germplasm of kiwifruit for long-term storage. However, very few reports on survival have been published so far despite many attempts of cryopreservation, with slow freezing method (Liu et al. 1998), ED (Bachiri et al. 2001; Suzuki et al. 1997; Suzuki et al. 1994; Suzuki et al. 1996; Wu et al. 2000) and vitrification (Xu et al. 2006b). Moreover, the method of droplet vitrification that has been tested successfully in many species in recent years (Panis 2009) has not been attempted on kiwifruit. Therefore, in this research, droplet vitrification was attempted with and without different pre-treatments to understand the protective role of each step and their combined effect.

Understanding the biochemical status and ultrastructural changes in cells during cryopreservation is important to improve the method further. Hence, antioxidant marker assays and transmission electron microscopy (TEM) were performed at different stages of cryopreservation to study the cellular changes. Although DV is considered to be an evolving generic method (Panis et al. 2009), it needs to be optimised for different species. Cold acclimation, sucrose preculture (Reed 1996), antioxidant treatments (Uchendu et al. 2010b) and the time of dehydration (Panis et al. 2009) are all-important steps in DV. Therefore, this research attempted to optimise CA, pre-treatment with sucrose and incorporation of ASA in media for *A. chinensis*.

AIMS

1. To study the application of DV in *Actinidia chinensis* and to develop a DV protocol for the conservation of kiwifruit germplasm.
2. To determine the significance of CA and sucrose preculture in the DV of *A. chinensis* and to maximise regrowth after cryopreservation by optimising mPVS2 treatment period and testing the effect of ASA during preculture and in subsequent steps of DV.
3. To investigate antioxidant metabolism at different stages of DV using antioxidant markers.

4. To investigate the ultrastructural changes/damages of kiwifruit cells during cryopreservation.

2 CRYOPRESERVATION OF *A. chinensis* ‘Hort16A’ BY DROPLET VITRIFICATION

2.1 INTRODUCTION

Actinidia is a diverse genus in the family *Actinidaceae* consisting of perennial, climbing or scrambling, generally deciduous vines (Monette 1995). It is vegetatively propagated horticultural crop (Bachiri et al. 2001), which has 55 species and about 76 taxa widely distributed in Eastern and Southern parts of Asia (Li et al. 2009). It was introduced to New Zealand from its native China about 1906 (Heatherbell 1975) and the first commercial orchards were established in New Zealand by about 1930 (Ferguson & Seal 2008). In 1999, *A. chinensis* cultivar ‘Hort16A’ was developed by Plant and Food Research (PFR), New Zealand through controlled hybridisation (Datson & Ferguson 2011; Wang & Gleave 2012). PFR is the only research provider to the New Zealand Kiwifruit Research Consortium, jointly funded by the Ministry of Business, Innovation and Employment and ZESPRI® Group Ltd and having the largest *Actinidia* germplasm outside China (PFR 2015).

As in many other plant species, genetic erosion due to biological and climatic hazards is haunting kiwifruit (Bachiri et al. 2001). For example, in November 2010, the bacterial canker of kiwifruit, *Pseudomonas syringae* pv. *actinidae* (Psa) was detected in Bay of Plenty regions that resulted in its spread to more than 1200 New Zealand orchards and is still causing widespread vine deaths (Beatson et al. 2012). This outbreak has triggered many breeding programs and conservation strategies. Preservation of an extensive gene pool for *Actinidia* species can ensure the availability of a wide variety of desirable alleles for breeding programmes (Monette 1995) and moreover, the conservation of *Actinidia* is critical for future kiwifruit industry development (Li et al. 2014b).

Different cryopreservation methods have been tested for the development of long-term conservation of *Actinidia* germplasm. Liu et al. (1998) and Li and Guo (1996) applied slow freezing method to cryopreserve *A. arguta* and *A. deliciosa* respectively. Xu et al. (2006b) and Hakozaiki et al. (1996) used PVS2-vitrification method for the cryopreservation of *A. chinensis* shoot tips and seedling-derived hypocotyl calli of *A. deliciosa* cv. ‘Hayward’ respectively. Bachiri et al. (2001) utilized ED method to cryopreserve nine *Actinidia* species. *A. deliciosa* (Suzuki et al. 1994; Wu et al. 2000) and *A. chinensis* (Wu et al. 2000) were also successfully cryopreserved by ED method. Thus researchers have tested slow freezing

method, ED and vitrification to cryopreserve *Actinidia* species, but there are no reports on the use of DV in *Actinidia*. This part of the project was aimed at optimizing a DV protocol for *A. chinensis*, using ‘Hort16A’ as a model. Shoot tips of *in vitro* plantlets containing the apical meristem was chosen as the explant for the experiments.

2.2 MATERIALS AND METHODS

2.2.1 TISSUE CULTURE MEDIA USED

Media were prepared using stock solutions (20x for macronutrients and 200x for micronutrients, iron source and vitamins. All the chemicals were supplied by Sigma Aldrich®, New Zealand, unless otherwise mentioned.

- Basal medium (BM): Murashige and Skoog (1962) (MS) macronutrients, MS micronutrients and B5 vitamins (Gamborg et al. 1968), 0.09 M sucrose and 7.5 g/L agar (Davis) - Bacteriological grade (NZ manuka Bioactives, Opotiki, New Zealand). Approximately 50 mL of media were used per 290 mL clear wide-mouth disposable polystyrene tissue culture tub.
- Elongation medium: BM media supplemented with 0.492 μ M Indole-3-butyric-acid and 0.912 μ M Zeatin. Approximately 50 mL of media were used per 290 mL clear wide-mouth disposable polystyrene tissue culture tub.
- Preculture medium: Half-strength MS macronutrients, MS micronutrients and Linsmaier and Skoog (1965) vitamins, solidified with agar (Davis) 7.5 g/L containing different sucrose levels such as 0.09, 0.25, 0.5, 0.75 and 1.0 M. Approximately 25 mL of media were used in each 90x15 mm disposable polystyrene Petri plate.
- Loading solution: Liquid basal medium with half-strength MS macronutrients containing 2 M glycerol and 0.4 M sucrose was filter-sterilized. The total molarity of the solution was 2.4. Approximately 20-30 mL was taken in a sterile 100 mL glass beaker for treating each replicate.
- Vitrification solution: The original PVS2 (Sakai et al. 1990) was modified by replacing MT medium with MS medium and 0.4 M sucrose. The solution constitutes of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol and 15 % (w/v) DMSO in MS medium. The modified PVS2 (mPVS2) was filter-sterilized after adjusting to pH 5.8. The total molarity of the solution was 7.8. Approximately 20-30 mL was taken in a sterile 100 mL glass beaker for treating each replicate.
- Recovery solution: Liquid basal medium with half-strength MS macronutrients containing 1.2 M sucrose was filter sterilized. The total molarity of the solution was 1.2. Approximately 40-50 mL was taken in a sterile 100 mL glass beaker for treating each replicate.

- Recovery media: BM having half MS macronutrients with 0.58 M sucrose. Approximately 25 mL of media were used per 90x15 mm disposable polystyrene petriplate.
- Regeneration medium: BM supplemented with 0.537 μ M 1-Naphthaleneacetic acid, 4.439 μ M 6-Benzylaminopurine and 13.686 μ M Zeatin solidified with phytagel 2.5 g/L. Approximately 25 mL of media were used per 90x20 mm disposable polystyrene Petri plate.

For all the media, pH was adjusted to 5.8 using NaOH or HCl prior to autoclaving at 120 °C for 20 min or filter sterilization. The loading and recovery solution and mPVS2 were filter-sterilised using 0.45 μ m nitrocellulose MFTM Membrane Filters (Merck Millipore Ltd., Tullagreen, Carrigtwohill Co. Cork Ireland). The filtration unit was set up with a vacuum pump. Filter sterilized ASA (0.4 mM) (Uchendu et al. 2010a) was added to the media after autoclaving the media or after filter sterilizing the solutions. Sterile Whatman Qualitative Grade 2 filter papers were used for holding explants on solid media in plates after their excision and until plant regeneration. The preculture was initiated by transferring the filter paper holding the explants to the preculture media with 0.09 M sucrose; this was followed by daily transferring the filter paper with shoot tips to increasing concentration of sucrose media until 0.75 M or 1.0 M sucrose. Sterile aluminium foil strips of size 5x25 mm were used for holding shoot tips for freezing and the samples were stored in LN for at least 1h during freezing step.

2.2.2 PLANT MATERIAL

A. chinensis ‘Hort16A’ stock plants were multiplied and grown in elongation media and maintained in culture rooms at PFR, Palmerston North. The shoot tips with 3-4 leaves from 6-7 weeks old plants were subcultured and maintained in tubs with elongation media. The temperature of the culture rooms were maintained at 24 $\pm$ 1 °C and a 16 h photoperiod was supplied at a photosynthetic flux density of 40 μ mol s⁻¹ m⁻² is provided by Philips cool-white 18 W fluorescent tubes. All the tissue culture procedures and the experiments were carried out under aseptic conditions using a horizontal flow laminar air hood.

2.2.3 EXPERIMENTS CONDUCTED FOR DEVELOPING A DROPLET VITRIFICATION PROTOCOL FOR *A. chinensis* 'Hort16A'

Several experiments were conducted to understand the response of 'Hort16A' shoot tips to dehydration and cryopreservation and to optimise pretreatment conditions. The preconditioning treatments used were CA at 4 ± 1 °C for 7 days and/or preculture in preculture media with increasing sucrose concentrations until 0.75 M or additional 1.0 M with or without ASA. The shoot tips 1.5-2 mm length excised from 4-6 weeks old *in vitro* plantlets were used in the experiments.

2.2.3.1 Testing the tolerance of shoot tips to dehydration with mPVS2

- (a) The buds were excised from the plantlets taken from the culture room and held on a filter paper placed on preculture media with 0.09 M sucrose.
- (b) The excised buds were treated for 20 min in loading solution at room temperature.
- (c) The explants were then dehydrated in mPVS2 at 0 °C for 0, 20, 30 and 40 min.
- (d) Dehydrated explants were mounted with a drop of mPVS2 on a sterile aluminium foil strip placed on a Petri plate on a gel ice pack. The aluminium strip with the droplet was quickly immersed in LN. The control consisted of shoot tips that were not dipped in LN after 4 treatment periods in mPVS2.
- (e) After a minimum of 1 h, the frozen tissues were thawed by quickly transferring the foils with the buds into recovery solution and treated for 20 min at room temperature. The recovery solution was changed twice to remove mPVS2.
- (f) The washed buds were incubated in solid recovery media for 24 h in dark.
- (g) The buds were then transferred to the solid regeneration media and placed in dark under culture room conditions. After 7 days in dark, the plates with shoot tips were exposed to light in culture room.
- (h) The survival was recorded as the formation of green tissue developing from the buds and regeneration was recorded when a minimum of two leaves arise from the emerging bud. Final count was recorded after 8 weeks on regeneration medium.

2.2.3.2 Testing the tolerance of buds to sucrose preculture with different mPVS2 treatment periods

- (a) The buds excised from plantlets were pretreated in preculture media supplemented with 0.09, 0.25, 0.5 and 0.75 M sucrose. The explants were precultured by daily increasing sucrose concentration until 0.75 M on day 4.
- (b) The explants were then treated in loading solution for 20 min at room temperature.
- (c) The buds were then dehydrated in mPVS2 for 25 min or 35 min.

Steps (d) – (h) were repeated as in 2.2.3.1

The control treatments consisted of shoot tips that were not dipped in LN.

2.2.3.3 Response of 'Hort16A' buds to mPVS2 toxicity

- (a) The buds were excised from the *in vitro* plantlets growing in culture room. The buds were held in four different plates containing basal media.
- (b) The explants were then treated in loading solution for 20 min at room temperature.
- (c) They were then subjected to dehydration in the vitrification solution at different timings- 0, 20, 30, 40 and 50 min.
- (d) After each timing, the buds were taken out from the solution in batches and washed in recovery solution for 20 min at room temperature.
- (e) Each batch was transferred to a separate recovery plate and incubated in dark at 24 °C for 24 h.
- (f) Then the filter paper with the buds were transferred to regeneration plates and maintained in the dark for 7 days and later exposed to light in the culture room.
- (g) The toxicity of mPVS2 was determined by counting the number of regenerated plantlets compared to the total number of buds cultured.

2.2.3.4 Comparison of the effect of combining cold acclimation with sucrose preculture

- (i) Cryopreservation with non-pretreated samples.
 - (a) The buds were isolated from plantlets grown in culture room.
 - (b) The explants were then treated in loading solution for 20 min at room temperature.

(c) The buds were then dehydrated in mPVS2 for 30 min.

Steps (d) – (h) were repeated as in 2.2.3.1

(ii) Cryopreservation after cold acclimation.

(a) The donor plantlets were maintained in cold chamber set at 4 ± 1 °C for 7 days and the buds were excised from the acclimated plantlets.

(b) The explants were then treated in loading solution for 20 min at room temperature.

(c) The buds were then dehydrated in mPVS2 for 30 min.

Steps (d) – (h) were repeated as in 2.2.3.1

(iii) Cryopreservation with the combination of cold and sucrose treatments.

(a) The donor plantlets were maintained in cold chamber set at 4 ± 1 °C for 7 days. The buds were excised from the acclimated plantlets and passed through sucrose series with daily increasing sucrose concentrations until 0.75 M.

(b) The explants were then treated in loading solution for 20 min at room temperature.

(c) The buds were then dehydrated in mPVS2 for 30 min.

Steps (d) – (h) were repeated as in 2.2.3.1

2.2.3.5 Testing the effect of sucrose preculture and ascorbic acid with and without cold acclimation on viability after cryopreservation

(i) Cryopreservation with ascorbic acid in treatment solutions after sucrose preculture (without cold pre-treatment of stock plants).

(a) The buds excised from the plantlets growing in culture room were pretreated on sucrose-supplemented media until 0.75 M by daily increasing sucrose concentration.

(b) The explants were treated in loading solution supplemented with ASA for 20 min.

(c) The buds were then dehydrated in mPVS2 supplemented with ASA for 30 min at room temperature.

(d) Then the explants were mounted with a drop of mPVS2 on an aluminium foil strip placed on a Petri plate on a gel ice pack. The aluminium strips were then directly plunged into LN.

- (e) The frozen tissues were thawed by rinsing the buds in recovery solution supplemented with ASA for 20 min at room temperature.
- (f) The washed buds were incubated on solid recovery media for 24 h in dark at 24 °C.
- (g) The buds were then transferred to the regeneration media, maintained in dark for 7 days and transferred to light in the culture room.
- (h) The survival was recorded as the formation of green tissue developing from the buds and regeneration was recorded when a minimum of two leaves arise from the tissue.

(ii) Cryopreservation with ascorbic acid in treatment solutions after sucrose preculture following cold acclimation.

- (a) The donor plantlets were maintained in a cold chamber at 4±1 °C for 7 days. The buds were excised from these acclimated plantlets and passed through sucrose series with daily increasing sucrose concentrations until 0.75 M.

Steps (b) – (h) were repeated as in 2.2.3.5 (i).

2.2.3.6 Pretreatment effect on plant growth

The effect of cold and ASA in combination with the sucrose media on ‘Hort16A’ was studied by comparing the growth of shoot tips from each treatment. The treatments selected for this study were:

- (i) Control: The shoot tips were excised from plantlets taken from culture room conditions and grown on regeneration medium in Petri plates for 2 weeks followed by transferring them to multiplication medium in tubs.
- (ii) Sucrose: The shoot tips were excised from culture room-grown plantlets and were precultured in media by daily increasing sucrose concentration (0.09, 0.25, 0.5 and 0.75 M). The Petri plates containing sucrose media holding shoot tips were maintained in dark in culture room during preculture period. After the 24 h incubation in preculture media supplemented with 0.75 M sucrose, the shoot tips were transferred to regeneration media for 2 weeks followed by growing in multiplication medium in tubs.
- (iii) Sucrose + ASA: The procedure in 2.2.3.6 (ii) was repeated except that the preculture media were supplemented with ASA.

(iv) CA + sucrose: The donor plantlets were cold acclimated at 4 ± 1 °C for 2 weeks followed by the preculture and regeneration as in 2.2.3.6 (ii).

(v) CA + sucrose + ASA: The donor plantlets were cold acclimated at 4 ± 1 °C for 2 weeks followed by the preculture and regeneration as in 2.2.3.6 (iii).

The growth and morphology of the explants on regeneration media was compared after 5 weeks in all the treatments.

2.2.3.7 Effect of sucrose in combination with cold acclimation and ascorbic acid during droplet vitrification

As sucrose concentration at 0.75 M for one day did not inhibit the growth of explants, this experiment was designed to test the effect of higher sucrose concentrations on regeneration ability of shoot tips. The final concentration of sucrose in the preculture media at 0.75 M for one day was compared with, 0.75 M for 3 days and an additional 1.0 M for one day.

Explants excised from cold acclimated (4 ± 1 °C for 2 weeks) plantlets were treated with the three different sucrose preculture concentrations. The media were supplemented with ASA throughout from 0.09 M sucrose preculture plates until recovery media. The procedure of DV was repeated as mentioned earlier

2.2.4 OPTIMISING THE mPVS2 TREATMENT PERIOD FOR DROPLET VITRIFICATION OF 'Hort16A'

To develop a robust droplet vitrification protocol, the response of shoot tips to optimised pre-treatment conditioning achieved in preliminary experiments was tested against the control shoot tips excised directly from the plantlets growing under culture room conditions (pre-treatment control). The pre-treatment included CA, sucrose preculture and supplementation of ASA as described below in detail. The treatment duration in loading solution, mPVS2, recovery solution, recovery plate and regeneration plate was same for both the pretreated samples and pre-treatment control samples. The experiments were replicated thrice and a minimum of 10 shoot tips plated on a single Petri dish represented a replicate.

2.2.4.1 Preconditioning of explants

- Cold Treatment: In order to cold acclimate the donor plantlets, 4-6 weeks old *in vitro* plantlets in tissue culture tubs were maintained in a cold chamber provided with constant 4 ± 1 °C and 10 h photoperiod supplied at a photosynthetic flux density of $25 \mu\text{mol s}^{-1}\text{m}^{-2}$. The plantlets were maintained under these conditions for 2 weeks.
- Preculture: Apical buds, about 2 mm length containing shoot tips along with two leaf primordia were dissected from the cold acclimated *in vitro* plantlets. The excised shoot tips were laid on preculture media with 0.09 M sucrose and the preculture was performed by daily increasing sucrose concentration as described in preliminary experiments [Experiment 6 (iii)] until the treatment with 1.0 M sucrose on 5th day for 24 h. During the preculture period, the explants were incubated in a cold chamber (4 ± 1 °C) in dark.

2.2.4.2 Loading treatment

Approximately 30 mL of ice-cold loading solution was taken in a 100 mL glass beaker maintained on ice. ASA was added and mixed. The pretreated samples were then transferred to this solution and treated for 40 min. The solution was swirled every two minutes to prevent the settling of the glycerol and sucrose at the bottom. The pre-treatment control samples were excised directly from culture room-grown plantlets without any pre-treatment and incubated

in preculture media with 0.09 M sucrose for 24 h in dark at 24 °C prior to loading treatment without the supplementation of ASA.

2.2.4.3 Optimisation of mPVS2

The loading solution was removed with a 5 mL pipette and approximately 30 mL of thoroughly mixed, ice cold mPVS2 supplemented with ASA was quickly added. The pretreated samples were treated in the vitrifying solution for different periods (0, 30, 40, 50 and 60 min) with swirling every 2 min. Pretreatment controls were treated in the same way as pretreated samples except the addition of ASA to mPVS2.

The two controls consisted of shoot tips either pretreated or not pretreated were transferred to recovery solution after dehydration in mPVS2.

2.2.4.4 Freezing

The freezing was carried out in LN taken in chilli bins. Two chilli bins were used to freeze both +LN samples (pretreated samples- +LN and pre-treatment control samples- +LN) separately. Prior to freezing, 2-3 shoot tips were placed on a sterile aluminium foil strip and mounted with a droplet of vitrifying solution. The strips were maintained ice-cold by placing them on Petri plates on a gel-ice pack. The strips holding the explants were carefully picked up with forceps and quickly immersed in LN without disturbing the droplet of mPVS2 mounted on the samples. The samples were kept in frozen state for a minimum of 1 h.

2.2.4.5 Thawing

The thawing step was done by washing the explants in recovery solution for 20 min. The solution was supplemented with ASA for treating pretreated samples.

The -LN samples were washed twice with fresh recovery solution taken in a glass beaker. The explants were treated at room temperature with gentle mixing of the medium.

For thawing +LN samples, a glass beaker containing recovery solution was held half immersed in a water bath set at 35 °C for few minutes prior to the addition of frozen samples. After a minimum of one hour of freezing, aluminium strips holding +LN samples were picked from LN with forceps and quickly immersed in the warmed recovery solution. Once all the

strips were transferred to the washing solution, the beaker was swirled gently for one minute. Then the beaker was removed from the water bath and maintained at room temperature inside the laminar hood for a further 19 minutes. During this period, the aluminium strips were removed and the LN⁺ samples were rinsed twice with fresh recovery solution with gentle swirling to ensure proper distribution of the concentrate.

2.2.4.6 Recovery

The recovery plate treatment was same for all the samples except that the recovery agar media plates were not supplemented with ASA for the treatment of pre-treatment control samples (+LN and -LN). A sterile filter paper was laid over solid recovery media in each Petri plates. The shoot tips were gently picked from the recovery solution with forceps and placed on the filter paper. The Petri plates were sealed and wrapped with aluminium foil to keep them in dark at culture room temperature. The samples were incubated on recovery plates for 24 h.

2.2.4.7 Regeneration

The filter papers holding the samples were transferred to the plant growth regulator-enriched regeneration media. The regeneration plates were wrapped with aluminium foil in order to incubate the samples in dark for 7 days at 24 °C. After the dark incubation, the aluminium foil was removed and explants were exposed to normal culture room conditions. The survival was recorded as the formation of green tissue developing from the buds and regeneration was recorded when a minimum of two leaves arise from the emerging bud. Final count was recorded after 8 weeks on regeneration media.

2.2.5 STATISTICAL ANALYSIS

For assessment of plant regeneration after cryopreservation experiments, a minimum of 10 shoot tips was used per replicate, and three replicates per treatment in a randomised block design were established and analysed as a binomial generalised linear model (GLM) with a logit link. The proportion of explants that successfully regenerated was analysed for different treatments in the preliminary experiments. For the final experiment (as described in

section 2.3.2 below), the effect of mPVS2 duration on the proportion of explants that successfully regenerated was analysed separately for unfrozen samples and frozen samples. As none of the cryopreserved shoot tips that were not pretreated survived (pre-treatment control +LN – 0 % survival), and all the non-pretreated shoot tips that were not cryopreserved (LN control) grew in regeneration media (100 % survival), these two control treatments were excluded from the final analysis. The regeneration response after two months in different mPVS2 treatment times was analysed for the pretreated LN control and cryopreserved treatments. The null hypothesis tested was that there is no significant effect of mPVS2 duration on the proportion of explants that survive after cryopreservation in shoot tips from CA plantlets that were pretreated with sucrose and ASA. The statistical software GenStat 17th edition (VSN International) was used for all analyses. When the treatment effect was significant, tests of mean separation between all the treatment times were conducted using contrasts and analysis of deviance.

2.3 RESULTS

2.3.1 TESTING OF DIFFERENT PRE-TREATMENTS FOR THE DEVELOPMENT OF A DROPLET VITRIFICATION PROTOCOL FOR *A. chinensis* 'Hort16A'

The regeneration data recorded from Experiment 2.2.3.1 showed that shoot tips dehydrated for periods from 0 – 40 min in mPVS2 without sucrose preculture and cold acclimation resulted in only 0 – 2.8 % regeneration after DV (Figure 2.1).

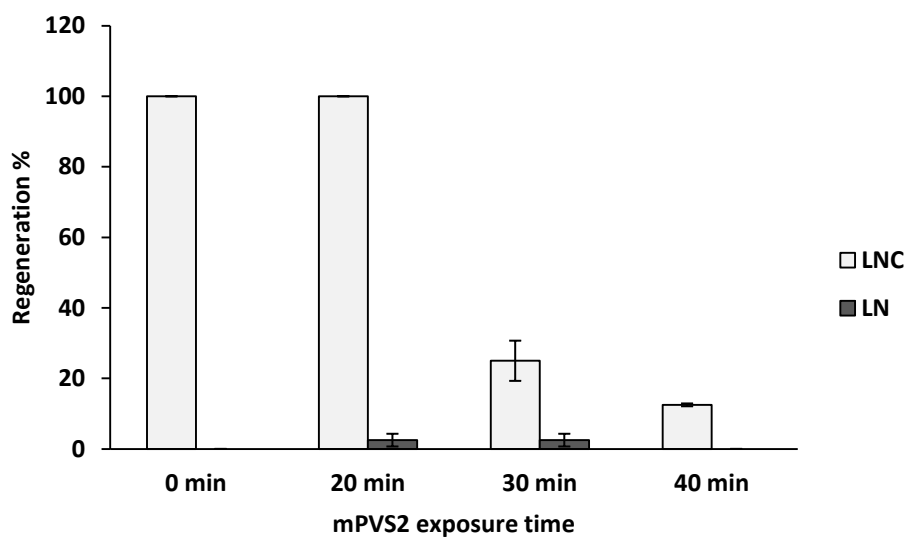


Figure 2.1. Regeneration percentage of cryopreserved 'Hort16A' shoot tips in response to mPVS2 exposure

Regeneration percentage after droplet vitrification of *A. chinensis* 'Hort16A' shoot tips excised from the plantlets directly from culture room in response to mPVS2 dehydration periods at 0, 20, 30 and 40min. Error bars are $\pm$ standard error of the mean and the bars with no standard error indicate the identical values of replicates; $n = 2$ (+LN in text is represented as LN and -LN is represented as LNC).

Because of poor regeneration results without pre-treatment, sucrose preculture was introduced with two time periods in mPVS2 in Experiment 2.2.3.2. The treatment of shoot tips in a series of increasing sucrose concentrations (0.09, 0.25, 0.5, 0.75 M over 4 days) resulted in a considerable increase in plant regeneration after DV (Figure 2.2), especially after 35 min in mPVS2.

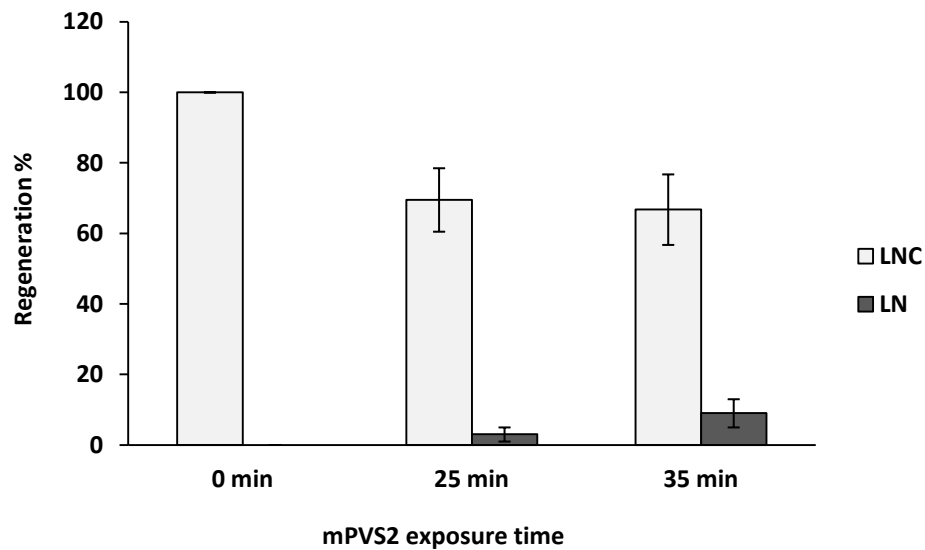


Figure 2.2. Regeneration rate after droplet vitrification of 'Hort16A' shoot tips in response to 0.75 M sucrose preculture and mPVS2

Regeneration rate after droplet vitrification of *A. chinensis* 'Hort16A' shoot tips after final preculture in 0.75M sucrose in response to mPVS2 dehydration periods at 25 and 35 min. Error bars are $\pm$ standard error of the mean and the bars with no standard error indicate the identical values of replicates; $n = 4$ (+LN in text is represented as LNC and -LN is represented as LN).

As 35 min treatment in mPVS2 resulted in an improvement in recovery after cryopreservation, to understand the response of shoot tips to the toxicity of mPVS2, an experiment was performed (Experiment 2.2.3.3) by exposing shoot tips to further higher duration in mPVS2 (0, 20, 30, 40 and 50 min) after pretreating the explants with 0.09, 0.25, 0.5, 0.75 M sucrose over a 4 day period. The regeneration result was inversely proportional to the length of mPVS2 time (Figure 2.3).

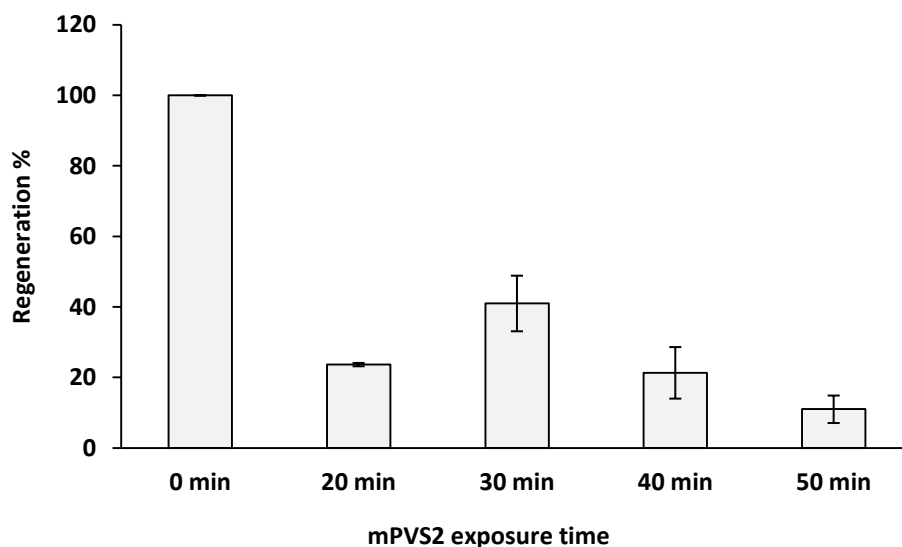


Figure 2.3. Regeneration rate of ‘Hort16A’ shoot tips after preculture in 0.75M sucrose in response to different mPVS2 dehydration periods

Regeneration rate of *A. chinensis* ‘Hort16A’ shoot tips after preculture in 0.75M sucrose in response to mPVS2 dehydration periods at 0, 20, 30, 40 and 50 min. Error bars are $\pm$ standard error of the mean and the bars with no standard error indicate the identical values of replicates; $n = 3$ (+LN in text is represented as LN and -LN is represented as LNC).

The 30 min mPVS2 time was selected for the next experiment (2.2.3.4) to compare the effect of cold acclimation prior to sucrose treatment on post-cryo regeneration. The combination of cold and sucrose treatments resulted in better regeneration than with cold alone. Cold with sucrose preculture were superior to control (Figure 2.4).

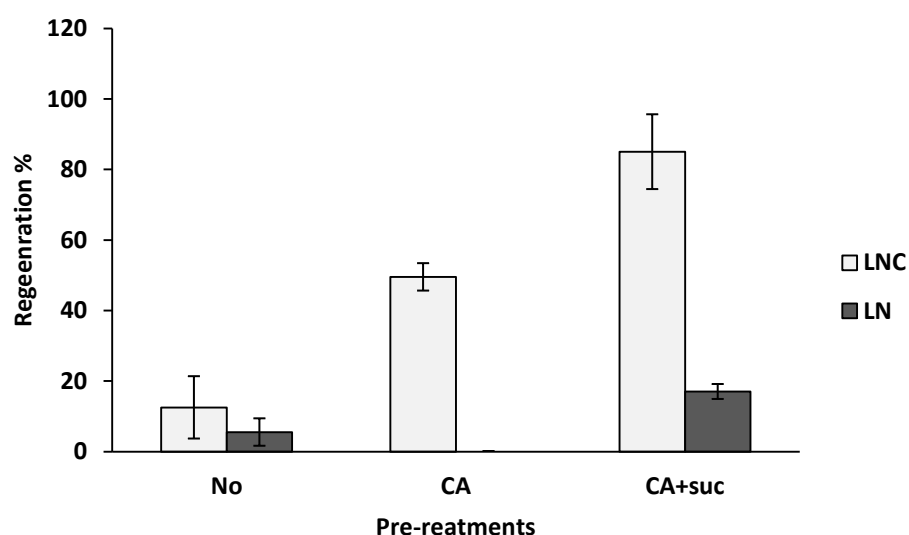


Figure 2.4. Regeneration rate after droplet vitrification of ‘Hort16A’ shoot tips in response to cold acclimation and its combination with sucrose preculture.

Regeneration rate of *A. chinensis* ‘Hort16A’ shoot tips after droplet vitrification, to compare the effect of cold acclimation (CA) with combination of cold acclimation followed by sucrose preculture (CA+suc) prior to cryopreservation with 30 min dehydration in mPVS2. No represents shoot tips with neither cold acclimation nor sucrose pre-treatment. Error bars are $\pm$ standard error of the mean; n = 2 (+LN in text is represented as LN and -LN is represented as LNC).

In the next experiment (2.2.3.5), ASA was included in the treatment solutions, and the effect of cold and sucrose treatments in combination with ASA supplementation on plant regrowth after cryopreservation was compared. Despite the inclusion of antioxidants, a notable regeneration difference after DV was not detected (Figure 2.5) suggesting that cold acclimation for 1week is not adequate for preconditioning of ‘Hort16A’ shoot tips for cryopreservation. Therefore, for the subsequent experiments, 2 weeks of cold acclimation was selected.

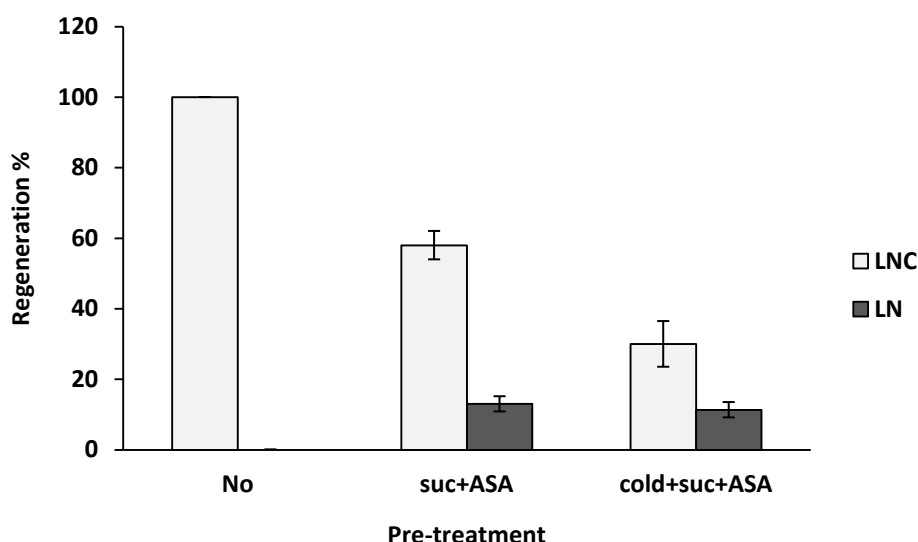


Figure 2.5. Regeneration rate after droplet vitrification of ‘Hort16A’ shoot tips in response to sucrose and ascorbic acid pre-treatment and cold acclimation.

Regeneration rate of *A. chinensis* ‘Hort16A’ shoot tips after droplet vitrification to test the efficiency of cold acclimation with sucrose preculture and ascorbic acid. Error bars are $\pm$ standard error of the mean and the bars with no standard error indicate the identical values of replicates, n = 3 (+LN in text is represented as LN and -LN is represented as LNC). No – no pretreatment, suc+ASA – pre-treatment with sucrose and ascorbic acid, cold+suc+ASA – pre-treatment with sucrose and ascorbic acid after cold acclimation at 4 °C for 2 weeks.

In Experiment 2.2.3.6, the effect of sucrose and ASA pretreatments were tested alone or in combination after 2 weeks of cold acclimation. The experiment was designed to understand the stress imposed by these treatments by studying the morphology of regenerated plants. The Figure 2.6 shows that most of the regenerated plantlets were struggling to grow

even after 5 weeks of recovery from sucrose preculture alone (Figure 2.6B). The severely stressed plantlets showed abnormal growth even later. The supplementation of ASA in the sucrose preculture media slightly reduced the stress level (Figure 2.6C), but not as much as with the cold acclimation prior to sucrose preculture (Figure 2.6D). In contrast, the combination of cold acclimation along with the ASA supplementation in the sucrose media showed notable difference in the plant growth (Figure 2.6E). All the regenerated plantlets regained normal growth within 4weeks and were morphologically similar to control plantlets (Figure 2.6A).

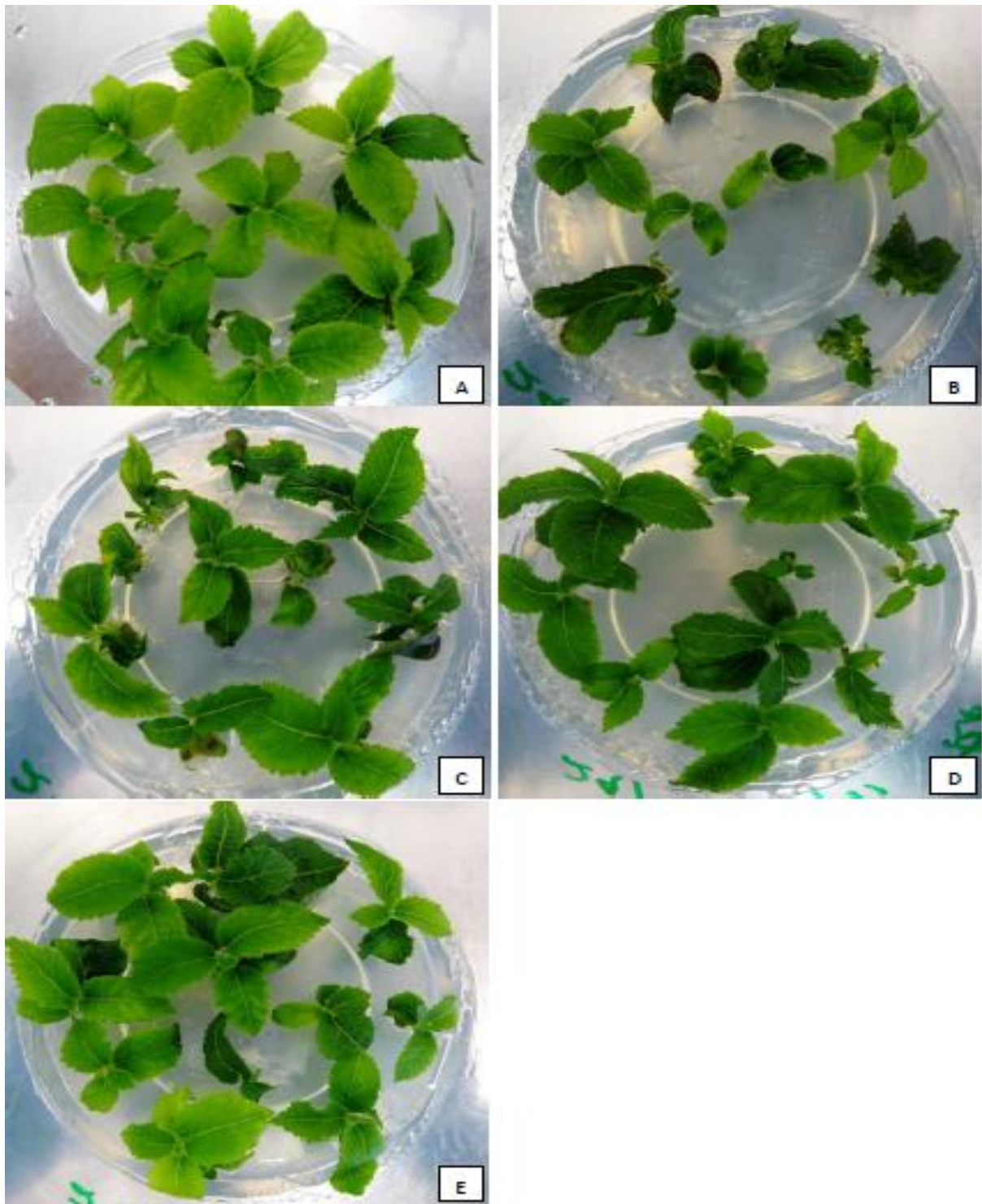


Figure 2.6. Regeneration of ‘Hort16A’ after different pre-treatments.

Regenerated plants after 5 weeks from preconditioning treatments with 0.25, 0.5 and 0.75 M sucrose in combination with or without cold acclimation and/or sucrose treatments. A– Control (shoot tips from culture room plantlets); B– After preculture in final 0.75 M sucrose media; C– After preculture in final 0.75 M sucrose media supplemented with ASA; D– After cold acclimation followed by preculture in final 0.75 M sucrose media; E– After cold acclimation followed by preculture in final 0.75 M sucrose supplemented with ASA (n=4).

As the preculture with 0.75 M sucrose along with cold acclimation and ASA supplementation did not show any suppression of growth, the sucrose treatment was extended to 0.75 M for 3 days or up to 1 M (2.2.3.7). The preculture with 1.0 M sucrose in combination with cold acclimation and ASA supplementation was found to be better than 0.75 M sucrose for 1 day or 3 days in post-cryo regeneration (Figure 2.7). Therefore, CA in combination with 1.0 M sucrose and ASA supplementation was used in the final experiment.

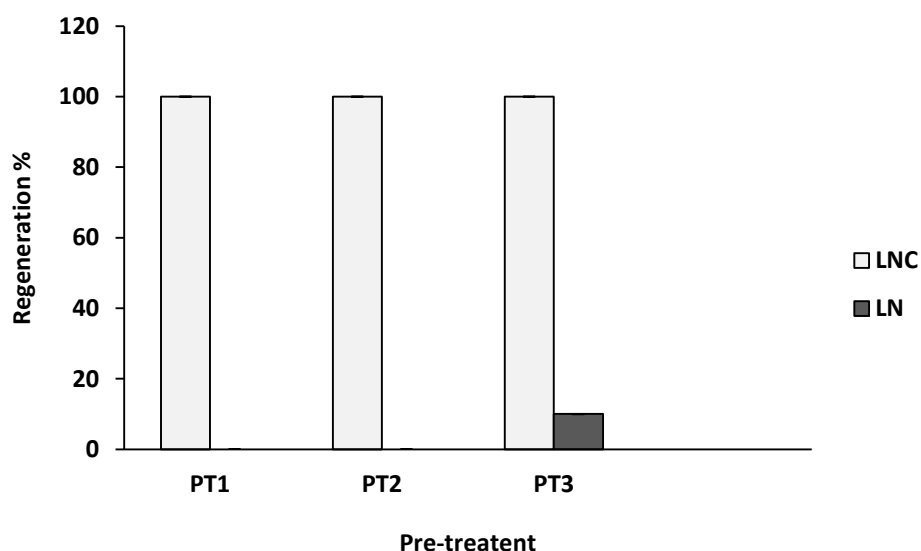


Figure 2.7. Regeneration percentage of cryopreserved ‘Hort16A’ shoot tips subjected to cold acclimation and different sucrose preculture regimes supplemented with ascorbic acid

The effect of the different sucrose treatments in combination with cold acclimation and ascorbic acid on regeneration rate after droplet vitrification of *A. chinensis* ‘Hort16A’ shoot tips after 30 min treatment in mPVS2. PT1- cold acclimation and preculture in final 0.75 M sucrose media supplemented with ascorbic acid for one day; PT2- cold acclimation and preculture in final 0.75 M sucrose media supplemented with ascorbic acid for three days; PT3- cold acclimation and preculture in final 1.0 M sucrose media supplemented with ascorbic acid for one day. Error bars are $\pm$ standard error of the mean and the bars with no standard error indicate the identical values of replicates, $n = 2$ (+LN in text is represented as LN and -LN is represented as LNC).

2.3.2 CRYOPRESERVATION RESPONSE OF *A. chinensis* 'Hort16A' SHOOT TIPS IN RESPONSE TO COLD ACCLIMATION FOR 2 WEEKS COMBINED WITH ASA AND SUCROSE PRE-TREATMENTS USING DIFFERENT MPVS2 DEHYDRATION TIMES

The final regeneration percentage of the shoot tips treated in mPVS2 (0-60 min) was recorded after 8 weeks of recovery. None of the cryopreserved shoot tips that were not pretreated survived (pre-treatment control +-LN 0 % survival), and all the non-pretreated shoot tips that were not cryopreserved (pre-treatment control -LN) grew in regeneration media (100 % survival) (Figure 2.8). These two control treatments were therefore excluded from the final analysis

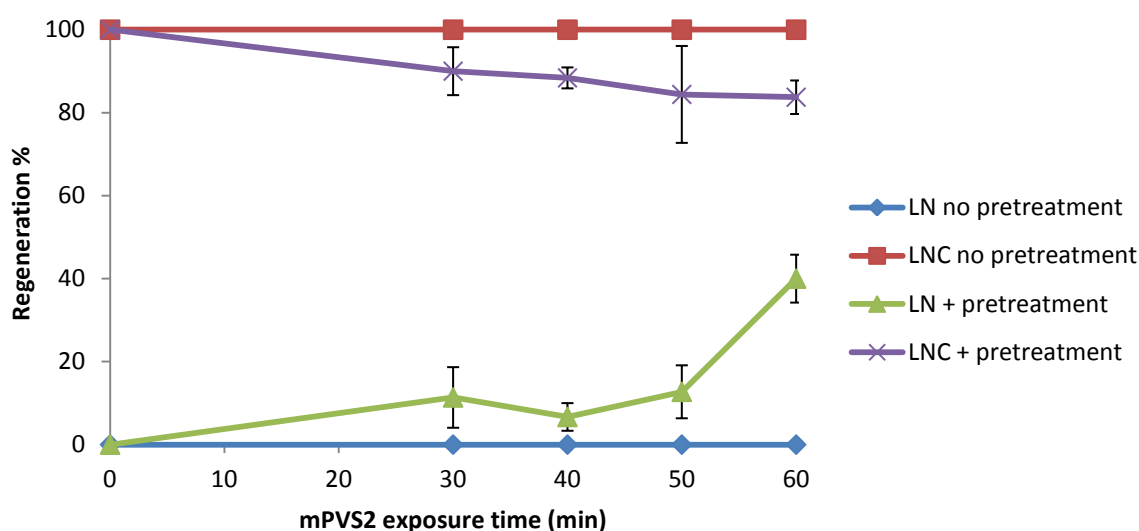


Figure 2.8. Regeneration of pretreated and non-pretreated shoot tips after cryopreservation in response to different mPVS2 dehydration periods .

Regeneration of cryopreserved shoot tips from plantlets of *A. chinensis* 'Hort16A' cold acclimated for 2 weeks at 4 °C followed by pre-treatment of shoot tips with sucrose (0.09, 0.25, 0.5, 0.75 and 1 M over five days) and 0.4 mM ascorbic acid. The shoot tips cryopreserved after different treatment periods with mPVS2 and compared with non-cryopreserved non-pretreated shoot tips (LNC no pre-treatment), non-cryopreserved pretreated shoot tips (LNC+ pre-treatment) and cryopreserved non-pretreated shoot tips (LN no pre-treatment). Error bars are $\pm$ standard error of the mean, n = 3 (+LN in text is represented as LN and -LN is represented as LNC).

All the pretreated shoot tips showed a slight decrease in regeneration with the increase in mPVS2 exposure time in control cultures without LN treatment (Figure 2.8). The pretreated shoot tips when cryopreserved showed an increase in regeneration percentage with increase in mPVS2 incubation times (Figure 2.8). The regeneration percentage after

cryopreservation (+LN) using different mPVS2 incubation periods was statistically significant (Table 2.1) at a probability of <0.001.

Table 2.1. Summary of analysis of regeneration percentage after cryopreservation of pretreated shoot tips.

Source	d.f.	deviance	mean dev	dev. ratio	P value
Regression	4	23.11	5.777	5.78	<0.001
Residual	10	10.64	1.064		
Total	14	33.75	2.411		

Pair-wise contrasts of mPVS2 treatment periods in cryopreserved pretreated shoot tips showed that 0 min treatment differed from that of the 30, 50 and 60 min treatments, but not the 40 min treatment (Table 2.2). The regeneration of cryopreserved shoot tips after 60 min treatment in mPVS2 was significantly greater than other mPVS2 durations tested (Table 2.2). However, the pretreated shoot tips that were not cryopreserved showed a significant difference only with 0 min mPVS2 treatment (Table 2.3). The mPVS2 treatment at 40, 50 and 60 min significantly differed from 0 min, but not 30 min (Table 2.3).

Table 2.2. Comparison of post-cryo regeneration of pretreated shoot tips of ‘Hort16A’ in response to different mPVS2 timings

Comparison of regeneration of cryopreserved shoot tips of cold acclimated *A. chinensis* ‘Hort16A’ subjected to pretreatment with sucrose (0.09, 0.25, 0.5, 0.75 and 1 M over five days) and 0.4 mM ascorbic acid among the mPVS2 duration groups. Contrasts with P-values (chi pr.) <0.05 were considered significant.

Source	deviance	d.f.	mean dev.	dev. ratio	chi pr.
0 min v 30 min	4.533	1	4.533	4.53	0.033
0 min v 40 min	2.142	1	2.142	2.14	0.143
0 min v 50 min	4.571	1	4.571	4.57	0.033
0 min v 60 min	19.999	1	19.999	20.00	<0.001
30 min v 40 min	0.507	1	0.507	0.51	0.476
30 min v 50 min	0.008	1	0.008	0.01	0.927
30 min v 60 min	7.213	1	7.213	7.21	0.007
40 min v 50 min	0.620	1	0.620	0.62	0.431
40 min v 60 min	11.029	1	11.029	11.03	0.001
50 min v 60 min	6.623	1	6.623	6.62	0.010

Table 2.3. Comparison of regeneration of the pretreated ‘Hort16A’ shoot tips before cryopreservation in response to different mPVS2 time periods

Comparison of shoot tips of cold acclimated *A. chinensis* ‘Hort16A’ subjected to pretreatment with sucrose (0.09, 0.25, 0.5, 0.75 and 1 M over five days) and 0.4 mM ascorbic acid among the mPVS2 duration groups. Contrasts with P-values (chi pr.) <0.05 were considered significant.

Source	deviance	d.f.	mean dev.	dev. ratio	chi pr.
0 min v 30 min	3.333	1	3.333	3.33	0.068
0 min v 40 min	4.533	1	4.533	4.53	0.033
0 min v 50 min	7.161	1	7.161	7.16	0.007
0 min v 60 min	7.199	1	7.199	7.20	0.007
30 min v 40 min	0.051	1	0.051	0.05	0.821
30 min v 50 min	0.579	1	0.579	0.58	0.447
30 min v 60 min	0.648	1	0.648	0.65	0.421
40 min v 50 min	0.295	1	0.295	0.29	0.587
40 min v 60 min	0.348	1	0.348	0.35	0.555
50 min v 60 min	0.003	1	0.003	0.00	0.959

The droplet vitrification protocol developed for ‘Hort16A’ shoot tips that resulted in a 40 % post-cryo regeneration success is presented schematically in Figure 2.9.

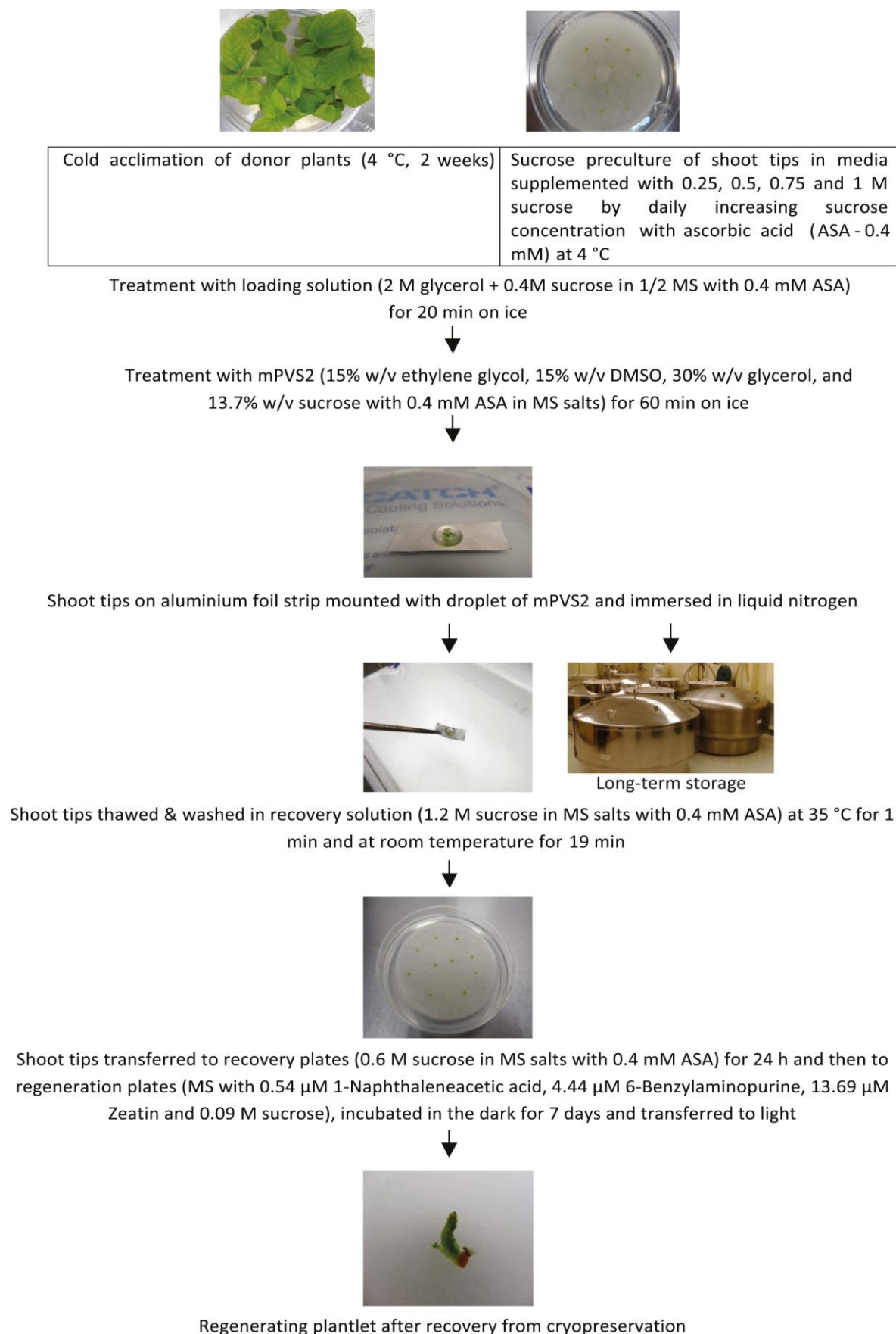


Figure 2.9. Droplet vitrification protocol developed for *A.chinensis* ‘Hort16A’

2.4 DISCUSSION

In this work, the possibility of successful application of DV technique for the cryopreservation of *A. chinensis* 'Hort16A' was confirmed. In addition, this is the first DV protocol successfully applied to *Actinidia* species. Shoot tip was preferred as the explant as it has meristematic-rich region that gives rise to direct shoot formation during regeneration without any transitory callus which may lead to mutations (Kaczmarczyk et al. 2012) and maintains genetic stability by the direct regrowth (Engelmann et al. 2008). Choice of shoot tips as explants also allows the initiation of cryoprotective treatments even from culture stage (Benito et al. 2004). Another advantage of shoot tips as explants is that the meristematic region that regenerates after cryopreservation has very little chance of being infected with endophytic microorganisms carried by the donor plant (Wang et al. 2009; Wang & Valkonen 2009b). Therefore it allows regeneration of high-health planting material after cryopreservation.

Preconditioning has a pronounced role in enhancing survival after cryopreservation. Based on the results of initial experiments, preconditioning showed a positive effect on the post-cryo regeneration against the control. Hence, further experiments were focused on different concentrations and durations of pre-treatments, and their combinations. These different combinations resulted in a notable difference in the morphology of regenerated plants after the treatments. The severely stressed plants after sucrose preculture alone showed that plants were sensitive to high sucrose concentrations. However, the cold acclimation of plantlets prior to preculture or preculture in sucrose media with the supplementation of ASA reduced the stress level induced by sucrose dehydration. Cold acclimation is very effective, especially for temperate plants for improving recovery after cryopreservation (Kushnarenko et al. 2009), and its combination with sucrose also resulted in successful survival in several temperate and tropical species (Yamamoto et al. 2011). Preculturing in high sucrose media combined with cold acclimation increased the survival of shoot tips of *Rubus* after cryopreservation from 0 % to 75 % (Chang & Reed 2001). The supplementation of ASA in the treatment media triggered early regrowth in the shoot tips of the tropical species *Garcinia mangostana* after cryopreservation (Ibrahim & Normah 2013). In this research, the combination of the three treatments helped the plants to recover normal physiological status similar to control plants. This clearly shows the combination of cold and ASA with sucrose treatment have a vital role in reducing the high osmotic and oxidative stresses that the shoot tips encounter during DV.

The duration of cold acclimation also seems to be critical for successful cryopreservation in *Actinidia* spp. This study shows that the cold acclimation for one week that was used in the earlier part of the research does not impart sufficient protection from the dehydration effect of high sucrose on the shoot tips, but 2 weeks of cold acclimation enhanced the pre-treatment effect even after the concentration of sucrose was increased to 1.0 M. Suzuki et al. (1994) achieved 80 % survival after ED of *A. deliciosa* when the plantlets were acclimated for 6 weeks at 5 °C. In contrast, preculture either with sucrose or glycerol or its combination could replace cold acclimation in *Prunus* (Barraco et al. 2012). In this research, when the three treatments were combined, the explants were able to survive even 1.0 M sucrose in preculture, which resulted in the highest regeneration after cryopreservation when treated with mPVS2 for 60 min (hence 60 min mPVS2 treatment was chosen for further studies in third chapter). In order to maintain the cold temperature, the donor plants and shoot tips were maintained in cold conditions until freezing. This included the precultured with sucrose in cold chamber and thereafter the treatments in loading solution and mPVS2 were also carried out on ice.

The non-cryopreserved pre-treatment control sample that gave 100 % regeneration even after 60 min in mPVS2 shows that ‘Hort16A’ shoot tips can tolerate the toxicity of mPVS2. This tolerance depends on the size of the explants (Niino et al. 1992) and could depend on the texture of shoot tips as well. The shoot tips smaller than the explants taken for this project may show less tolerance to mPVS2 as it would take less time for the mPVS2 to penetrate the cells and dehydrate the shoot tips. The shoot tips of ‘Hort16A’ falls under the category of hairy explants (which is a characteristic feature of *Actinidia*), which causes high surface tension that may prevent the easy penetration of the cryoprotectants into the cells (Li et al. 2013). A slight decrease in the pretreated samples’ survival with the increase in the mPVS2 exposure period could be observed in -LN samples. This might be because some weakened explants that struggled to surpass the high osmotic stress lost their defence against the toxic effect of cryoprotectants in the loading solution and mPVS2. The preculture in sucrose for five days might have also decreased the surface tension, so that it made easier for the toxic cryoprotectants (e.g. glycerol and DMSO in LS and mPVS2) to enter into the cells, decreasing the survival. Nevertheless, the shoot tips that were subjected to pre-treatment and cryopreserved recorded a relatively high survival, with the highest survival in samples treated longest in mPVS2, with a significantly higher survival after 60 min (the longest treatment period). The control samples that were not pretreated and cryopreserved recorded no re-growth at all after cryopreservation. Researchers have previously observed a sudden improvement in regeneration after cryopreservation in samples treated for longer periods in

PVS2, similar to the results in this research (statistically significant increase in 60 min treatment against 50 min). For example, Wang et al. (2013) observed a positive shift in cryo-survival of potato at 30 min from 20 min of PVS2 treatment during DV. Similar increase in survival was observed in *Lilium* from 1-2 h (Yin et al. 2014) and in apple between 60-80 min (Sakai & Engelmann 2007). In the final experiment, the 40 % plant regeneration in cryopreserved *Actinidia* shoot tips from pretreated samples in contrast to non-survival in any of the shoot tips of non-pretreated samples clearly shows the vital need of pre-treatment in cryopreservation of this species. Similar to results of this research, Plessis et al. (1993) reported successful cryopreservation of grapevine shoot tips after pre-treatment with 1.0 M sucrose, whereas cold hardening of the plants prior to sucrose preculture improved the survival of date palm meristems (Fki et al. 2013) and Thymus (Ozudogru & Kaya 2012). Uchendu et al. (2010a) successfully cryopreserved blackberry shoot tips with the supplementation of antioxidants, vitamin C and vitamin E in addition to the cold acclimation and preculture in DMSO media. Suzuki et al. (2006) applied a two step preculturing with sucrose to improve the survival after cryopreservation of gentians.

In the present research, all the survived shoot tips regenerated into plantlets without any callus formation. This suggests that the media used for plant regeneration are optimal for *A. chinensis* 'Hort16A'. However, the results couldn't conclude 60 min treatment in mPVS2 as the optimal time for DV of *A. chinensis* as longer periods were not tested due to time limitations. Hence future research may be directed at testing longer periods in mPVS2.

Increasing number of reports on DV show that testing of various pre-treatments in different concentrations and different timings is important for developing successful cryopreservation protocols for various species. The optimization of each treatment is needed for different species, but few species such as cassava (Escobar et al. 2014), *Colocassia* (Hong et al. 2014), *Malus* (Condello et al. 2011a), *Musa* (Agrawal 2014) and *Rubus* (Vujovic et al. 2011).have been successfully regenerated after cryopreservation without any pre-treatment. Present research clearly shows that *Actinidia* spp. requires optimisation of the parameters of pre-treatments for regeneration after cryopreservation and presented in this thesis is the first successful droplet vitrification protocol developed within the genus *Actinidia*.

3 ULTRASTRUCTURAL AND BIOCHEMICAL CHANGES AT DIFFERENT STAGES OF CRYOPRESERVATION

3.1 INTRODUCTION

Understanding the mode of action of cryopreservation in plant cells is needed to improve the protocols available for germplasm conservation (Helliot et al. 2003). Cryoprotectants are the key to successful cryopreservation, and in the absence of cryoprotectants, cells cannot survive ultra-freezing (Gnanapragasam & Vasil 1992). However, the high concentrations used as well as the toxicity of some of the chemicals used in cryoprotectants can result in damage to cells, despite minimizing the damage caused by ice crystallisation during the freeze-thaw processes of cryopreservation (Arakawa et al. 1990; Kaczmarczyk et al. 2012). Freezing causes severe structural damage to cells, including membrane rupture, caused by the ice crystallisation, membrane denaturation and cell wall degradation and the loss of enzyme activities (Talens et al. 2002). Cryopreservation also imposes a series of stresses to the plant cells, including osmotic and temperature related stresses, which can result in increased oxidative stress (Reed et al. 2012).

The combination of mechanical damage and oxidative stress can result in poor cell survival. Often mechanical cell damage and oxidative stress are related. Ultrastructural studies using transmission electron microscopy (TEM) can reveal damage to cells at the whole cell and sub-cellular levels (Helliot et al. 2003), whereas biochemical assays, including biomarkers of antioxidant metabolism and the markers of oxidative damage, can be used to link structural damage to metabolic disruption or vice versa (Blokchina et al. 2003).

This chapter presents an investigation of ultrastructural changes and assays of oxidative stress markers at critical steps during the DV cryopreservation and recovery of *A. chinensis* ‘Hort16A’ shoot tips and also highlights the importance of whole plant pretreatment for the successful DV by comparison of pretreated samples with non-pretreated samples. The critical steps of DV of pretreated and non-pretreated shoot tips selected for the study include stock plant controls, after mPVS2 treatment, after rewarming (in the recovery solution) and 24 h after rewarming (on the recovery plate). The ultrastructural study was carried out with TEM using shoot tips from the stages described above. Enzymatic and non-enzymatic markers of antioxidant metabolism were measured, and the degree of oxidative damage to proteins and lipids was determined. The enzymatic antioxidant assays included SOD, CAT, APX, GPX

and GR, and the levels of non-enzymatic antioxidant ascorbate and glutathione were also measured. Protein carbonyls and lipid peroxides were used as the markers of oxidative damage to proteins and lipids respectively.

3.2 MATERIALS AND METHODS

Samples for ultrastructural and biochemical studies were collected from pretreatment and cryopreservation steps as mentioned in the second chapter and were carried out in PFR, Palmerston North.

3.2.1 ULTRASTRUCTURAL STUDY USING TRANSMISSION ELECTRON MICROSCOPY

- i. The primary fixation was done at PFR, Palmerston North. After each critical stage of DV viz. control, mPVS2, recovery solution and recovery plate of pretreated and non-pretreated shoot tips, 7-10 samples were immediately fixed in 4 % glutaraldehyde in 0.2 M phosphate buffer, pH 7.3. The fixation was done inside a fume hood and the samples in glass vials were kept at room temperature for 30 min and then at 4 °C overnight.
- ii. The samples were rinsed thrice in ice-cold 0.2 M phosphate buffer taken from fridge and stored in phosphate buffer at 4 °C until further steps were carried at the Otago Centre for Electron Microscopy, University of Otago, Dunedin.
- iii. The samples were washed with cold 0.2 M Cacodylate buffer for three times. Each wash extended for 15 min on rotator inside the fume cupboard.
- iv. The post-fixation was done with 2 % osmium tetroxide in 0.1M Cacodylate buffer on rotator at 2 g in the fridge for an overnight.
- v. The samples were washed thrice in ice-cold Cacodylate buffer and left in the buffer at 4 °C until ready to process.
- vi. The samples were then washed thrice with double distilled water; each wash lasted for 10 min.
- vii. Tertiary fixation and *en bloc* staining were done with 1 % uranyl acetate (UA) in double distilled water. The tubes were wrapped with tin foil to exclude light as UA is photo reductive (Drobne 2013) and will precipitate in the presence of light hindering proper sectioning. The tubes were kept on a rotator for 60 min in fume hood.
- viii. They were then washed twice with double distilled water, 10 min for each wash.
- ix. The samples were then subjected to dehydration through a series of ethanol and propylene oxide (PO). The ethanol dehydration started with 50 % ethanol (15

min) with increasing concentrations; such as 15 min each in 70 % and 95 %, followed by 100 % ethanol dehydration thrice at 10, 15 and 20 min respectively. Then the samples were dehydrated in PO for 20 min, which was repeated twice before resin treatment.

- x. The samples were then treated with PO and resin in the ratio 3:1 for 1.5 h, followed by the treatment at 1:1 ratio for 1.5 h and kept in 100 % resin overnight.
- xi. After 16 h, the resin in which the samples were stored was replaced with fresh resin twice after 3 h in each.
- xii. Then fresh resin was embedded in embedding moulds and polymerised at 60 °C for 48 h, and the samples were labelled.
- xiii. Semi-thin sections (1 µm) were cut on glass knives, mounted on glass slides and stained with the monochromatic stain Methylene Blue/Azur 1 and Borax. These sections were examined in the light microscope to assess fixation and embedding quality, and to locate the region of interest.
- xiv. Once the region of interest was located the block face was trimmed down to approximately 0.5 mm by 0.5 mm ready for ultrathin sectioning.
- xv. Ultrathin sectioning was performed using a Diatome diamond knife (Biel, Switzerland) at a 35-degree cutting angle. Section thickness was 80 nm.
- xvi. Ultrathin sections were collected on Formvar coated single slot copper grids.
- xvii. Contrasting was performed in a LKB Ultrastain automatic grid stainer (LKB-Produkter AB, Bromma, Sweden) using 1 % uranyl acetate in water and the Reynolds lead citrate stain.
- xviii. Ultrathin sections were examined using a Philips CM100 BioTwin transmission electron microscope (Philips/FEI Corporation, Eindhoven, Holland) fitted with MegaView III digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

3.2.2 BIOCHEMICAL ASSAYS

The biochemical analyses were carried out in the Department of Botany, University of Otago, Dunedin. Samples were transferred by overnight courier in dry ice. The assays for enzymatic antioxidants and the oxidative damage markers were independently replicated nine times and the non-enzymatic antioxidant assays three times.

3.2.2.1 Determining oxidative damage

3.2.2.1.1 Protein Carbonyls

Total protein was extracted in a buffer consisting of 100 mM potassium phosphate (pH 7.3), 1 mM K₂EDTA, 1 mM ascorbate and 1 % (w/v) Polyclar AT (SERVA Chemicals Ltd). Tissue was ground in LN until a fine powder and total protein was extracted by adding ice-cold enzyme extraction buffer to frozen ground tissue as at a ratio of 5:1 (v:w), followed by mixing with a vortex-mixer. The homogenate was then centrifuged for 10 min at 10,000 g (Eppendorf 5417R, Eppendorf South Pacific Pty. Ltd., North Ryde, NSW, Australia) at 4 °C.

Vivaspin 500 spin columns (Sartorius Stedim Biotech, GmbH 37070, Goettingen, Germany) (10KD molecular weight cut-off) were used for ultra-filtration, to remove small molecules that could interfere with subsequent assays and for protein concentration, of the supernatant according to the manufacturer's instructions. After centrifugation for 10 min at 10,000 g at 4 °C, the sample was reconstituted with 100 mM potassium phosphate (pH 7.3). Purified protein extracts were divided into aliquots and stored at -80 °C. The protein content of each extract was determined using the modified Lowry protein assay (Fryer et al. 1986).

PCs were determined in purified protein extracts via reaction with 2,4-dinitrophenylhydrazine (DNPH) according to Reznick and Packer (1994), adapted for measurement in a microplate reader. Levels of PCs were determined by measuring the absorbance at 370 nm using a Li 1420 multilabel counter (Perkin Elmer, San Jose, California, USA), controlled by a PC, and fitted with temperature control cell, set to 25 °C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, USA). PCs content in nmol was determined using the extinction coefficient of DNPH at 370 nm ($0.022 \mu\text{M}^{-1} \text{cm}^{-1}$), corrected for the calculated path-length of the solution (0.6 cm). The protein content of the extracts was determined using a Lowry

protein assay (Fryer et al. 1986) and protein carbonyl content expressed as nmol protein carbonyl/mg protein.

3.2.2.1.2 Lipid Peroxides

Tissue was ground in LN until a fine powder. Tissue (50 mg) was homogenized in 150 μ L of methanol:chloroform (2:1 v/v) and left for 1 min. Chloroform (100 μ L) was added and mixed for 30 sec. Then 100 μ L of deionised water was added and mixed for 30 sec. Centrifugation was done at 10000 g for 1 min to separate the phases, and the lower chloroform phase was transferred to a new tube.

LPs were determined using the ferric thiocyanate (Mihaljević et al. 1996) adapted for measurement in a microplate reader. Levels were determined by measuring the absorbance at 500 nm using a Li 1420 multilabel counter (Perkin Elmer, San Jose, California, USA), controlled by a PC, and fitted with temperature control cell, set to 25 °C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, USA). A calibration curve with t-butyl hydroperoxide was used and lipid hydroperoxide content calculated as nmol LP per g FW.

3.2.2.2 Determining enzymatic antioxidants

Purified protein extracted as detailed above for the PC assay were diluted as required for the enzyme assays detailed below. All assays were carried out using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, USA.), controlled by a PC, and fitted with a temperature control cell, set to 25 °C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software (Perkin Elmer, San Jose, California, USA.).

3.2.2.2.1 Superoxide Dismutase

SOD was assayed using microplate assay described by Banowetz et al. (2004) with minor modifications. Briefly, 50 μ L of extract, diluted extract or standard prepared from bovine liver SOD (Sigma-Aldrich, St. Louis, MO, USA.) was mixed with 125 μ L of freshly prepared reaction solution containing piperazine-1,4-bis (2-ethanesulfonic acid) (Pipes) buffer, pH 7.8, 0.4 mM o-dianisidine, 0.5 mM diethylenetriaminepentaacetic acid (DTPA), and 26 μ M riboflavin. One unit of SOD corresponded to the amount of enzyme that inhibited

the reduction of cytochrome c by 50 % in a coupled system with xanthine oxidase at pH 7.8 and 25 °C. The absorbance at 450 nm (A_{450}) was measured immediately ($t = 0$ min), then the samples were illuminated with an 18 W fluorescent lamp placed 12 cm above the plate for 30 min ($t = 30$) and the A_{450} was measured again. A regression analysis was used to prepare a standard line relating SOD activity to the change in A_{450} , and SOD activities in the extracts were calculated with reference to the standard line, and expressed as units SOD per mg of total protein.

3.2.2.2.2 Catalase

CAT was determined using the method of Summermatter et al. (1995) as adapted by Gillespie et al. (2011) for 96-well microplates. Briefly, 50 μ L of diluted extract was mixed with 50 μ L of 50 mM sodium phosphate buffer (pH 7.0), after which 50 μ L of 35 mM H_2O_2 was added to each well. Duplicate samples of each extract were incubated at 25 °C and the reactions were stopped after 1 min in one set of samples by the addition of 50 μ L of 15 % (w/v) trichloroacetic acid (TCA). After a further 2 min, the reactions were stopped in the second set of samples by the addition of TCA as detailed above. The H_2O_2 remaining after incubation with CAT was determined by mixing 5 μ L of the reaction mix with 100 μ L of H_2O_2 determination reagent (1 g l⁻¹ 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABST)), 0.8 U mL⁻¹ horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA.)) in the well of a new microplate. The plate was incubated for 10 min at 20 °C, and the absorbance was measured at 410 nm. The amount of H_2O_2 was determined using standard curves obtained with known concentrations of H_2O_2 . Purified bovine liver CAT (Sigma-Aldrich, St. Louis, MO, USA) in homogenization buffer was used as a positive control. CAT activities in the extracts are expressed as μ M of H_2O_2 consumed per minute per mg of total protein.

3.2.2.2.3 Ascorbate Peroxidase

APX activity was assayed according to Nakano and Asada (1981) with minor modifications for use in a microplate reader (Schweikert & Burritt 2012), by following the decrease in the absorbance at 290 nm (A_{290}) as ascorbate disappeared. Briefly, 50 μ L of diluted extract was mixed with 200 μ L of 100 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM ascorbate, 0.2 mM H_2O_2 and 0.1 mM EDTA in a UV transparent microplate (Greiner bio-one GmbH, Kremsmunster, Austria) and the absorbance was measured at 30 sec intervals for 3 minutes. If required, corrections were made for the

oxidation of ascorbate in the absence of H_2O_2 and for the low, non-enzymatic oxidation of ascorbate by H_2O_2 . APX activity (expressed as $\mu\text{mol min}^{-1}$) was calculated using an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$, corrected for the calculated path-length of the solution (0.6 cm).

3.2.2.2.4 Glutathione Peroxidase

GPX activity was measured according to the spectrophotometric method of Paglia and Valentine (1967) with modifications for use with a microplate reader. Briefly, 50 μL of extract, diluted extract or standard (GPX from bovine erythrocytes, Sigma-Aldrich, St. Louis, MO, USA., in extraction buffer) was mixed with 170 μL of assay buffer containing 50 mM Tris-HCl buffer (pH 7.6), 5 mM ethylene diamine tetra-acetic acid, 0.14 mM NADPH, 1 mM GSH and 3 units mL^{-1} GR (from wheat germ, Sigma-Aldrich, St. Louis, MO, USA). The reaction was initiated by the addition of 20 μL t-butyl hydroperoxide to give a final concentration of 0.2 mM. The consumption of NADPH was monitored at 340 nm (A_{340}) every 30 seconds for 3 min, with the plate shaken automatically before each reading. GPX activities in the extracts were calculated with reference to a standard line constructed with GPX purified from bovine erythrocytes. Data are expressed as μmoles or nmoles per min per mg of total protein.

3.2.2.2.5 Glutathione Reductase

GR was assayed using the method of Cribb et al. (1989) with minor modifications. Briefly, 50 μL of extract, diluted extract or standard (GR from wheat germ, Sigma-Aldrich, St. Louis, MO, USA, in homogenization buffer) was mixed with 150 μL of 100 mM sodium phosphate buffer (pH 7.6) containing 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 10 μL of NADPH (12 mM). The reaction was initiated by the injection of 10 μL of oxidised GSSG (3.25 mM) and the absorbance at 415 nm (A_{415}) was measured every 30 seconds for 3 min, with the plate shaken automatically before each reading. The rate of increase in A_{415} per minute was calculated and a regression analysis was used to prepare a standard line relating standard GR activities to the change in A_{415} . GR activities in the extracts, calculated with reference to the standard line were expressed as μmoles or nmoles of GSSG reduced per minute per mg of total protein.

3.2.2.3 Determining non-enzymatic antioxidants

3.2.2.3.1 Glutathione

The tissue was ground in LN until a fine powder. It was homogenized in ice-cold 5 % (w/v) sulfosalicylic acid at a ratio of 1:4 (w/v) powdered tissue to 5 % sulfosalicylic acid and then centrifuged at 1000g for 15 min at 4 °C. GSH and GSSG were measured using the enzymatic recycling method using the microplate assay described by Rahman et al. (2006). Assays were carried out in PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, USA), controlled by PC, and fitted with temperature control cell, set to 25 °C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software (Perkin Elmer, San Jose, California, USA).

3.2.2.3.2 Ascorbate

Ascorbate was extracted by homogenizing ground tissue in ice-cold 5 % (w/v) metaphosphoric acid at a ratio of 1:4 (w/v) powdered tissue to 5 % metaphosphoric acid and then centrifuged at 1000 g for 15 min at 4 °C.

Oxidised and total ascorbate levels were measured using the enzymatic recycling method; a microplate assay described by Gillespie and Ainsworth (2007). Assays were carried out in Li 1420 multilabel counter (Perkin Elmer, San Jose, California, USA), controlled by PC, and fitted with temperature control cell, set to 25 °C, and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software (Perkin Elmer, San Jose, California, USA).

3.2.3 STATISTICAL ANALYSIS

Statistical analysis was performed using GenStat 17th edition (VSN International) for all assays. Differences among the response of the non-pretreated and pretreated samples as well as the difference between the treatments were analysed with two-way ANOVA and the means were compared using Fisher's protected least significant difference test. LPs and PCs data was log transformed. P-values of <0.05 were considered to be significant.

3.3 RESULTS

3.3.1 COMPARISON OF CHANGES IN CELL ULTRASTRUCTURE OF PRETREATED AND NON-PRETREATED SHOOT TIPS DURING CRYOPRESERVATION

To study the effect of pretreatments and DV on the cell ultrastructure of *A. chinensis* ‘Hort16A’ kiwifruit shoot tips TEM was performed and photomicrographs of the pretreated shoot tips were compared with those of the non-pretreated shoot tips. The cells of the shoot tips from *in vitro* plantlets that did not receive any pre-treatments were compactly arranged at the meristematic part, with many actively dividing cells (Figure 3.1: A). The plasma membrane covering the cytoplasm maintained the same shape as the cell wall without any protrusions or invaginations. The cytoplasm of these cells contained a prominent large nucleus with uniform heterochromatinisation and a round dense nucleolus. The cells maintained a high nucleo-cytoplasmic ratio. The vacuoles varied in their size, but consumed less space compared to the more mature cells. After the cold acclimation of the plants and preculture of shoot tips in high sucrose media supplemented with ASA, some minor changes could be observed in the cells such as plasmolysis (in some cells), which caused some shrinkage of cytoplasm and nucleus, along with plasma membrane (Figure 3.1: B). The vacuoles of these cells occupied comparatively more space than in non-pretreated control cells.

After treatment in mPVS2 for 60 min, only minor changes were observed in non-pretreated cells (Figure 3.1: C), which included increased vacuolisation and the presence of some dark globular particles in the cytoplasm. In contrast, severe plasmolysis and fragmentation of vacuoles was observed in most of the cells of pretreated shoot tips after 60 min treatment in mPVS2 solution (Figure 3.1: D); vacuoles occupied most of the cell space. Undulation of the plasma membrane was common in all cells and rupture of the plasma membrane was noted in some cells. In addition to strong heterochromatinisation at the nuclear periphery in some cells of the pretreated shoot tips, the nucleus became denser and nucleolus appeared irregular in shape.

Freezing and warming caused plasmolysis in non-pretreated cells (Figure 3.1: E), and further fragmentation of vacuoles was common in all cells. In pretreated shoot tips, though some cells show plasmolysis and plasma membrane rupture, some cells preserved the integrity of plasma membrane and cell structure (Figure 3.1: E). In some cells, rupture of

plasma membrane, leakage of cytoplasm and strong heterochromatinisation was observed in both non-pretreated and pretreated shoot tips.

After 24 h recovery following cryopreservation, large differences between shoot tips from the two treatments were observed. The plasma membranes of most non-pretreated cells were ruptured (Figure 3.1: G), resulting in the loss of the cytoplasmic contents. The plasmodesmatal connections between cells were damaged and the cells had shrunk and lost contact with their neighbouring cells, and appeared dead. In contrast, some more intact and apparently viable cells could be seen in pretreated shoot tips (Figure 3.1: H). Surviving cells maintained a normal cell structure with no serious injuries and had intact plasma membranes with well-distributed cytoplasm containing dense round nuclei (Figure 3.1: H).

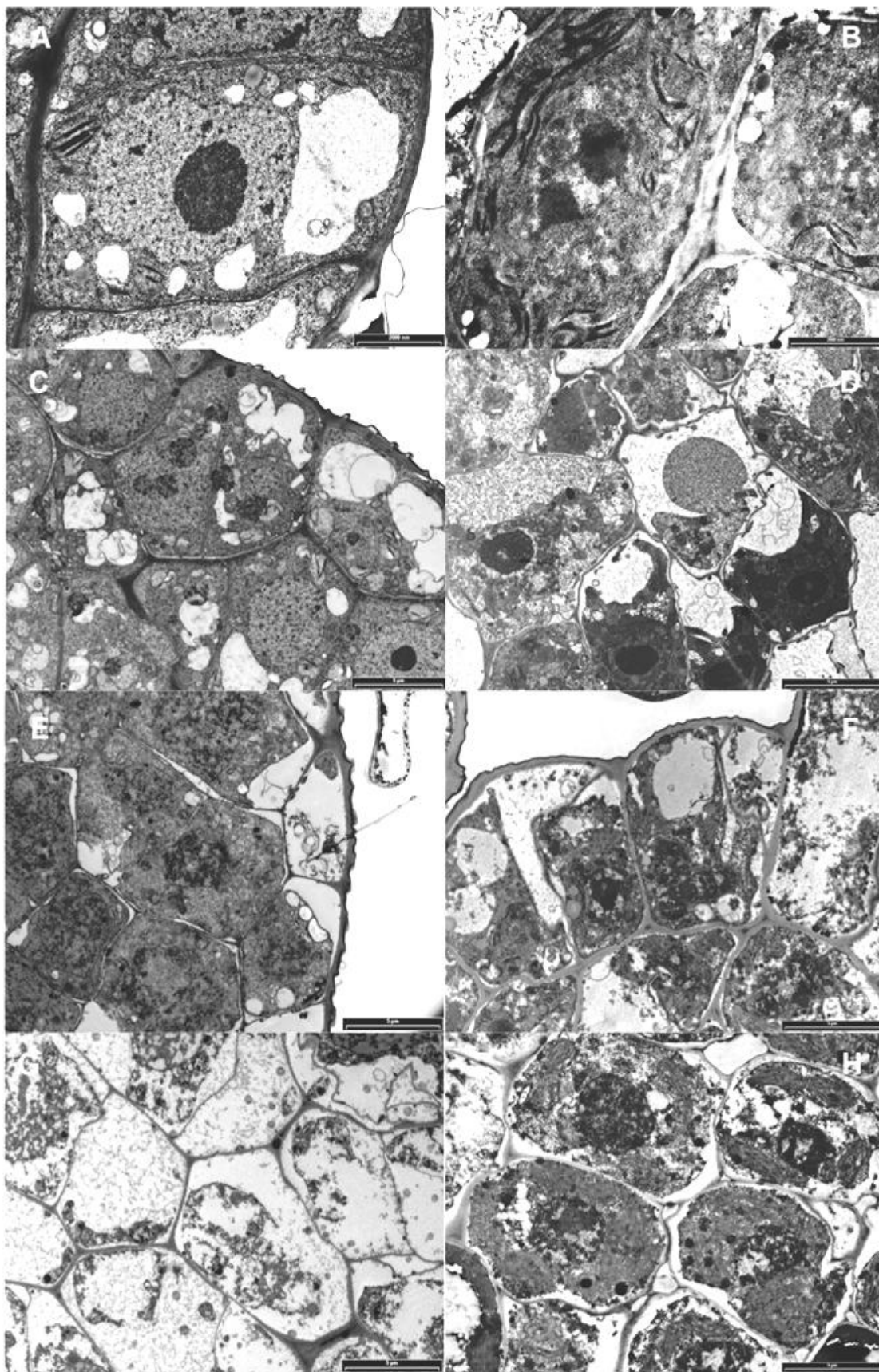


Figure 3.1. (Legend is given in the following page).

Figure 3.1. Ultrastructural changes in non-pretreated and pretreated shoot tips of ‘Hort16A’ during droplet vitrification.

Ultrastructural changes in non-pretreated (**A**, **C**, **E**, and **G**) and pretreated shoot tips (**B**, **D**, **F**, and **H**) of *A. chinensis* ‘Hort16A’ during droplet vitrification. **A**: shoot tips excised from plantlets grown in culture room conditions (non-pretreated control), **B**: shoot tips from 2 weeks cold acclimated plants precultured in 1 M sucrose supplemented with ascorbic acid (pretreated control), **C**: non-pretreated shoot tips after 1 h mPVS2 treatment, **D**: pretreated shoot tips after 1 h mPVS2 treatment, **E**: cryopreserved non-pretreated shoot tips after 20 min in recovery solution, **F**: cryopreserved pretreated shoot tips after 20 min in recovery solution, **G**: non-pretreated shoot tips after 24 h on recovery plate, **H**: pretreated shoot tips after 24 h on recovery plate. Bars: **A** and **B** = 2000 nm; **C** – **H** = 5 μ m.

3.3.2 USE OF OXIDATIVE MARKERS TO STUDY THE ANTIOXIDANT MECHANISMS DURING DIFFERENT STEPS OF CRYOPRESERVATION

3.3.2.1 Oxidative damage markers

3.3.2.1.1 Protein Carbonyls

In general, there was a significant increase in PCs (oxidative damage to proteins) as the shoot tips progressed through the different steps of the DV process in both non-pretreated and pretreated shoot tips (Figure 3.2). The non-pretreated and pretreated samples showed no notable difference between them in the degree of damage before mPVS2 treatment (control). However, they differed significantly in their damage level from the mPVS2 treatment onwards (Figure 3.2). The pretreated samples showed more damage than non-pretreated samples after treatment with mPVS2 and when in the recovery solution ($P < 0.001$; Table 3.1). The degree of damage increased drastically 24 h after rewarming (recovery plate), at this stage non-pretreated shoot tips showed significantly greater damage than pretreated samples ($P < 0.001$; Table 3.1); the damage increased by 73 % in non-pretreated shoot tips, whereas pretreated samples showed only 45 % increase damage.

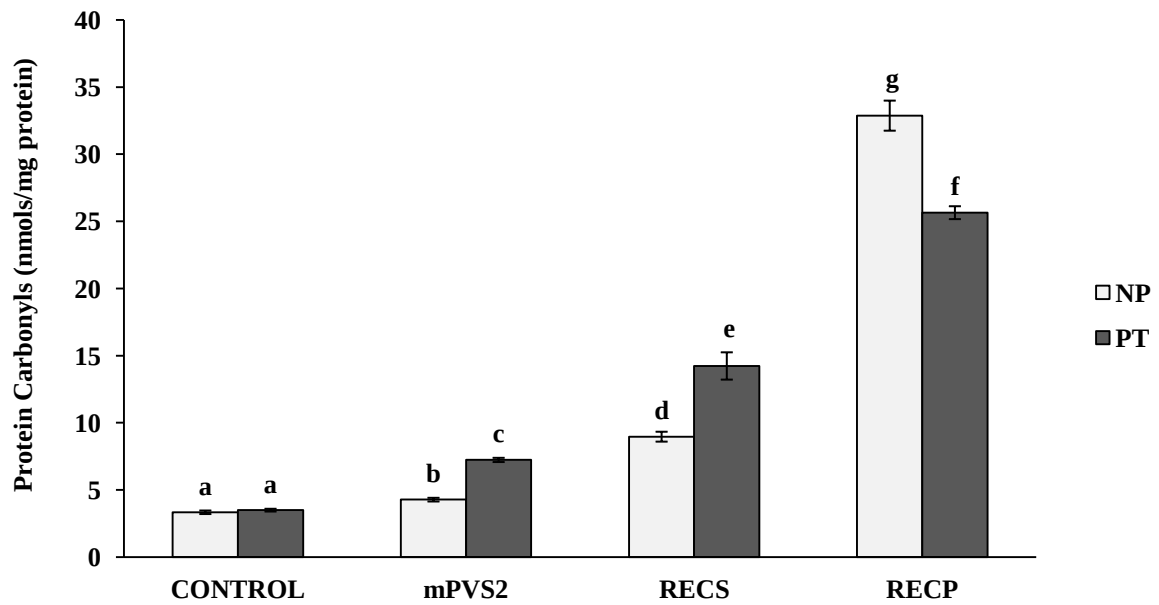


Figure 3.2. Protein carbonyls assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of protein carbonyl content in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.1. Analysis of variance of protein carbonyls content of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of protein carbonyl contents in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	0.001945	0.001945	0.98	0.325
mPVS2	1	0.233918	0.233918	117.85	<0.001
RECS	1	0.177183	0.177183	89.27	<0.001
RECP	1	0.051613	0.051613	26.00	<0.001

3.3.2.1.2 Lipid Peroxides

LPs showed a similar trend to PCs with control shoot tips not showing a significant difference whether they are pretreated or not, but showing increased damage at each consecutive steps of DV (Figure 3.3). The pretreated samples showed more damage than non-pretreated samples after treatment with mPVS2 and when in the recovery solution ($P < 0.001$: Table 3.2) A sudden rise in LPs was observed after 24 h on the recovery plates and resulted in significantly higher damage in non-pretreated samples than pretreated samples. Non-pretreated shoot tips recorded a 73 % increase in LPs after 24 h on recovery plates compared to the control, whereas pretreated samples showed only a 46 % increase.

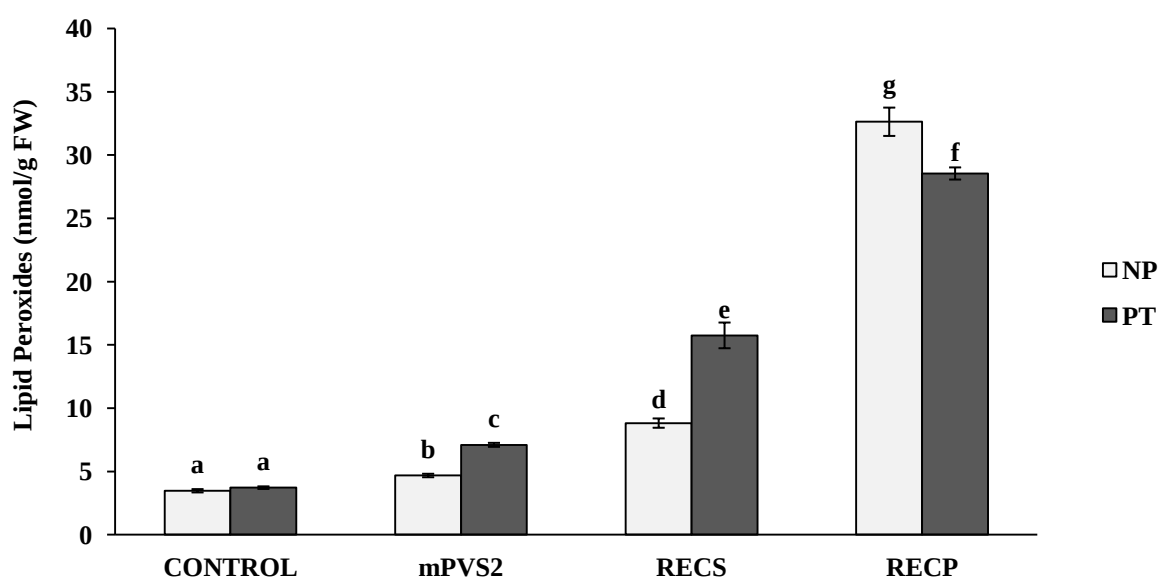


Figure 3.3. Lipid peroxides assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of lipid peroxides content in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.2. Analysis of variance lipid peroxides contents of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of lipid peroxides contents in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	0.004285	0.004285	2.04	0.157
PVS2	1	0.149217	0.149217	70.96	<0.001
RECS	1	0.277239	0.277239	131.84	<0.001
RECP	1	0.014373	0.014373	6.84	0.011

3.3.2.2 Enzymatic antioxidant markers

The results with enzymatic antioxidant markers showed a similar trend in the variation of enzyme levels in pretreated and non-pretreated shoot tips. The trend was also similar at different stages of the cryopreservation process, with higher level of antioxidants at the beginning and increasingly lowers levels as the shoot tips passed through mPVS2 treatment, recovery solution and recovery plates. The differences in the levels between the non-pretreated and pretreated samples for all antioxidant were highest in the control and recovery plates ($P < 0.001$) whereas, mPVS2 treatment showed significant difference in all assays except APX. However, treatment with recovery solution showed no significant difference between the samples in all assays except SOD.

3.3.2.2.1 Superoxide Dismutase

Compared to non-pretreated samples, the pretreated samples maintained a high level of SOD activity in all the treatments (Table 3.3), especially in the control where SOD activity in pretreated shoot tips was 29 % higher than non-pretreated shoot tips ($P < 0.001$: Table 3.3). The SOD activity in pretreated shoot tip controls was significantly higher than in all other treatments with a significant loss in SOD activity after mPVS2 treatment in both non-pretreated and pretreated shoot tips (Figure 3.4). In the pretreated samples, this loss was 32 % and the difference was not significantly different compared with non-pretreated controls. Compared to the mPVS2 treatment, the recovery step did not show a notable change in SOD activity in both non-pretreated and pretreated samples. A drop by 41 % in SOD activity was observed in the non-pretreated samples after 24 h of rewarming against 12 % decrease in pretreated samples. In both non-pretreated and pretreated shoot tips, the SOD activity in shoot tips on the recovery plate showed a significantly lower activity compared to the controls. Nevertheless, the pretreated shoot tips maintained 38 % more SOD activity than non-pretreated shoot tips after 24 h on the recovery plates ($P < 0.001$: Table 3.3).

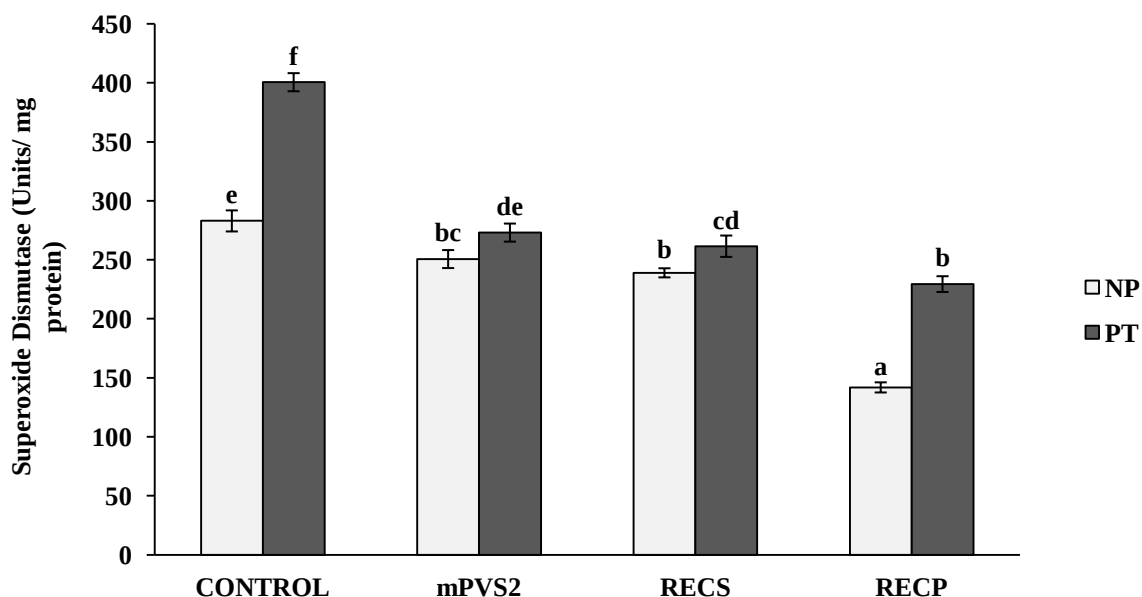


Figure 3.4. Superoxide dismutase assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of superoxide dismutase levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.3. Analysis of variance of superoxide dismutase of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of superoxide dismutase levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	62186.9	62186.9	120.05	<0.001
PVS2	1	2266.9	2266.9	4.38	0.040
RECS	1	2289.4	2289.4	4.42	0.039
RECP	1	34496.9	34496.9	66.59	<0.001

3.3.2.2.2 Catalase

The pretreated shoot tip samples maintained a high level of CAT activity in all the treatments compared to non-pretreated samples. This difference was significant in all the steps of cryopreservation except in the recovery solution step (Figure 3.5). In the control, pretreated shoot tips had 28 % higher CAT activity than non-pretreated shoot tips and this was statistically different ($P < 0.001$; Table 3.4). CAT activity declined after mPVS2 treatment in both non-pretreated and pretreated shoot tips. This loss was greater in pretreated samples (29 %) (Figure 3.5). A steep decrease by 35 % in the CAT activity was observed in the non-pretreated samples after 24 h on recovery plates, whereas, the pretreated samples maintained almost same level of CAT activity as they had after the recovery solution treatment (Figure 3.5). After 24 h on recovery plates, both non-pretreated and pretreated shoot tips had significantly lower CAT activity than their respective controls. After the recovery plate step, the pretreated samples showed 36 % more CAT activity than non-pretreated samples ($P < 0.001$; Table 3.4).

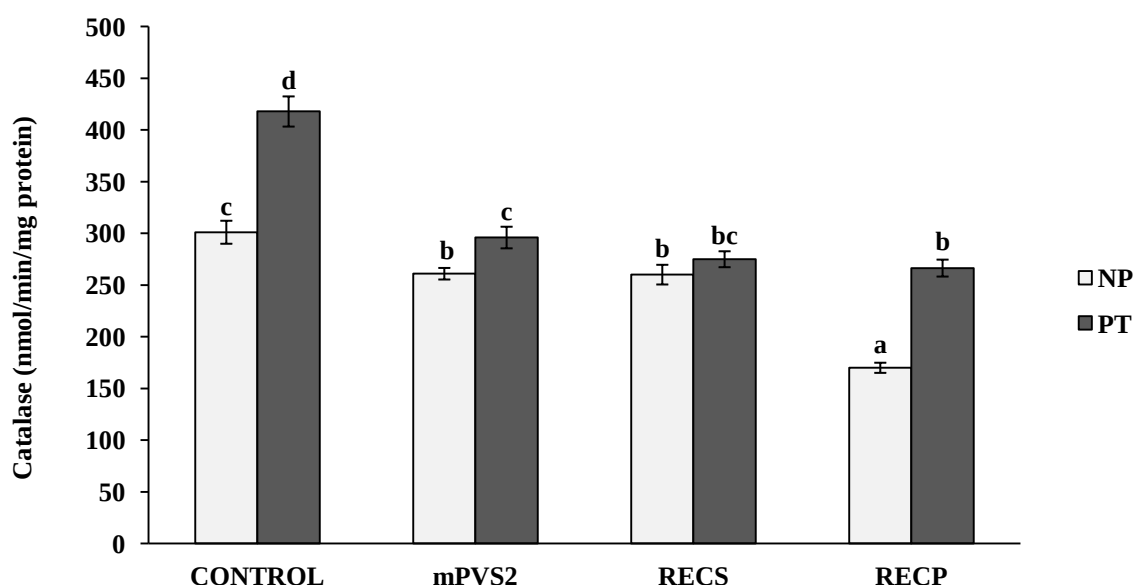


Figure 3.5. Catalase assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of catalase levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.4. Analysis of variance of catalase of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of catalase levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	61366.7	61366.7	76.80	<0.001
PVS2	1	5512.5	5512.5	6.90	0.010
RECS	1	997.6	997.6	1.25	0.267
RECP	1	41856.9	41856.9	52.38	<0.001

3.3.2.2.3 Ascorbate Peroxidase

Pretreated shoot tips generally maintained a higher level of APX activity than non-pretreated samples ($P < 0.001$; Table 3.5; Figure 3.6). The pretreated shoot tips showed 34 % higher APX activity than non-pretreated shoot tips (controls) (Figure 3.6). A decline in APX activity was found after mPVS2 treatment for both pretreated and non-pretreated shoot tips. The APX activity of the pretreated samples after mPVS2 treatment was similar to the level of activity in non-pretreated control shoot tips. After the recovery solution, there was no notable change the APX levels in both non-pretreated and pretreated samples. Thereafter a steep decline of 37 % in APX activity was observed in the non-pretreated samples after 24 h on recovery plate, whereas the pretreated samples maintained almost the same level of APX activity. The APX activity in both sample types after 24 h on recovery plates differed significantly from their controls, but the pretreated samples showed 38 % more APX activity than non-pretreated samples. The APX activity of pretreated samples after 24 h on recovery plates was only 15 % less than the activity measured in non-pretreated controls.

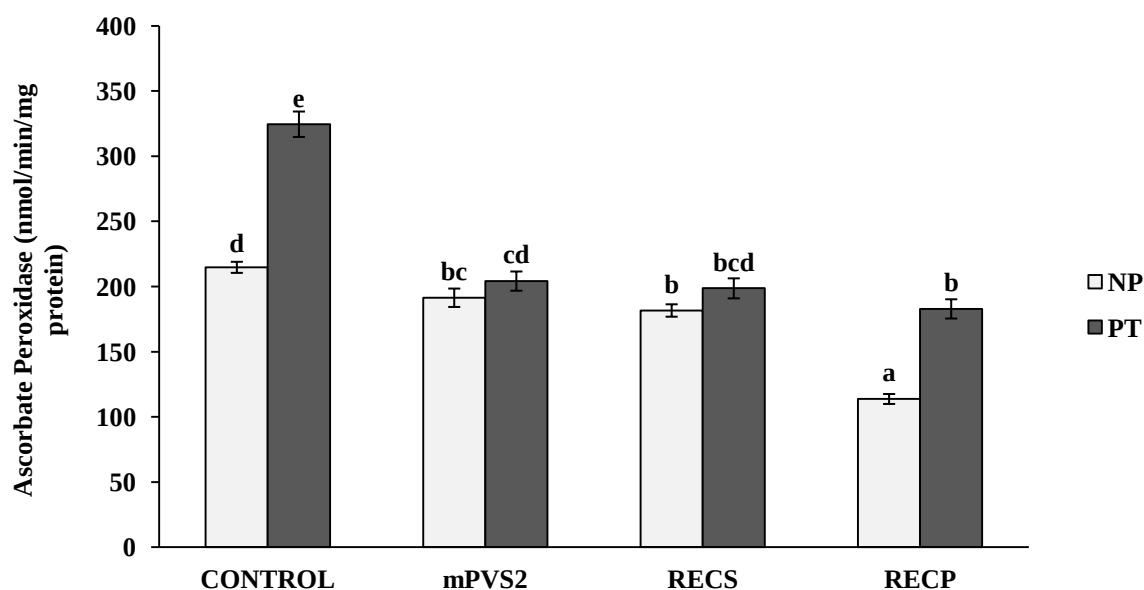


Figure 3.6. Ascorbate peroxidase assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of ascorbate peroxidase levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.5. Analysis of variance of ascorbate peroxidase of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of ascorbate peroxidase levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	54230.2	54230.2	142.89	<0.001
PVS2	1	734.7	734.7	1.94	0.168
RECS	1	1300.5	1300.5	3.43	0.068
RECP	1	21493.6	21493.6	56.63	<0.001

3.3.2.2.4 Glutathione Peroxidase

GPX activity was higher in pretreated shoot tips at all treatment stages than in non-pretreated shoot tips (Figure 3.7). The highest activity was in pretreated controls, which was 28 % more than in the non-pretreated controls, and it differed significantly ($P < 0.001$; Table 3.6) from all of the other treatment stages. A loss of GPX activity was found after mPVS2 treatment in both non-pretreated and pretreated samples. A greater loss of GPX activity was observed in pretreated samples (30 %) and GPX activity was not significantly different from the GPX activity in the non-pretreated control samples. There was no significant difference in the levels of GPX activity between shoot tips that were treated with mPVS2 and recovery solution in the respective pretreated and non-pretreated samples. A significant decline of 36 % in the GPX activity was measured in the non-pretreated samples after 24 h on recovery plate, whereas, the pretreated samples maintained almost the same level of GPX activity found after the recovery solution treatment. Both non-pretreated and pretreated shoot tips showed a significant loss of GPX activity when compared to controls, but the pretreated samples showed 35 % more GPX activity after 24 h on recovery plates.

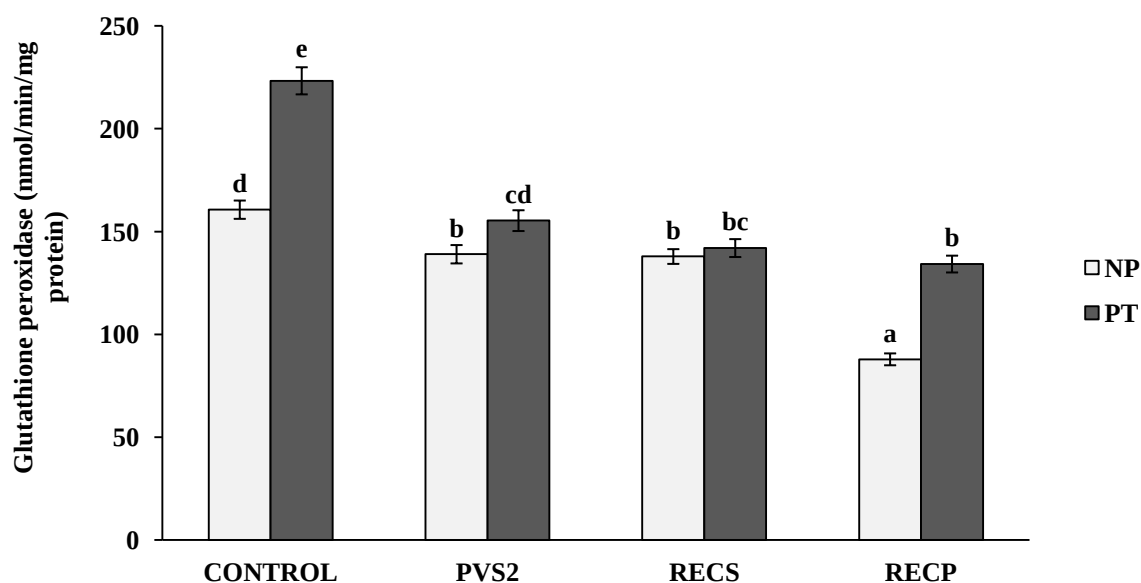


Figure 3.7. Glutathione peroxidase assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of glutathione peroxidase levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.6. Analysis of variance of glutathione peroxidase of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of glutathione peroxidase levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P
Control	1	17672.0	17672.0	80.83	<0.001
PVS2	1	1200.5	1200.5	5.49	0.022
RECS	1	76.1	76.1	0.35	0.557
RECP	1	9660.5	9660.5	44.18	<0.001

3.3.2.2.5 Glutathione Reductase

Pretreated shoot tips again maintained a higher level of GR activity than non-pretreated samples in all the treatments, especially in control ($P < 0.001$; Table 3.7) where the GR activity in pretreated shoot tips was 28 % higher than in non-pretreated shoot tips (Figure 3.8). However a significant loss in GR activity was noted after mPVS2 treatment for both pretreated and non-pretreated shoot tips (Figure 3.8). Although this decline in pretreated samples was much higher (33 %), the GR activity of pretreated shoot tips after mPVS2 treatment was not significantly different from the activity in non-pretreated control shoot tips. The GR activity of both non-pretreated and pretreated shoot tips after recovery solution did not significantly change from the level of activity after mPVS2 treatment. A highly significant drop by 34 % in the GR activity was observed in the non-pretreated samples after 24 h on recovery plates, whereas the pretreated samples maintained the same GR level as before. (Figure 3.8) The shoot tips held for 24 h on recovery plates showed a highly significant difference in GR activity from their controls in both samples, but the pretreated samples showed 35 % more GR activity than non-pretreated samples at this stage ($P < 0.001$; Table 3.7). A 17 % difference in GR activity was detected between non-pretreated control shoot tips and pretreated shoot tips after 24 h on recovery plates.

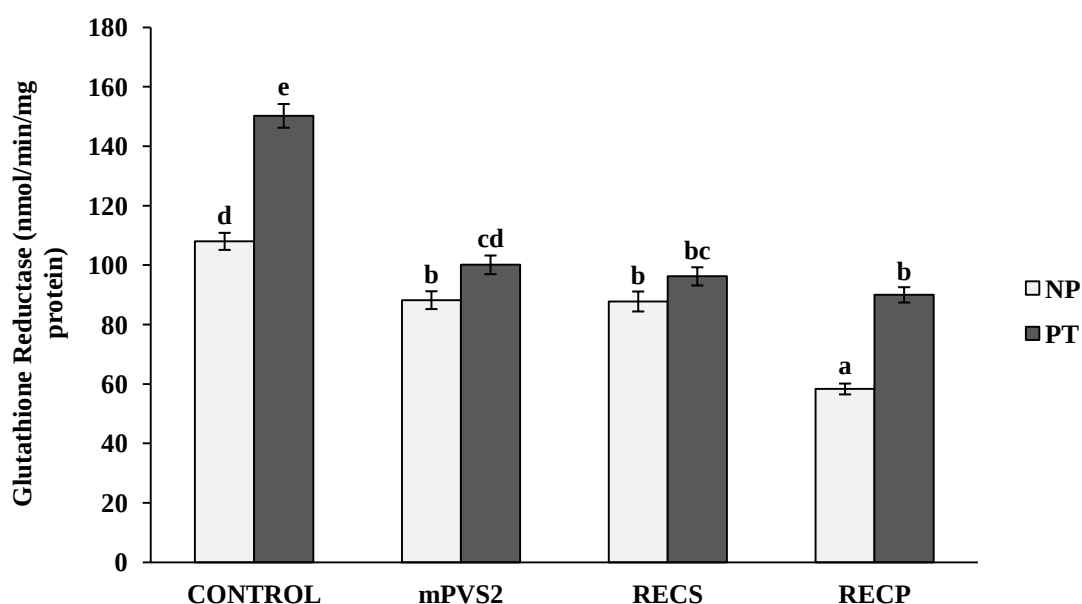


Figure 3.8. Glutathione reductase assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of glutathione reductase levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

Table 3.7. Analysis of variance of glutathione reductase of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of glutathione reductase levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	8022.22	8022.22	93.21	<0.001
PVS2	1	636.06	636.06	7.39	0.008
RECS	1	320.89	320.89	3.73	0.057
RECP	1	4512.50	4512.50	52.43	<0.001

3.3.2.2.6 The influence of pretreatment components on the enzymatic markers in shoot tips

To understand which pretreatment components had the greatest influence on activity of the antioxidant enzymes, cold, sucrose and the combination of cold, sucrose and ascorbic acid treated shoot tips were compared with non-pretreated shoot tips. For all five enzymes the pretreated shoot tips (treatment of interest) which were subjected to sucrose preculture supplemented with ASA and cold acclimation had higher levels of antioxidant enzymes and was significantly different from all the other three treatments (Figure 3.9). It appears that the combination of sucrose, ASA and cold was required to stimulate an increased antioxidant response.

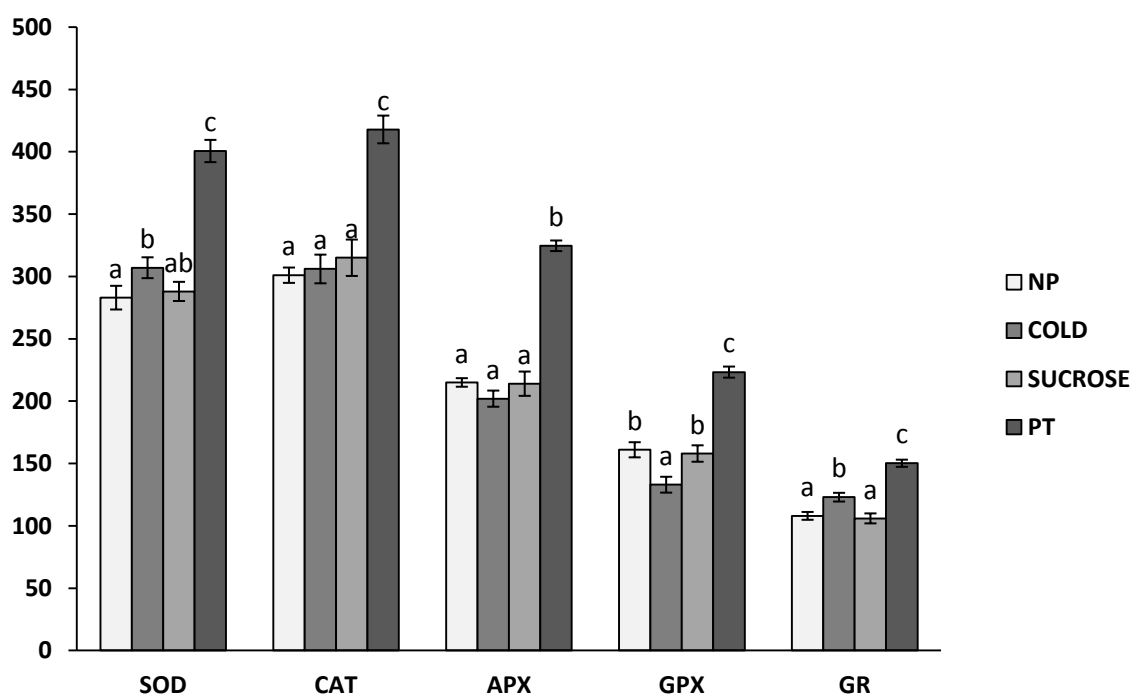


Figure 3.9. Antioxidant enzyme assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of antioxidant enzyme activity among non-pretreated shoot tips and shoot tips from different pretreatments. NP – shoot tips from culture room grown plantlets without any treatment; COLD – shoot tips from cold acclimated plantlets; SUCROSE– shoot tips after sucrose preculture without prior cold acclimation and PT–shoot tips from cold acclimated plantlets after sucrose preculture with supplementation of ASA. SOD – superoxide dismutase; CAT – catalase; APX – ascorbate peroxidase; GPX – glutathione peroxidase; GR – glutathione reductase. The bars indicate the standard error of mean. Means with the same letter for each enzyme are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 9$).

3.3.2.3 Non-enzymatic antioxidants

3.3.2.3.1 Total Ascorbate

The pre-treated shoot tips generally showed higher levels of total ascorbate than non-pretreated samples (Figure 3.10). The pretreated control shoot tips showed a greater total ascorbate level (28 % more than non-pretreated samples) ($P < 0.001$: Table 3.8), but this declined significantly after mPVS2 treatment and was not significantly different from non-pretreated controls. The non-pretreated samples showed no significant difference in the level of total ascorbate either in the mPVS2 or recovery solution from their control, but showed a significant decline after 24 h on the recovery plates (Figure 3.10). The total ascorbate level in pre-treated shoot tips after 24 h on recovery plates was not different to the levels observed in shoot tips in the recovery solution, and this was 39 % more than non-pretreated shoot tips on the recovery plates (Figure 3.10) ($P < 0.001$: Table 3.8).

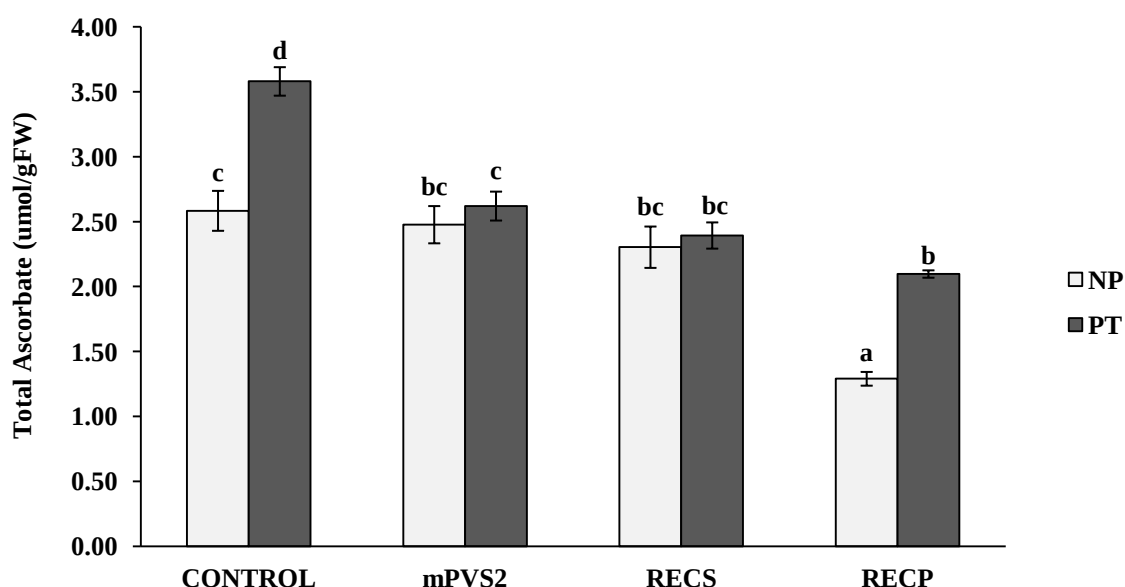


Figure 3.10. Total ascorbate assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of total ascorbate levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 3$).

Table 3.8. Analysis of variance of total ascorbate of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of total ascorbate levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	1.49002	1.49002	21.36	<0.001
PVS2	1	0.03082	0.03082	0.44	0.514
RECS	1	0.01215	0.01215	0.17	0.681
RECP	1	0.97607	0.97607	13.99	0.001

3.3.2.3.2 Reduced Ascorbate (% ASA)

In general, a decrease in the % of reduced ascorbate was observed at each consecutive DV treatment (Figure 3.11). The analysis of variance of % ASA in pretreated shoot tips showed no significant differences in control, after mPVS2 treatment and after recovery solution between non-pretreated and pretreated shoot tips (Table 3.9). The differences were marginally significant after recovery plates (Table 3.9). Mean separation by Fisher's protected least significant difference test gave a significant difference only at the recovery plate stage (Figure 3.11). The control treatment of both pretreated and non-pretreated shoot tips showed similar percentage of reduced ASA, but non-pretreated shoot tips decreased % ASA (loss by 28 %) after mPVS2 treatment in whereas, the pretreated shoot tips maintained the level with no significant difference to its control. The pretreated shoot tips that lost the reduced ASA levels after freeze-warming step maintained the same percentage even after 24 h on recovery plate (Figure 3.11). In contrast to this, a significant loss of % ASA was observed in non-pretreated shoot tips after 24 h on recovery plate. This decline resulted in a 24 % more % ASA in pretreated shoot tips than non-pretreated shoot tips after recovery plate (Figure 3.11).

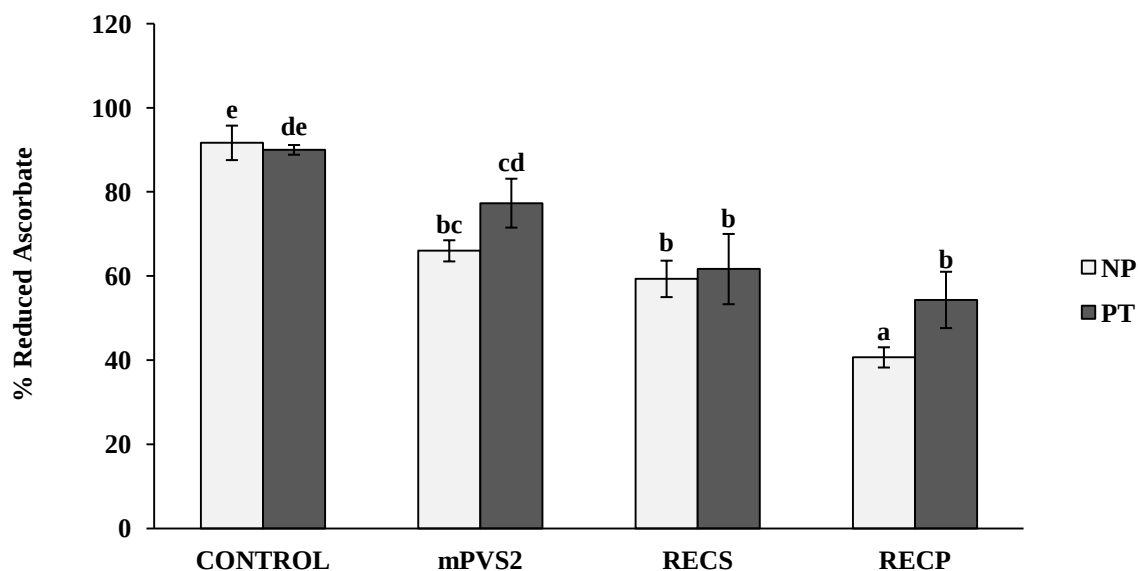


Figure 3.11. Reduced ascorbate assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of reduced ascorbate percentage in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 3$).

Table 3.9. Analysis of variance of reduced ascorbate of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of reduced ascorbate percentage in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	4.17	4.17	0.07	0.799
PVS2	1	192.67	192.67	3.09	0.094
RECS	1	8.17	8.17	0.13	0.721
RECP	1	280.17	280.17	4.49	0.047

3.3.2.3.3 Total Glutathione

The pre-treated shoot tips showed significantly higher levels of total ascorbate than non-pretreated samples in all treatments except recovery solution (Table 3.10). The pretreated control shoot tips showed a greater level (35 % more) of total glutathione than non-pretreated samples ($P < 0.001$; Table 3.10), but a significant decline was noted in pretreated shoot tips after mPVS2 treatment that reached a level equal to the control of non-pretreated shoot tips (Figure 3:12). Both pretreated and non-pretreated shoot tips significantly lost total glutathione content after recovery solution and recovery plate (Figure 3:12), but pretreated shoot tips showed 30 % more total glutathione level than non-pretreated shoot tips after recovery plate (Figure 3:12).

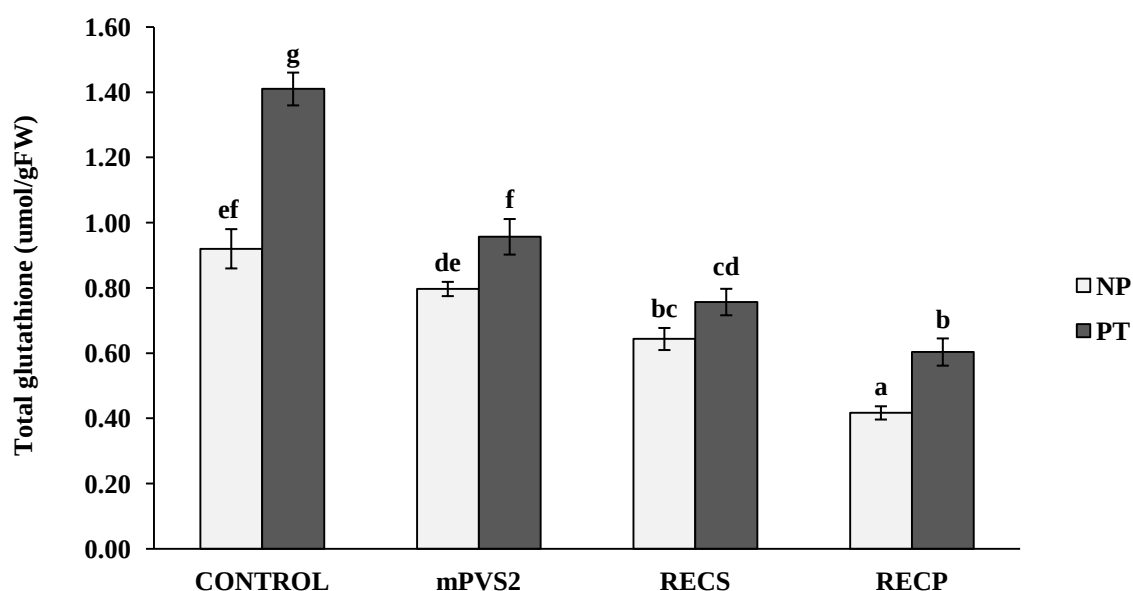


Figure 3.12. Total glutathione assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of total glutathione levels in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 3$).

Table 3.10. Analysis of variance of total glutathione of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of total glutathione levels in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	0.360150	0.360150	53.62	<0.001
PVS2	1	0.038400	0.038400	5.72	0.027
RECS	1	0.019267	0.019267	2.87	0.106
RECP	1	0.052267	0.052267	7.78	0.011

3.3.2.3.4 Reduced Glutathione (% GSH)

The % GSH in pretreated and non-pretreated shoot tips did not show any notable difference between them until recovery solution treatment, but a significant difference was observed after 24 h on recovery plate (Table 3.11). Mean separation by Fisher's protected least significant difference confirmed this (Figure 3.13). Though the % GSH declined after mPVS2 treatment in non-pretreated shoot tips, the pretreated shoot tips after mPVS2 treatment did not show any significant difference in % GSH to its control. However, after recovery solution and recovery plate treatment, a notable decrease in the level of % GSH in both pretreated and non-pretreated shoot tips were observed as in total glutathione assay. However, the pretreated shoot tips recorded significantly greater % GSH level (26 %) than non-pretreated samples ($P < 0.01$; Table 3.11; Figure 3.13).

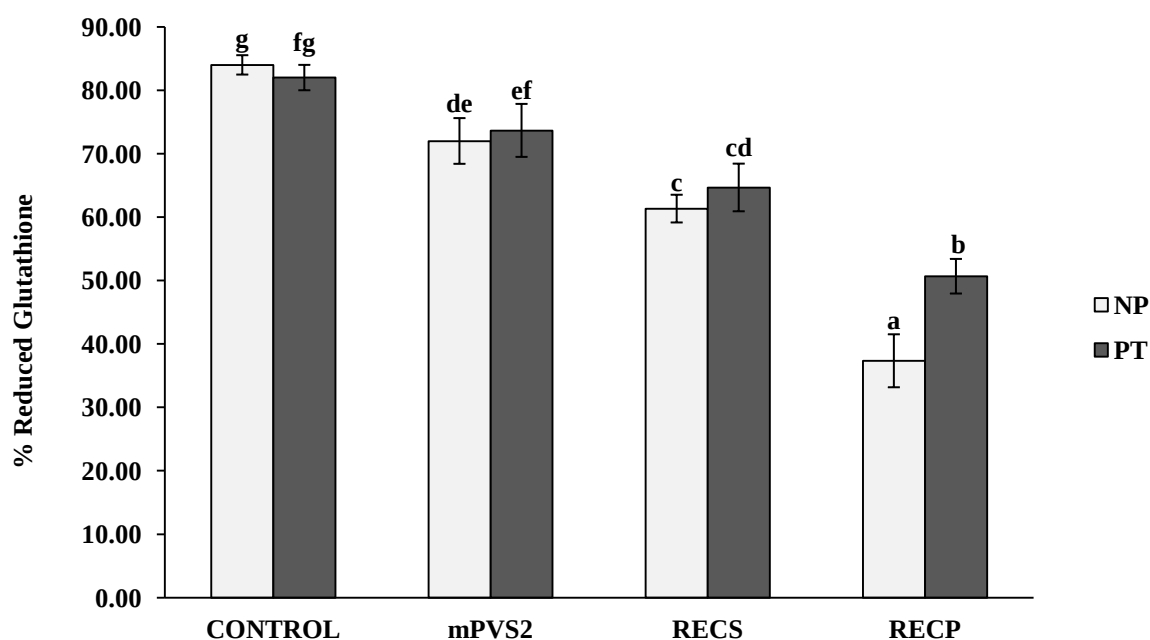


Figure 3.13. Reduced glutathione assay of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Comparison of reduced ascorbate percentage in *A. chinensis* ‘Hort16A’ shoot tips [non-pretreated (NP) and pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid (PT)] in Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate. The bars indicate the standard error of mean. Means with the same letter are not significantly different according to Fisher’s protected least significant difference test at $P < 0.05$ ($n = 3$).

Table 3.11. Analysis of variance of reduced glutathione of non-pretreated and pretreated ‘Hort16A’ shoot tips during droplet vitrification

Analysis of variance of reduced glutathione percentage in *A. chinensis* ‘Hort16A’ non-pretreated shoot tips and shoot tips pretreated by cold acclimation and serial dehydration in sucrose with ascorbic acid. Control– before treating with loading solution; mPVS2– after treatment in mPVS2 solution; RECS– shoot tips after cryopreservation and rewarming in recovery solution and RECP– after 24 h on recovery plate.

Treatments	d.f.	s.s.	m.s.	v.r.	P.
Control	1	6.00	6.00	0.23	0.637
PVS2	1	4.17	4.17	0.16	0.694
RECS	1	16.67	16.67	0.64	0.434
RECP	1	266.67	266.67	10.20	0.005

3.4 DISCUSSION

The results presented in this chapter reveal that the variations in the oxidative damage markers and antioxidant levels were proportional to the amount of damage caused at each step in the DV process, as observed by the ultrastructural study. In general, antioxidant levels decreased with increasing cell damage. This observation provides some evidence that there is a relationship between the cellular damage and a reduction/loss of the ability of cells to cope with oxidative stress at each step of the DV cryopreservation process.

Comparison, in this study, of ultrastructural changes of the untreated shoot tips with those serially dehydrated in increasing sucrose concentrations in the media supplemented with ASA after cold acclimating the stock plantlets for 2 weeks at 4 °C (pretreatment) showed that there is less lethal damage in many cells of pretreated shoot tips. However preculture on sucrose media did induce some ultrastructural changes such as fragmentation of vacuoles, swelling of the endoplasmic reticulum, accumulation of starch and undulation of plasma membrane due to partial plasmolysis (Helliot et al. 2003). Ristic and Ashworth (1993) and Zhang et al. (2005) observed greater changes to the ultrastructure in leaves in response to CA, such as invagination of plasma membrane, irregular shape of chloroplasts and nucleus, vascularisation of plasma membrane and chloroplast membrane, fragmentation of endoplasmic reticulum and formation of darkly stained globules on plasma membrane and organelle membranes, but no reports are available on the ultrastructure of shoot tips in response to CA.

Cold acclimation and sucrose preculture have been found to enhance the antioxidant activity of plant cells, thus protection against oxidative stress. For example, sucrose enhanced GR in olive somatic embryos (Lynch et al. 2011) and CAT activities in *Morinda citrifolia* adventitious root suspension cultures, but also induced the accumulation of H₂O₂ and MDA (Baque et al. 2012). Cold acclimation increased the levels of APX and total glutathione, but also produced MDA as the by-product of lipid peroxidation in *Arabidopsis thaliana* (O'Kane et al. 1996). The results of the present study show that sucrose preculture or cold acclimation alone did not cause a significant increase in the activities of the antioxidant enzymes measured. In contrast, the combination of cold acclimation of stock plants with sucrose pretreatment of shoot tips in ASA supplemented media resulted in a significant rise in the levels of all the antioxidants measured. The higher antioxidant status, more the protection plant cells have against ROS damage (Bela et al. 2015; Johnston et al. 2007; Prasad 1996). It is also notable that pretreated shoot tips, in this study, showed PCs and LPs contents similar to

shoot tips freshly excised from culture room grown-plants, which have not experienced any additional stresses. This might be the reason for less ultrastructural damage in the pretreated shoot tips in the present study in contrast to several other reports showing greater ultrastructural damage, when cold acclimation or sucrose preculture were used separately. The maintenance of shoot tips in the cold (4 °C) and in the dark during sucrose dehydration might have also protected them from severe ultrastructural changes, as no photosynthetic electron transport would occur.

The TEM images showed that the 60 min treatment in mPVS2 at 4 °C did not result in severe ultrastructural changes in non-pretreated shoot tips, although some plasmolysis and fragmentation of the vacuoles did occur. However, pretreated shoot tips suffered plasmolysis and even rupturing of the plasma membrane in some cells. Osmotic stress or water stress in plant cells induces the production of ROS (Mittler 2002) that subsequently can cause damage to membranes and macromolecules of the cell (Sharma et al. 2012). Uchendu et al. (2010a) hypothesized that the accumulation of MDA, which damages proteins and DNA, is caused by osmotic stress induced by the sucrose and glycerol treatment of blackberry shoot tips undergoing cryopreservation. ROS mediated oxidative stress can cause oxidation of lipoproteins of the membranes, glycoxydation and oxidation of DNA (Bandyopadhyay et al. 1999). In stressed tissues, a significant proportion of proteins are carbonylated (Møller & Kristensen 2004). These findings support the results reported in the present research and suggest that the high molarity of the loading and mPVS2 solutions might have resulted in increases in the PCs and LPs contents of both non-pretreated and pretreated shoot tips after mPVS2 treatment, even though the DMSO in mPVS2 has some antioxidant properties (Sanmartín-Suárez et al. 2011; Uchendu et al. 2010a). The significant increase in oxidative damage in the pretreated shoot tips compared to the non-pretreated shoot tips might be because of the additional treatment of pretreated shoot tips in high concentrations of sucrose that might have resulted in a combined synergistic stress effect, upon exposure to mPVS2. Li et al. (2010) observed that exposure to 15 min in DMSO containing solution inhibited GR activity in common carp sperm and they also suggested that DMSO altered the lipid bilayer structure of the membrane. Gale et al. (2014) also reported a significant increase in the levels of PCs and LPs along with a decrease in the total glutathione level after treatment of oocytes of greenshell mussels with a cryoprotectant solution. Pang et al. (2002) observed that the lipid peroxidation by-product, MDA is inversely proportional to the SOD and CAT activity in wheat seedlings under lead-induced stress. Although there are no reports on individual antioxidant markers during cryopreservation of plant tissue, the results from animal systems

described above suggest that one of the reasons for the increased LP content in this study might have been caused by the decrease in CAT activity during DV.

It has been reported that the various stressors can cause a decrease in the activity of antioxidant enzymes (Ali & Alqurainy 2006; Dionisio-Sese & Tobita 1998; Meloni et al. 2003; Zhang & Kirkham 1994). Similarly, in the present study, the levels of all the enzymatic antioxidants decreased as the stress levels of shoot tips increased at each step in the DV process, with the loss in activity greater in non-pretreated shoot tips than in pretreated shoot tips, which corresponded to greater cell damage. In contrast to the significant decrease in the level of enzymatic antioxidants after mPVS2 treatment in shoot tips, the non-pretreated shoot tips did not show any significant decrease in total ascorbate and glutathione levels, but a significant increase in their oxidised status was noted. Despite the reduction in ascorbate and glutathione levels in pretreated shoot tips after mPVS2 treatment, they maintained percentage of reduced form (high GSH/GSSG ratio) as in control (i.e. before loading treatment). This shows that the pretreatment helps to maintain a healthy antioxidant status in the surviving cells.

After incubating the frozen shoot tips in recovery solution at room temperature, lethal cell damage could be seen in many cells for both non-pretreated and pretreated shoot tips that could cause tissue death. Although the levels of PCs and LPs rose significantly at this stage in both non-pretreated and pretreated shoot tips, the enzymatic antioxidants did not show any significant change from the levels achieved after mPVS2 treatment. Gale et al. (2014) also reported a large increase in PCs and LPs level after rewarming the cryopreserved green shell mussel oocytes. The maintenance of high concentrations of GSH is important for the overall redox balance of plant cells (Foyer et al. 1997). Under most conditions, glutathione is found largely in its reduced form accounting for 90-99 % of the total glutathione content of the cell (Foyer et al. 1991). Under normal conditions, the conversion of GSH to GSSG by GPX and the conversion of the oxidised form (GSSG) back to its reduced form (GSH) by GR are tightly regulated (Lushchak 2012), but severe stress can increase the concentration of GSSG (Strid 1993). Strid et al. (1994) observed a drastic reduction of total glutathione along with the accumulation of GSSG under UV stress (Foyer et al. 1997). A similar observation was noted in both non-pretreated and pretreated shoot tips in the present research also. GSSG can inactivate enzymes by forming mixed disulphides, but their formation is usually prevented by a high GSH/GSSG ratio, but a fall in this ratio can lead to the formation of mixed disulphides (Foyer et al. 1997). The accumulation of GSSG in this study suggests that the freeze-warming step might have resulted in the production of a large amount of ROS that could have altered GSH regeneration, synthesis or both. In addition, ice crystallisation may have physically

damaged the cell organelles, for example the mitochondria, increasing ROS production and GSH utilisation. Decreased GSH levels can cause hypersensitivity of the cells to stress (Apel & Hirt 2004).

Severe ultrastructural damage to almost all cells was apparent after 24 h on the recovery plate resulting in death of 100 % of non-pretreated shoot tips. In contrast many cells in the pretreated shoot tips showed less ultrastructural damage and survived the potentially lethal effects of cryopreservation. Muldrew and McGann (1994) hypothesised that the pressure developed during freezing, induced by the water flux, ruptures the plasma membrane. The TEM results of this study showed loss of the cell contents in most damaged cells as a result of plasma membrane rupture. The rupture of plasma membrane is considered to be the main cause of freezing injury along with the overproduction of ROS (Kendall & McKersie 1989). For example ultra-freezing during cryopreservation caused substantial increase in the production of ROS in human sperm cells (Mazzilli et al. 1994; Wang et al. 1997) and bovine spermatozoa (Chatterjee & Gagnon 2001), and resulted in poor function after thawing. The oxidative stresses that occur during the different stages of cryopreservation result in reduced viability of the tissues after recovery (Uchendu et al. 2010a). The higher levels of ROS can damage proteins, lipids and DNA (Goodarzi et al. 2010; Møller et al. 2007; Rowe et al. 2008; Sharma et al. 2012). Møller et al. (2007) reported that ROS can also oxidize and modify amino acids like cysteine and thereby prevent the proper functioning of enzymes such as GPX, which contains cysteine in their active site (Bela et al. 2015). Bilodeau et al. (2000) reported decreased SOD and GSH levels after cryopreservation of bovine sperm. Lasso et al. (1994) reported a significant decrease in SOD levels after cryopreservation of human sperm was the result of plasma membrane damage. Increased amounts of MDA, a breakdown product of LPs, was detected in frozen/thawed rice cells compared to the unfrozen cells, suggesting that freezing injury can cause lipid peroxidation (Benson et al. 1992). Alvarez and Storey (1992) reported a decrease in SOD after cryopreservation of human sperm and they hypothesized that the enhanced lipid peroxidation observed after recovery is mediated by the loss of SOD activity. These results along with the observation in this study suggest that the decrease in the antioxidant levels along with the increased oxidative damage are might contribute to the loss of cell viability. This could in part be due to the loss of antioxidants from cytosol as an aftermath of plasma membrane rupture. In this study, both pretreated and non-pretreated shoot tips showed high PCs and LPs levels and a decline in all the antioxidants. However, non-pretreated shoot tips showed much greater loss of antioxidant activity and higher levels of oxidative damage.

Addition of antioxidants during the cryopreservation process reduces oxidation and results in improved regrowth of both control and cryopreserved plant tissues (Reed et al. 2012). The supplementation of antioxidants enhanced protection of the membrane with an increased SOD activity and lower MDA production in bovine semen cryopreservation (Beconi et al. 1993). Hence the addition of ascorbic acid to media during pretreatment may have been the cause of the increase in total ascorbate observed in the pretreated controls and this may have helped to protect the pretreated shoot tips during cryopreservation.

Very little research has been conducted investigating oxidative stress and antioxidant metabolism during plant cryopreservation. Johnston et al. (2007) measured the non-phenolic antioxidants and total antioxidants at different stages of ED of *Rubus* shoot tips. They also studied the production of $\cdot\text{OH}$ during cryopreservation by measuring the level of methane (a marker of $\cdot\text{OH}$). Uchendu et al. (2010a) measured MDA to study lipid peroxidation during cryopreservation of blackberry shoot tips using PVS2 vitrification method. In general, the results presented in this chapter show that cold acclimating the donor plants and preculturing the shoot tips in media with increasingly high sucrose concentrations along with ascorbic acid supplementation protect the shoot tips from oxidative stress during cryopreservation. Cells that survived in the pretreated shoot tips may have retained a sufficiently high antioxidant status to counteract the oxidative damage resulting from the freezing and osmotic injury. This study is, to the best of my knowledge, the first to investigate oxidative stress during cryopreservation in plants utilizing the three categories of oxidative biomarkers viz. enzymatic antioxidants, non-enzymatic antioxidants and oxidative damage markers, combined with an ultrastructural investigation. In addition, this study has demonstrated the biomarkers of oxidative stress and antioxidant metabolism, which include enzymatic and non-enzymatic antioxidants as well as markers for oxidative damage, combined with ultrastructural investigations can be very helpful to understand the degree of damage occurring during cryopreservation and thus could help to more effectively develop protocols for the cryopreservation of plant germplasm.

4 GENERAL DISCUSSION

Cryopreservation is currently the only method available to preserve cells or tissues for long periods of time. Plant cryopreservation is essential to provide germplasm for breeding programmes and other research as well as to overcome the loss of germplasm erosion caused by disease, pests or natural calamities. Plant cells are more difficult to cryopreserve than animal or bacterial cells, often regenerate poorly after cryopreservation due to the presence of vacuoles with high water content and rigid cell walls (Heine-Dobbernack et al. 2008). Plant species that produce recalcitrant seeds or are vegetatively propagated, like kiwifruit, (Bachiri et al. 2001; Monette 1995) often rely on cryopreservation for long-term conservation (Altman & Hasegawa 2012; Engelmann 2004).

Though successful plant cryopreservation began in the late 1950s (Sakai 1960), the first cryopreservation of kiwifruit was reported in 1989 when Jian and Sun (1989) successfully cryopreserved stem segments of kiwifruit by two-step freezing. Since then successful regeneration after cryopreservation has seldom been reported in kiwifruit despite the nutritional (Antunes et al. 2010; Ferguson & Ferguson 2002; Tavarini et al. 2008) and economic importance of this fruit plant (Balestra et al. 2009; Benelli et al. 2013; Lorestani & Tabatabaeefar 2006). In addition, there is no collection of kiwifruit conserved in any of the cryopreserved gene banks in the world (Table 1.1). Since the work of Jian and Sun (1989) using slow freezing, the cryopreservation techniques applied to *Actinidia* species include: slow freezing (Li & Guo 1996; Liu et al. 2003; Liu et al. 1998), vitrification (Cai et al. 2006; Hakoziaki et al. 1996; Xu et al. 2006a; Xu et al. 2006b) and ED (Bachiri et al. 2001; Suzuki et al. 1994; Suzuki et al. 1996; Wu et al. 2000; Zhai et al. 2003). Though researchers have tried to cryopreserve *Actinidia* species, in nearly half of the reports, no mention was made of successful regeneration (Table 4.1), suggesting that regeneration of *Actinidia* species after cryopreservation is difficult to achieve, especially when vitrification solution-based vitrification protocols were used. Genetic fidelity after cryopreservation is important, and reports to date suggest that cells of *A. deliciosa* that survive cryopreservation were phenotypically (Li & Guo 1996) and genetically similar (Zhai et al. 2003) to controls. Hakoziaki et al. (1996) developed a vitrification protocol for the cryopreservation of hypocotyl callus from *A. deliciosa* ‘Hayward’ as a model, probably because it was the only cultivar known outside China. Liu et al. (2003) optimized a freezing solution for the cryopreservation of the lateral buds of *A. arguta* by slow freezing. Cai et al. (2006) and Xu et al. (2006a) investigated the ultrastructural changes in shoot tips of *A. deliciosa* and *A. chinensis* respectively during cryopreservation by vitrification.

Table 4.1. Application of cryopreservation reported in *Actinidia* species

Species	Explant used	Technique applied	Key findings/results	Reference
<i>A. chinensis</i>	Stem segments	Slow freezing	Survival after LN: 75 %	(Jian & Sun 1989)
<i>A. deliciosa</i>	Callus	Slow freezing	The survived cells after cryopreservation showed no significant phenotypic differences	(Li & Guo 1996)
<i>A. arguta</i>	Lateral buds	Slow freezing	Survival after LN: 50 %	(Liu et al. 1998)
<i>A. arguta</i>	Lateral buds	Slow freezing	Optimised freezing solution for cryopreservation	(Liu et al. 2003)
<i>A. deliciosa</i>	Shoot tips	ED	Regeneration after LN: 80 %	(Suzuki et al. 1994)
<i>A. deliciosa</i>	Shoot tips	ED	Pretreated with ABA or proline before cryopreservation; survival of 30 % and 23 % respectively	(Suzuki et al. 1997)
<i>Actinidia</i>	Shoot tips	ED	Survival after LN: 22 %-56 %	(Wu et al. 2000)
<i>Actinidia</i> spp.	Shoot tips	ED	Survival after LN: 70-95 % in 9 genotypes	(Bachiri et al. 2001)
<i>A. deliciosa</i>	Shoot tips	ED	Using RAPD, the genetic stability of the cryopreserved apex confirmed	(Zhai et al. 2003)
<i>A. deliciosa</i> 'Hayward'	Hypocotyl callus	PVS2-Vitrification	Method of cryopreservation described. Calli viable after cryopreservation.	(Hakozaki et al. 1996)
<i>A. deliciosa</i>	Shoot tips	PVS2-Vitrification	Investigated ultrastructural changes during cryopreservation	(Cai et al. 2006)
<i>A. chinensis</i>	Shoot tips	PVS2-Vitrification	Investigated ultra structural changes during cryopreservation	(Xu et al. 2006a)
<i>A. deliciosa</i>	Shoot tips	PVS2-Vitrification	Regeneration after LN: 51.6 %	(Xu et al. 2006b)

While the findings of the previous research on kiwifruit cryopreservation have helped to understand the processes underlying the different techniques used, most of the work did not report the efficiency of their protocols with respect to regeneration of plants after cryopreservation. It is also noteworthy that even valuable *Actinidia* germplasm collections are at a high risk of threatening diseases like Psa, there has been no successful cryopreservation reported in *Actinidia* species for the last nine years and no new methods have been attempted other than the reported three methods.

The most successful kiwifruit cultivar in recent years has been ‘Hort16A’ belonging to *A. chinensis* (Yahia 2011). The only reports of the cryopreservation of this species have been limited to Xu et al. (2006a) and Jian and Sun (1989). Xu et al. (2006a) studied ultrastructural changes during cryopreservation without mentioning the rate of success of regeneration and Jian and Sun (1989) used stem segments as explants. Shoot tips and meristems are considered ideal for cryopreservation as they are capable of producing virus free plants at high frequency and also produce genetically identical progeny (Vasil & Thorpe 1994). The largest *Actinidia* collection outside China is in New Zealand (Ferguson & Seal 2008; PFR 2015) and as a first step towards the establishment of a cryopreserved collection, the present research was aimed at developing a robust protocol for *A. chinensis* ‘Hort16A’ using droplet vitrification, which is becoming a generic method of plant cryopreservation (Panis et al. 2009).

In the present research, a series of experiments were conducted with different treatments to study the response of *A. chinensis* ‘Hort16A’ to the vitrification solution, sucrose dehydration, CA and to supplementation of media with ASA. The shoot tips survived DV, only when they were pre-treated before treating with loading solution and mPVS2 dehydration. A combined pre-treatment was more effective than a single treatment when post-cryo regeneration was considered. The combination of CA of donor plants and preculturing the shoot tips in sucrose containing media has been shown to increase the survival after cryopreservation in several plant species such as *Thymus* (Coelho et al. 2014; Ozudogru & Kaya 2012), sugarcane (Fki et al. 2013), kiwifruit (Wu et al. 2000), chrysanthemum (Yamamoto et al. 2011) and potato (Sallam et al. 2013). Addition of ASA to the treatment medium has also improved post-cryo survival of blackberry shoot tips (Uchendu et al. 2010a) and triggered early regrowth in *Garcinia mangostana* shoot tips after cryopreservation (Ibrahim & Normah 2013). ASA is a potent antioxidant and helps to protect plants against environmental stresses, playing a central role in removing H_2O_2 and $O_2^{\cdot -}$ (Ahmad 2014). Cold acclimating donor plants at 4 °C for 2 weeks and preculturing with daily increasing sucrose concentrations (up to 1 M) supplemented with ASA was chosen as the best pre-treatment.

While many studies investigating cryopreservation concentrate largely on method development, in the present study ultrastructural and biochemical changes associated with each step of cryopreservation and regeneration were also investigated. The results of biochemical analyses and observations of the ultrastructure presented in the third chapter help to shed some light on the biological reasons for the regeneration results presented in Chapter two. The pre-treated shoot tips recorded the highest regeneration when treated in mPVS2 solution for 60 min at 0 °C. This treatment provided the highest antioxidant activity and lesser

ultrastructural damages when compared with non-pretreated shoot tips that gave 0 % regrowth after DV.

Despite being exposed to stressors, oxidative damage level in the pretreated shoot tips was similar to that in non-pretreated shoot tips, probably because of the increased antioxidant activity in the pretreated shoot tips after pre-treatment. Helliot (2003) reported severe plasmolysis and subcellular changes when shoot tips of banana were pre-cultured on sucrose-enriched media. In this study, such changes were comparatively less, suggesting that either CA or ASA supplementation or their combined action reduced the osmotic damage caused by exposure to high sucrose concentrations. ASA might have played a vital role in maintaining the higher antioxidant status in the cells. Similar levels of PCs and LPs observed in both pre-treated and non-pre-treated shoot tips show that the pre-treatment did not cause significant oxidative damage to the shoot tips. However, the regeneration of pre-treated shoot tips was slower compared to the shoot tips that did not receive any pre-treatments. This indicates that even though the pretreatment did not cause severe damage to shoot tips, cold stress and sucrose dehydration, even with ASA, weakened/stressed them. However, after DV, only pre-treated shoot tips survived, whereas, shoot tips that did not receive any pretreatment did not survive ultra-freezing and all died. This demonstrates that shoot tips require CA and sucrose dehydration, with ASA, in order to tolerate ultra freezing temperatures. CA minimises the concentration of toxic solutes, decreases the sensitivity of plasma membrane to mechanical stress induced by osmotic contraction and expansion and also stabilizes plasma membrane during freezing (Steponkus 1984), whereas, sucrose (Venediktov & Krivoshejeva 1983) and ASA (Bartoli et al. 2004) are known for their abilities to protect proteins against denaturation and oxidative stress.

The next critical step chosen in protocol development was the mPVS2 treatment, which follows after the loading solution treatment. The non-pretreated shoot tips in the current study showed 100 % viability before cryopreservation. After mPVS2 treatment they showed mild ultrastructural changes and a slight decrease in antioxidant levels. It is difficult to cryopreserve 'hairy' explants because of their high surface tension, which prevents efficient treatment with cryoprotectants (Li et al. 2013). Hence, 'Hort16A' shoot tips, which have epidermal hairs, are comparatively more tolerant to mPVS2 than many other genotypes, which require less time in PVS2 for successful cryopreservation (Jin et al. 2012; Kiran et al. 2012; Le Bras et al. 2014; Uchendu et al. 2013, 2014). Furthermore, the high surface tension created by the air bubbles formed around the hairs on 'Hort16A' shoot tips might slow down the uptake of cryoprotectants into the shoot tip tissue.

In contrast to the non-pretreated shoot tips, the pretreated shoot tips after mPVS2 treatment showed notable changes in terms of regeneration, ultrastructure, oxidative damage and level of antioxidants. The osmotic stress induced by sucrose and glycerol can cause lipid peroxidation (Uchendu et al. 2010a). In the present research, the pretreated shoot tips would have been more stressed with an additional preculture in high sucrose media. Compared to the non-pretreated shoot tips, less air bubble formation was observed in pretreated shoot tips at the time of treatment with loading solution and mPVS2. The additional sucrose dehydration may have reduced the turgidity of the shoot tip tissues, particularly in the epidermal cells and hairs, facilitating easy penetration of cryoprotectants. Thus, some of these severely stressed and flaccid pretreated shoot tips (dehydrated for 4 days on increasingly high-sucrose preculture) further weakened by the osmotic stress induced by loading solution, and the subsequent treatment with toxic mPVS2 solution (7.8 M) may have died before cryopreservation. Though the DMSO in PVS2 has antioxidant properties (Uchendu et al. 2010a), prolonged exposure of explants to PVS2 can cause injury to cells/tissues due to chemical toxicity or strong osmotic stress (Li & Palva 2012; Towill & Bajaj 2002; Volk et al. 2013). The regeneration of shoot tips decreases with increased exposure to PVS2 as a general rule (Choudhary et al. 2014; Ibrahim & Normah 2013; Lin et al. 2014a; Marković et al. 2014). The regeneration of pretreated shoot tips significantly decreased with an increase in the duration of mPVS2 treatment and was highest at the extreme duration of 60 min tested in this study; however 60 min dehydration resulted in the highest regrowth and was chosen for the ultrastructural and biochemical studies. Although PVS2 has a damaging effect on the cells, it also dehydrates the cells, thus reducing lethal ice formation during freezing/thawing (Volk & Walters 2006).

Ultrastructural studies on banana (Helliot et al. 2003) and kiwifruit (Xu et al. 2006a) have shown severe damage after PVS2 treatment that resulted in decreased regeneration. In this study, the ultrastructural and biochemical results could be linked to regeneration percentage. The toxic effect of the mPVS2 might have caused lethal to the cell organelles, which in turn caused cell death that is reflected in 16-17 %, decrease in regeneration after 60 min mPVS2 treatment. Thus the higher amount of dead tissue among the pretreated shoot tip samples after mPVS2 treatment (even before LN treatment) could be the reason for the decrease in antioxidant levels and the increased oxidative damage in the form of lipid peroxidation and protein oxidation, when compared to pre-treatment controls. The results of the study show that only 82 % of the total shoot tips survived before immersion in LN.

The effectiveness of cryopreservation cannot be determined immediately after thawing from liquid nitrogen (Naaldijk et al. 2013). Cryopreservation-induced apoptosis, which plays an important role in freeze-thaw injuries associated with cryopreservation (Borderie et al. 1998; Johnston et al. 2009) can be caused by oxidative stress (Kannan & Jain 2000; Sinha et al. 2013). The most common form of cell death found in cultured plant cells and tissues, that has many similarities to animal apoptosis, is termed apoptotic-like programmed cell death (AL-PCD), and occurs during both developmental and hypersensitive response-related cell death (Petrov et al. 2015; Reape et al. 2008). As it is genetically programmed and occurs at certain phases of the cell cycle (Herbert et al. 2001; Kuthanova et al. 2008), AL-PCD is not an immediate phenomenon; a significant increase in cell death was observed after 24 h rather than immediately after thawing in cryopreserved human corneal keratocytes (Borderie et al. 1998). The shoot tips in this study did not show as much damage in the recovery solution compared to the recovery plate (24 h after thawing). The similar antioxidant activity of shoot tips after recovery solution compared to that after mPVS2 solution in the present study shows that the cells were still in similar metabolic states. The increase in the lipid peroxides and protein carbonyls observed in this study after treating the shoot tips with recovery solution indicate cell damage immediately after the freeze-thaw process.

The ultrastructural changes observed after 24 h incubation on recovery agar plates agree with the regeneration responses observed after cryopreservation. The ultrastructural observations indicate largely dead cells were present in non-pre-treated shoot tips in contrast to the pretreated shoot tips that possessed some apparently living cells that were able to maintain their structural integrity and metabolic functionality. The biochemical analysis showed a sharp decrease in the antioxidant activity along with a rise in the level of protein carbonyls and lipid peroxides in non-pretreated shoot tips at this stage. These biochemical data along with ultrastructural changes indicate severe damage to the cells of the shoot tips that did not receive pre-treatment and show reason for the 0 % regeneration of non-pretreated shoot tips after cryopreservation. In contrast, the antioxidant levels in pretreated shoot tips, after 24 h on the recovery plates, did not show a significant difference in these biomarkers compared to levels after the recovery solution, except for a slight decrease in SOD and glutathione. In general, levels of all the antioxidants in pretreated shoot tips were higher, and hence lower oxidative damage (i.e. PCs and LCs) was observed compared to non-pretreated shoot tips. The surviving cells, with less damage observed in the ultrastructural study of pretreated shoot tips appeared to have the capacity to undergo repair and retain regenerative potential after their re-culture, resulting in 40 % regeneration of the pre-treated shoot tips.

Hence, a pretreatment of plantlets with CA followed by preculture of shoot tips on sucrose media supplemented with ASA to increase their survival rate is recommended for *A. chinensis* 'Hort16A' droplet vitrification.

Due to time limitations, this research could not test further durations for mPVS2 exposure, higher concentrations of sucrose preculture, different antioxidant supplementations or prolonged CA. Introduction of techniques such as vacuum infiltration (Funnekotter et al. 2015) may require further changes to the timing in mPVS2. Further research on these parameters could improve the protection and increase the survival of shoot tips. A more focussed ultrastructural study on cell organelles would be helpful to understand the changes in each organelle. Study on organelle-wise ROS production and antioxidant synthesis would help in better understanding of cryopreservation-induced oxidative stress and thus improve the DV protocol developed in the present study. The cryopreservation response is highly genotype-specific in plants (Johnston et al. 2007; Kaczmarczyk et al. 2011; Niino & Arizaga 2015; Uchendu & Reed 2008; Wang & Valkonen 2009a) and therefore testing the applicability of this protocol to other *A. chinensis* genotypes and then to other *Actinidia* species followed by further research to improve it would pave the way for the establishment of a cryopreserved *Actinidia* collection in New Zealand.

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