AN ABSTRACT OF THE THESIS OF

Esther Eyiuche Uchendu for the degree of Doctor of Philosophy in Horticulture presented on August 27, 2009.

Title: Cryopreservation of Shoot Tips: Antioxidant Investigations with *Rubus* and Protocols for *Mentha* and *Vaccinium*.

Abstract approved: _____
Barbara M. Reed

Oxidative stress that occurs during cryopreservation may be reduced by the addition of antioxidants. Vitamin E (Vit E), Vitamin C (Vit C), glutathione (GSH), lipoic acid (LA), glycine betaine (GB) and polyvinylpyrrolidone (PVP) were applied at four steps of the PVS2 vitrification protocol (pretreatment, loading, rinsing, and regrowth). Shoot tips of *in-vitro* grown blackberry cultivars 'Chehalem' and 'Hull Thornless' were cryopreserved. Malondialdehyde (MDA), a lipid peroxidation product was higher in shoot tips at each step compared to controls. Shoot tips treated with Vit E had low MDA similar to the controls. GB, GSH, Vit E and Vit C significantly increased regrowth at all steps. Vit C added to regrowth medium with double Murashige and Skoog iron concentration decreased recovery; however, in iron-free medium Vit C improved regrowth. LA was not very effective in regrowth medium. PVP was not effective at any step. Regrowth was highest (up to 92%) with Vit C. This is the first report of vitamins C, and E, GB, and LA use in protecting cultures from oxidative damage during cryopreservation.

Mentha species [*M. canadensis* L., *M. australis* R.Br., *Mentha* x *piperita citrata* (Ehrh.) Briq. and *M. cunninghamii* Benth,] were cryopreserved using controlled cooling (CC), encapsulation dehydration (ED), and PVS2 vitrification (VIT).

Recovery of *Mentha* x *piperita citrata* and *M. australis* showed significant differences among protocols with CC > VIT > ED. *M. canadensis* and *M. cunninghamii* recovery with CC and ED was significantly better than VIT. All species responded to CC (93%) better than ED (71%) or VIT (73%).

Cranberry (*Vaccinium macrocarpon* Aiton) cultivars 'Wilcox' and 'Franklin' and blueberry (*Vaccinium corymbosum* L.) 'Berkeley', 'O'Neal' and 'Brigitta' were tested for desiccation tolerance and recovery after cryopreservation. Blueberry cultivars were desiccation tolerant after 7 h of drying under laminar air flow while cranberry cultivars were mostly dead by 3 h. Cryopreserved blueberry cultivars had 83% to 92% regrowth with ED. The results varied from 33% to 87% for VIT and from 50% to 67% for CC. Cranberry cultivars had poor regrowth with these protocols but improved by 20-30% with Vit C added at any of the four PVS2 steps.

CRYOPRESERVATION OF SHOOT TIPS: ANTIOXIDANT INVESTIGATIONS WITH *RUBUS* AND PROTOCOLS FOR *MENTHA* AND *VACCINIUM*.

by

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A THESIS

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented August 27, 2009

Commencement June 2010

Doctor of Philosophy thesis of Esther Eyiuche Uchendu presented on August 27, 2009.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Esther Eyiuche Uchendu, Author

ACKNOWLEDGEMENTS

My first gratitude goes to God Almighty who kept me alive till date. I thank God for HIS gifts of wisdom, patience and the perseverance to pursue my dream of attaining academic excellence.

I thank the following organizations that offered fellowships or scholarship awards for my academic and professional training; the Ford Foundation International Fellowship Program, the American Association of University Women, the P.E.O. International Peace Scholarship, the University Club of Portland Inc. and the Society for *In Vitro* Biology.

I thank my academic advisor, professional model and best friend, Dr. Barbara M. Reed for her patience and selfless service towards improving my academic performance and the quality of my life. Also, for her financial support towards my program and personal life. I am very grateful to Dr. Ralph Reed for the excellent review of all my manuscripts and much useful advice.

I thank all my committee members; Drs. Tony Chen, Maret Traber, John Lambrinos and James Osborne for their professional contributions towards my academic success.

My deep gratitude goes to Jeanine DeNoma whom I have learnt a lot from in the last few years and to Dr. K. Hummer, for supporting my program financially and also helping with my learning. To all staff of NCGR for their friendliness and support especially Joseph, Doug, Ted, April, Nahla and my fellow graduate students. To Scott Leonard, for assisting with my assays.

I am grateful to the following administrative staff of Horticulture department; Dr. Anita Azarenko, Viki, Lee Ann and Gina for providing help every time I asked them. Many thanks go to Dr. and Mrs. Ray William for their encouragement and support.

I appreciate all my special friends; Laurine Hutley, Jo and Jack Rich, Patrick Nwafor, Cathy and Chuck Zellmer, Chuck Traunweiser and Vida Atchulo who have made Corvallis a home away from home. I also thank Dr. Carol M. Stiff for her support.

My deepest gratitude goes to my mentors and acquired parents, Prof. and Prof. (Mrs) O. Oduntan and family for welcoming me into their family and lives. My academic progress was advanced with my first ever scholarship award from them at the University of Ibadan, Nigeria.

I thank my god-parents, Mrs Maria Obi and late Chief D.I. Obi and family for their love and support throughout my academic journey. I thank Rev. Fr. Dermot Connolly, Prof. D.C. Ugwuegbu, Dr. R. Asiedu, Prof. L. Lajide, Dr. M. Abang, and Dr. D. Okenu for their great support and guidance.

I thank all my family members especially my mother, Elizabeth Uchendu who gave up her resources and comfort in life caring for me over the many years that I was a very sick child and without medical help. To my brothers; Jerome, Stephen, Aloy and Okechukwu and sisters; Regina, Mary, Monica, Ngozi and Chinelo and all my nieces, nephews and in-laws, for their love and support throughout my life. I thank my husband Augustine U. Eze for his contributions.

CONTRIBUTION OF AUTHORS

Dr. Barbara M. Reed was involved in the planning, design and interpretation of all data. Dr. Maret Traber and Mr. Leonard Scott assisted with analysis of vitamin E and malondialdehyde in chapter 4. Dr. Sandhya Gupta contributed information on glutathione in chapter 5. Dr. Magfrat Muminova added data on polyvinylpyrrolidone studies in chapter 5.

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DEDICATION

This project is dedicated to the loving memory of my mother, Mrs Elizabeth Uchendu, and late father, Chief C. A. Uchendu, who both inspired me as a child to pursue education seriously as my means of survival in the society.

CRYOPRESERVATION OF SHOOT TIPS: ANTIOXIDANT INVESTIGATIONS WITH *RUBUS* AND PROTOCOLS FOR *MENTHA* AND *VACCINIUM*.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Plants are sources of food, pharmaceuticals and shelter. Over the years, there has been a dramatic reduction in plant biodiversity and genetic resources due to the activities of humans, animals, micro-organisms and climate change. Over two thousand species of higher plants are at risk of extinction (Bajaj, 1995b). Thus, there is need to conserve plants and their diversity.

Conservation strategies

Plant conservation traditionally started with maintaining plants in their natural place of origin or environment (*in situ*). For *ex situ* conservation, seed storage is a common method for plants with seeds that remain viable when dried or can withstand cold temperatures (orthodox seed). Dried seeds remained viable when stored at low temperature of -20 °C (Clark, 1993) and in liquid nitrogen (Popova et al., 2003). In recent years, field collections were developed by breeders to preserve or maintain genotypic variations. Field conservation requires a large space for plant cultivation, favorable climatic conditions and involves labor expenses. Green and screen houses offer additional means of protecting plants from insects, diseases and poor climatic conditions.

In vitro conservation

In vitro methods utilize tissue culture tools for maintenance of plants under aseptic and controlled growth conditions (Ashmore, 1997). *In vitro* methods are useful for handling plants that are difficult to multiply or maintain under natural conditions in a field gene bank and in screen or green houses. Disease-free plantlets can be obtained from diseased parents using *in vitro* techniques. Virus free plants are commonly produced from heat treated and meristemed plants that are regrown *in vitro*. *In vitro* options provide short, medium and long term protection of plant genetic resources.

Short-term options include maintaining plantlets with regular transfer of stock cultures to fresh media. The benefits associated with short-term options include immediate availability of plantlets and quick multiplication of plants within a short period. Micropropagation involves regular maintenance, the frequency of which sometimes exposes plants to the risk of human error.

Medium-term options involve storage of germplasm at cold temperature (~ -4 ºC) or storage at standard growth room conditions (Ashmore, 1997). The later is mostly used for tropical species that are sensitive to cold conditions. The growth environment and culture medium could be manipulated to slow growth via temperature adjustments and exclusion of hormones in the culture media. Medium-term options are known to keep plants maintained for as long as 3 to 5 years without reculture (Reed, 1993). This reduces the risk of handling error and saves labor cost.

The only long-term option is cryopreservation of germplasm under ultra low temperature conditions, usually involving liquid nitrogen (-196 °C) or its vapor phase (–160 ^{o}C) (Reed, 2008). Cryopreservation is not a substitute for field maintenance but it provides a suitable backup against the loss of valuable genotypes that may occur under field conditions. Cryopreservation is currently the only available long-term conservation technique that offers protection for unlimited period of time for clonally propagated plants. It is reliable for maintaining viability of germplasm under stable and low cost conditions, safe from diseases or environmental damage (Harvengt et al., 2004). Shoot tips, somatic and zygotic embryos, whole seeds, pollen, anther and buds can be cryopreserved and stored. A wide range of plant species have already been adapted to cryopreservation. They range from very hardy, moderately hardy, sub-tropical to tropical crop species (Bajaj, 1995a; Engelmann, 1997; Engelmann, 2004). The development of efficient cryopreservation protocols is of key importance to future crop development and the long-term availability of economic plants. Regrowth variation among diverse genotypes with a species is still a problem, thus cryopreservation research is a priority in most laboratories that maintain plant genetic resources.

Historical overview of cryopreservation

Cryopreservation as a science began in the mid 1940's when it was discovered that glycerol could protect spermatozoa from injurious freezing temperatures (Polge et al., 1949). A decade later, dimethyl sulfoxide (DMSO) was found to protect red blood cells at subzero temperature (-50 ºC) (Lovelock and Bishop, 1959). The discovery of these cryoprotectants unveiled many opportunities for optimizing other variables affecting recovery of biological materials exposured to subzero temperatures. Experiments were aimed at preserving cells of different organisms. During early 60's, research into the causes of freezing injury at subzero temperature provided a model that explains water and solutes permeability in cells and the freezing rates at which cells can effectively survive (Mazur, 1960; 1963). Sakai (1960) using cold hardy winter twigs of poplar (*Populus sieboldi* Miquel) and willow (*Salix koriyanagi* Kimura ex Goerz) demonstrated for the first time that plants could survive at ultra low temperature (-196 ºC) if slow freezing rate is applied and materials cooled to -30 ºC prior to plunging in liquid nitrogen. By the late 60's, sections of mulberry stems (*Morus bomibycis* Koidz) (Sakai, 1966; Sakai and Yoshida, 1967) and flax (*Linum usitatissimum* L.) cell suspension cultures (Quatrano, 1968) were successfully cryopreserved in liquid nitrogen using a combination of treatments including the duration of treatment, concentrations of ethylene glycol and DMSO, the rates of cooling and warming. Slow cooling was the first cryopreservation protocol for plant materials and is widely used for the storage of plant cell cultures as well as shoot tips and dormant buds.

Protocols have evolved through many decades of research, all aimed at improving growth or maintaining viability of stored germplasm after liquid nitrogen exposure. Notable among these is the development of vitrification procedure. Vitrification allows phase transition of water from liquid into amorphous glass. The theory of vitrification was initially proposed by Luyet (1937) and shown by Fahy et al. (1984) to circumvent the damaging effects of ice crystal formation with the presence of highly viscous cryoprotectant solutions that solidify into a glass phase upon rapid cooling. Glass formation stops metabolic reactions and promotes a state of dormancy

and stability in biological systems (Burke, 1986). Using a differential scanning calorimeter (DSC), Hirsh (1987) determined that the sugars raffinose and stachyose, and proteins in cold hardy woody plants formed an intracellular glass that protected cells from damage. Vitrification was applied for cryopreservation of cells and somatic embryo cultures of asparagus (*Asparagus officinalis* L.) with 50-65% recovery using vitrification solutions containing "(w/v) 22% glycerol, 15% ethylene glycol, 15% propylene glycol and 7% DMSO in Murashige-Skoog medium with 0.5 M sorbitol" (Uragami et al., 1989).

During the early 1990's, new vitrification-based protocols were created including encapsulation dehydration in which excised shoot tips were embedded in a calcium-alginate mixture that forms solid bead at ambient temperature (Dereuddre et al., 1990; Fabre and Dereuddre, 1990). This technique was derived from synthetic seed technology (Redenbaugh et al., 1986) and encapsulation dehydration allowed more plants to be cryopreserved particularly those from tropical origin. Additional plant vitrification solutions were formulated with inclusion of bovine serum albumin and a pretreatment step or loading materials in ethylene glycol prior to cryoprotectant exposure of protoplasts isolated from leaves of winter rye (*Secale cereale* L. cv Puma) (Langis and Steponkus, 1990). Sakai et al. (1990; Sakai et al., 1991) created vitrification mixtures for nucellar cells of orange (*Citrus sinensis* Osb. var. *brasiliensis tanaka*) of which, plant vitrification solution 2 (PVS2) containing "30% glycerol, 15% ethylene glycol and 15% DMSO in MS medium with 0.15 M sucrose" increased recovery to 80% and later became more popularly used for cryopreservation of diverse crop species. Towill (1990) applied ethylene glycol, DMSO and polyethylene glycol as cryoprotectants for mint shoot tips cryopreservation and recovery varied from 31-75%. Many vitrification protocols for cryopreservation of shoot tips of diverse taxa are now available and most of them are modifications of Sakai's original formulation (Matsumoto et al., 1994; Sant et al., 2008; Yamada et al., 1991). A new vitrification solution, VSL (vitrification solution L), containing 20% (w/v) glycerol, 30% (w/v) ethylene glycol, 5% (w/v) sucrose, 10% (w/v) DMSO and 10 mM $CaCl_2$ produced

comparable regrowth results to those with PVS2 using gentian buds as the experimental material (Suzuki et al., 2008).

Creating a protocol

The overall purpose of every cryopreservation procedure is to preserve the structure and function of cells after exposure to liquid nitrogen and subsequently under normal physiological conditions (Grout, 1995). Growth responses following cryopreservation differ among plant species and cultivars as well as cryopreservation protocols used. This could be attributed to differences in their normal physiological status (Chang et al., 1992), genotype response to low temperatures (Benson et al., 1996) and cryopreservation protocols (Reed et al., 2001). The cryopreservation of new types of plant materials often begins with researchers testing various combinations of factors in order to optimize a protocol for the desired plant. This is often a time consuming process that may not result in a useable technique. There are standard protocols that have been created for many plant genera. These existing standard protocols are good starting points and in some cases can be directly applied to plants with little cryopreservation history (Reed et al., 2003). Recalcitrant species may require manipulation of the standard protocols before they can be adapted to cryopreservation depending on the crop's genetic make-up (Uchendu and Reed, 2008). Protocols can be combined to improve regrowth of some species (Matsumoto et al., 1995; Sakai and Engelmann, 2007).

Standard techniques

Controlled rate cooling

This technique works through the assumption that intracellular water is removed during the cooling process. Controlled rate cooling involves the use of a programmable freezer or a standard laboratory freezer to cool the materials from 4 °C to very low temperatures, usually around - 40 °C, at a specified cooling rate before plunging the samples into liquid nitrogen at –196 °C. The materials are generally treated with a cryoprotectant mixture at 0 to 4 ºC for a certain period of time and then

slowly cooled at a rate ranging from 0.1 to 0.5 ºC/min depending on the type of plant materials. Ice formation is induced at ~ -9 ºC to avoid supercooling, at which point the cryoprotectant solution is already frozen and there is ice present at the extracellular spaces. As the cooling progresses, water moves out of the cells due to increased osmotic potential of ice outside the cells. Ice build-up in the cytosol or in the intracellular spaces could be very problematic to cells (Pearce and Ashworth, 1992), thus the dehydration process should be slow and steady as temperature decreases. It is thought that the reduction of freezable intracellular water allows compounds to concentrate in the cytoplasm and to vitrify upon contact with liquid nitrogen.

Controlled rate cooling is advantageous because of the efficient use of time especially when handling a large number of samples. Cooling rate is the crucial factor when adopting this technique. Cooling rate of 0.1 °C/min is known to be best for the recovery of many species including those of cryopreserved *Pyrus* and *Malus* (Chang et al., 1992; Reed, 1990). A fast cooling rate exposes materials to the dangers of inadequate desiccation or intracellular ice crystal formation which causes fatal damage or results in death of plants. Very slow cooling rate can cause excess desiccation of cells to occur. Controlled rate cooling generally involves the use of cryoprotectants. Cryoprotectants offer protection by osmotic control of cellular water.

Vitrification

Vitrification protocol works by achieving an ice-free intracellular environment. The freezable water content of cells, tissues or organs are dehydrated with highly concentrated solutions such that cell's liquid contents concentrate and form a glass upon contact with liquid nitrogen (rapid cooling). This is due to high viscousity of the solutions resulting in the avoidance of ice crystal formation that cause lethal damage (Sakai, 1993). Many formulations are currently available that protect plants in liquid nitrogen and allow recovery upon rewarming. These formulations were designed mostly based on the plant type, size of material, chemical sensitivity and plant response to different temperature conditions (Sakai, 1993; Sakai et al., 1990). Plant

vitrification solution 2 (PVS2) is well tested among different taxa of plants (Sakai et al., 2008).

The sensitivity of plant materials to cryoprotectant mixtures is a critical factor that can influence the choice of cryoprotectant for increasing survival following cryopreservation. Volk et al. (2006) examined individual chemical components of the PVS2 on mint shoot tips and found each chemical can be damaging to the tissues especially when they are combined. A careful timing of the exposure period or temperatures of exposure have resulted in substantial regrowth in many plant genera (Makowska et al., 1999; Wu et al., 1999). Blackberries are generally tolerant to PVS2. Gupta and Reed, (2006) found that regrowth of PVS2-treated shoot tips after cryopreservation ranged from 71 to 85% with exposure durations of 20 min at 25 °C. Wang et al. (2005) tested PVS2 on a raspberry genotype from 0 to 360 min and found that the highest regrowth (85%) was obtained after exposure for 180 min at 24 °C. A high regrowth of grape shoot tips (60%) was obtained after exposure to PVS2 for 80 min at 0 ºC prior to plunging in liquid nitrogen (Matsumoto and Sakai, 2003). However, Verleysen et al. (2004) noted that PVS2 had negative impact on Azalea tissues. They observed increased electrolyte leakage after incubation in PVS2 solution for 80 min. PVS3 [50% glycerol, 50% sucrose in MS basal medium] was successfully used on asparagus that was sensitive to PVS2 (Nishizawa et al., 1993). Zhao et al. (1995) tested Sakai's plant vitrification solutions on three *Malu*s cultivars under varying exposure times and found the best regrowth with PVS3 for 80 min, thus PVS3 is suggested as the best alternative to PVS2 for sensitive plant materials.

Encapsulation dehydration

This protocol is based on osmotic regulation of cellular water. Excised shoot tips are encapsulated in calcium-alginate mixture dissolved in MS medium with added sucrose. The alginate bead protects biological materials against osmotic shock which may occur when directly exposed to high sucrose concentration (Plessis et al., 1991). Also, the beads help achieve partial desiccation of the cell through osmosis due to the

initial higher solute concentration of the beads compared to those of the cytosol (Benson, 1990). Sucrose is loaded in the beads for 18 - 24 hrs during preculture in 0.75 – 1M sucrose solution. The exact timing of protocol depends on plant tolerance of high sucrose conditions. Encapsulated shoot tips are afterwards air dried in the laminar flow hood or over silica gel to reduce the freezable intracellular water and allow vitrification to occur when they are plunged in liquid nitrogen. This protocol works for hundreds of species (Engelmann et al., 2008).

The cellular water content optimal for survival following encapsulation dehydration and liquid nitrogen exposure is ~20% for most shoot tips, but depending on the dehydration tolerance of individual genotypes, some modifications may be needed. A 30% water content yielded regrowth in the range of 70 to 90% for encapsulated apple cultivars (Zhao et al., 1999). A 20% moisture content was best for shoot tips of *Vitis vinifera* L. cv. *Chardonnay* (Plessis et al., 1991). Twenty percent water content resulted in 60 to 100% recovery of *Rubus* species (Gupta and Reed, 2006). A range of 15.6 to 17.6% water content was best for cryopreservation of some grape cultivars (Wang et al., 2000a) while ~10% water content yielded 78-90% survival in gentian axillary buds (Suzuki et al., 1998).

Encapsulation dehydration is useful for plants originating from both temperate and tropical climates. The applicable cryoprotectant is mainly sucrose. A major disadvantage is that the beads must be handled individually, about 4-5 times during the entire process. This level of handling makes the protocol somewhat cumbersome or difficult when cryopreserving large number of samples. Also, some genotypes must be removed from the beads before reculture. This makes the protocol even more time consuming and predisposes samples to the risk of contamination.

Pretreatments

Many pretreatment options are available for preparing plant materials for cryopreservation. These options involve fortifying the culture medium for producing healthy and vigorous growing parent plants, adjustments of photoperiod and growth temperature for better acclimation or improved regrowth or use of protective chemical mixtures prior to cryopreservation operation to induce desiccation and low temperature tolerance. Pretreatments that produce optimal protection and regrowth usually work best if applied in combination with multiple treatment factors. Pretreatments reduce cell or tissue water content prior to freezing and condition materials to increase freezing tolerance (Chang and Reed, 1997; Dumet et al., 1993; Gonzalez-Arnao et al., 1998; Thierry et al., 1997). An osmotic agent such as sucrose reduces water content prior to freezing. This can be combined with anti-stress agents like proline or abscisic acid (Reed, 1996). In addition to reducing cellular water content, sucrose also enhances freezing tolerance. This was demonstrated with shoot tips of *Chrysanthemum cinerariaefolium* (Hitmi et al., 1999). Sucrose also maintains plasma membrane structure and function by substituting for water on the surfaces of membrane and binding with biomolecules in the membrane thus, providing stability during desiccation and cooling stresses (Crowe et al., 1987; Crowe et al., 1984; Santarius, 1973).

High sucrose levels within a plant cell protect against excessive dehydration and cell collapse that may result to lethal freezing damage (Hirsh, 1987; Koster, 1987). Fowler et al. (1981) found a significant correlation between increased cellular sucrose concentrations and improved freezing tolerance in winter wheat cultivars in the field. The freezing tolerance of apple and *Rubus* shoot tips was significantly improved with sucrose (Caswell et al., 1986; Palonen and Junttila, 1999) and cold acclimation (Chang and Reed, 2000).

Cold acclimation

Cold acclimation adapted for *in-vitro* grown plantlets was designed based on the appraisal of plant behavior under natural freezing conditions. Cold acclimation serves to improve tolerance to desiccation and increase cold hardiness of the tissues. It is known that physical and chemical changes occur within plant cells during cold acclimation including tissue dehydration, degradation and re-synthesis of various compounds (Levitt, 1972; Pomeroy and Siminovitch, 1971). Molecular tools show that plants accumulate special proteins including dehydrins and heat shock proteins (Neven et al., 1992) during cold acclimation. Heat shock proteins bind to and enable denatured proteins to refold and resume normal physiological activity following desiccation or freeze induced strain (Neven et al., 1992). Plants also up-regulate "cold regulate" genes in response to low temperatures leading to the acquisition of freezing tolerance (Lee and Chen, 1993; Naik et al., 2007). Intracellular sugars including glucose, fructose and sucrose levels increased during cold acclimation of shoot tips of sugar beets (Vandenussche et al., 1999). Trehalose and sucrose levels also increased during dehydration-induced stress (Bachiri et al., 2000; Scott, 2000). Dissolved solutes form hydrogen bonds with biomolecules such as phospholipids and proteins in the cellular system (Crowe et al., 1984; Taylor, 1987).

Cold acclimation was applied to minimize freeze-induced desiccation stress during freezing and increase survival following cryopreservation. Photoperiod cycles, temperature adjustments or use of anti-stress hormones such as absicsic acid (ABA) can also be applied to improve survival under low temperature treatments (Chen and Gusta, 1983). For cold-sensitive species, freeze tolerance can be induced by increasing the concentration of sucrose or other osmotica in the pretreatment medium (Dumet et al., 2000). An average of two weeks is required under cold acclimation conditions to increase cold hardiness of tissues and improve regrowth after cryopreservation in many species. Up to 3 weeks of cold acclimation significantly improved shoot regrowth of cryopreserved *Rubus parvifolius* L. from 25 to 75% (Chang and Reed, 1999). Longer cold acclimation periods (4 to 12 wks) are known to improve some

Pyrus and *Rubus* cultivars that had low regrowth with less cold acclimation (Chang and Reed, 1999; Chang and Reed, 2000).

Cryoprotectants

Chemical mixtures are applied during cryopreservation to avoid intracellular ice crystal formation or to prevent injury resulting from excessive dehydration. Cryoprotectants are mostly useful but can also be harmful. The main concern with its use is the risk of chemical toxicity on plant tissues (Finkle and Ulrich, 1979). Sucrose and DMSO are the most commonly used components of a cryoprotective mixture in plant tissue culture. Glycerol and DMSO are effective on most eukaryotic and microbial cell cultures (Lovelock and Bishop, 1959; Polge et al., 1949). Various sugars have dual benefits in cryopreservative mixtures. They serve as nutrients and also confer cryoprotection during cooling. Amino acids such as proline are among the components of some cryoprotective mixtures (Withers and King, 1980). Anti-freeze proteins inhibit ice formation or ice crystal growth and stabilize membrane structure (Devries and Lin, 1977).

Action of cryoprotectants

Volk and Walters (2006) used Differential Scanning Calorimetry (DSC) to monitor the mode of action of PVS2 (30% glycerol, 15% ethylene glycol and 15% DMSO and 0.4 M sucrose in MS medium). They observed that this mixture displaced intracellular freezable water, filled the entire cellular space and protected against shrinkage or cell collapse which would have occurred under severe water loss. They also observed that PVS2 changed the freezing and endothermic ice melting pattern in the cytosol and prevented the alteration of cellular pH. This study concluded that glasses can stop chemical reactions involving molecular diffusion. Cryoprotectants reduce the toxic impact of electrolytes and decrease osmotic shock. The extent of protection depends on the molar ratio of the cryoprotectant to intracellular solutes (Kiran et al., 2004). Cryoprotectants increase solute concentration inside the cell and decrease the amount

of freezable water available therein (Lovelock, 1953; Lovelock, 1954). Glycerol and DMSO decrease the freezing point of water and many biological fluids by colligative action; glycerol decreases it to ~-46 ºC and DMSO decreases it to ~-73 ºC (Meryman et al., 1977; Tsuru, 1973). DMSO also protects the fluidity of the membrane. Sugars (trehalose, sucrose) and glycerol protect against damage due to excessive water loss (Jochem and Korber, 1987).

Activities of cryoprotectants also depend on their permeability status. Most are permeable and some are semi-permeable or non-permeable. Permeable cryoprotectants accumulate in the cytosol. They lower intracellular freezing temperatures, stabilize the membranes, and protect against problems associated with excessive desiccation of cells. Semi-permeable cryoprotectants accumulate between cell walls and outside the cell membranes and form layers that prevent ice formation and excessive water loss (Hubalek, 2003). Non-permeable cryoprotectants accumulate on cell exterior surfaces around the cell walls. They form layering around the lattice of the cell wall to reduce ice production and also protect cells against mechanical damage (Ashwood-Smith and Warby, 1971; Meryman, 1974).

Classification of cryoprotectants

Cryoprotectants are grouped based on their molecular weights i.e. low molecular weight and high molecular weight compounds (Nash, 1966) or by their permeability potential. Cryoprotectants that readily pass through cell systems include methanol, ethanol, ethylene glycol, propylene glycol, dimethylformamide, methylacetamide and DMSO and those that penetrate cells slowly or do not penetrate at all include monosaccharides, oligosaccharides, and polysaccharides, mannitol, sorbitol, dextran, hydroxyethyl starch, methyl cellulose, albumin, gelatin, polyvinylpyrrolidone, polyethylene glycol, polyethylene oxide, and polyvinyl alcohol (Meryman, 1971; Tao and Li, 1986). Sugars including sucrose and trehalose penetrate the cell membrane and promote high viscousity for stable glass formation (Fuller, 2004; Koster, 1991).

Rewarming of specimen

Rewarming temperature is a critical factor. Devitrification or recrystalization of ice can adversely affect recovery of plant materials if it occurs (Grout, 1995). Rapid rewarming reduces the chance of ice crystal formation of vitrified liquids (Engelmann, 1991). Fast rewarming at temperatures of 25 °C to 45 °C is mostly used during cryopreservation (Bajaj, 1995a; Nishizawa et al., 1993; Reed, 1990; Sarkar and Naik, 1998). A fast rewarming at 40 ºC for 3 min produced highest regrowth of cryopreserved grape shoot tips (Wang et al., 2000b). Slow rewarming, might be safely used for some genotypes depending on the type of cryoprotectants applied (Marín et al., 1990; Withers, 1979). Slow rewarming could be used if the samples are guaranteed to be sufficiently dehydrated prior to immersion in liquid nitrogen such that no freezable water is left inside the cell that would cause ice to form or that would expose samples to the risk of devitrification. This is the case for encapsulation dehydration procedures. Otherwise, slow rewarming is usually avoided.

Post-cryopreservation treatments

Following rewarming, a rinse solution is immediately applied that dilutes cryoprotectants, minimizes osmotic shock on cells and tissues and prevents any toxic effects that might result from prolonged exposure of samples to the cryopreservative chemicals. The composition of reculture or regrowth medium can influence survival following cryopreservation. Removal of auxin (IBA) from regrowth medium (Chang and Reed, 1999) and the addition of activated charcoal (Schrijnemakers and Van Irene, 1995) are some of the options researchers use to optimize regrowth following cryopreservation. Adjustment of light intensity or photoperiod in the growth environment prevents or reduces the problem of oxidation and initiation of free radical chain reactions that damage cryopreserved tissue cultures (Benson and Noronha-Dutra, 1988).

Regrowth assessment

Recovery following cryopreservation depends on plant physiological status, type of plant material, pretreatments and the cryopreservation protocol used. A successful cryopreservation procedure produces observable signs of regrowth within a few days of reculture for vitrification protocols and within two weeks for controlled rate cooling. In the case of a 3-year old cryopreserved *Arabidopsis* suspension cells encapsulated in an alginate matrix, regrowth was observed in less than seven days following reculture (Bachiri et al., 2000). Greening is usually the first observable sign of regrowth with shoot cultures but direct production of new tissues (shoots, leaves, buds) from original cryopreserved material is the best indicator of a successful cryopreservation procedure (Duran-Villa, 1995). In the case of cell cultures, fluorescein diacetate (FDA) (Widholm, 1972) and 2,3,5-triphenyltetrazolium (TTC) (Steponkus and Lanphear, 1967) are routinely used staining tests for the assessment of viability after exposure to low temperature conditions.

Cryo-injury

Cryo-injury results from excessive intracellular dehydration or ice crystal formation (Mazur, 1960). Tissue browning is one of the early detectable signs and occurs as a result of low-temperature induced damage to plant tissues. This is commonly seen with woody plant species. Tissue browning caused by stress or cold, leads to loss of cellular compartmentation, leaking of phenolics and formation of insoluble brown complexes upon oxidation (Chalker-Scott et al., 1989). Freezing injury resulting in phospholipid degradation was observed in membranes of *Populus euramenricana* cortical tissues subjected to subzero temperatures (Heber, 1968; Yoshida and Sakai, 1974). Cryo-injury may also be caused by free radicals. A free radical has one or more unpaired and reactive electrons. These electrons readily pair with biomolecules with which they come into contact and form deleterious covalent bonds (Beckman and Koppenol, 1996; Halliwell and Gutteridge, 2006).

Oxidative stress

Reactive oxygen species (ROS) refers to both oxygen-derived radical and non-radical species (Halliwell, 2006; Halliwell and Gutteridge, 2006; Vanin et al., 2004). ROS include superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide and singlet oxygen (Asada, 2006). The hydroxyl radicals are the most highly reactive of all species (Evans et al., 2004; Von Sonntag, 1987). ROS cause damage by removing electrons from macromolecules such as lipids, proteins and nucleic acids resulting in structural modifications that alter their functions (Møller et al., 2007; Takahama, 2004). ROS attack lipids, proteins or nucleic acids and cause lipid peroxidation, protein denaturation and DNA or RNA changes (Martinez-Montero et al., 2002; Quartacci et al., 2000). Lipid hydroperoxides are formed during this damage. Hydroperoxides can break down to form aldehydes as secondary products, some of which crosslink with macromolecules (Esterbauer et al., 1991; Valenzuela, 1991). Chilling and freezing promote many changes in cell systems which can lead to increased ROS production and injury (Day et al., 2000).

ROS readily oxidize membrane lipids. Lipid peroxidation affects membrane structure and function which often results in tissue damage or death of cells. ROS primarily targets the polyunsaturated fatty acids (PUFA) in membranes (Esterbauer et al., 1991). Oxidation of PUFA may produce malondialdehyde (MDA) (Davey et al., 2005; Yamauchi et al., 2008). MDA is highly reactive and toxic when it binds to biomolecules found in organelles. Lipid oxidation can be estimated by the level of MDA found in specimens (Davey et al., 2005; Esterbauer and Cheeseman, 1990).

ROS are formed during respiration and photosynthesis in the mitochondria, peroxisomes and chloroplasts as byproducts of physiological reactions (Mittler et al., 2004). Plants have natural antioxidant defense systems to control ROS production (Quan et al., 2008). These involve antioxidant enzymes including superoxide dismutase, peroxidases, mono- and dehydroascorbate reductase, glutathione reductase and catalase (Elstner and Osswald, 1994). Intracellular antioxidant levels increased

during oxidative stress induced by low temperature (8 °C) in *Arabidopsis thaliana* (Havaux et al., 2005). Johnson et al. (2007) found increased antioxidants in stressed tissues of *R. nigrum*.

Abiotic factors including temperature and water or nutrient deficiencies can increase ROS production in cells (Mittler, 2002). Cold stress leds to increased production of ROS (Johnston et al., 2007; Tao et al., 1998). ROS production can exceed the antioxidant capacity of any given cell (Benson, 2000) or the antioxidant systems can breakdown due to physiological problems. Plants control damage induced by abiotic stress by increasing the levels of sugars, sugar alcohols, proteins, (Bachiri et al., 2000; Valluru and Van den Ende, 2008; Vandenussche et al., 1999) and antioxidants present in the system (Kytridis and Manetas, 2006; Takahama, 2004). Tocopherol controls MDA and phytoprostanes level in PUFA enriched seeds (Maeda and DellaPenna, 2007). These natural adaptive mechanisms provide clues for further developing protocols that protect *in vitro* plantlets from damage and death. Ascorbic acid was applied in growth medium to stop lipid peroxidation caused by salt stress in tomato seedlings (Shalata and Neumann, 2001) and also to stop damage caused by polyethylene glycol-induced stress in sorghum and sunflower seedlings (Zhang and Kirkham, 1996). Galactinol and raffinose were used in *Arabidopsis thaliana* transgenic cultures and these compounds protected against oxidative damage and countered the activities of $OH^{\bullet}$ (Nishizawa et al., 2008).

Antioxidants

Antioxidants prevent the process of oxidation or repair the resulting damage. They act by lowering the cellular level of damaging reactive species and allow species to perform necessary biological functions (Asada, 2006; Halliwell and Gutteridge, 2006). Antioxidants may be water soluble (hydrophilic), lipid soluble (hydrophobic) or insoluble and in bound form (Bonoli et al., 2004; Chu et al., 2002; Wang et al., 1999). Antioxidants are grouped based on their function. Two major categories are identified; the primary and secondary antioxidants. The primary antioxidants prevent the

occurrence of damaging oxidative reactions. These include antioxidant enzymes which quench reactive species or convert them into stable inactive products and preventing the formation of lipid peroxidation chain reactions. Secondary antioxidants include non-enzymatic antioxidant compounds which scavenge reactive species, inactivate toxic byproducts of metal ions, and stop the propagation of complex radical chain reactions (Jadhav et al., 1996; Ou et al., 2002; Wang, 2000). Antioxidant mixtures of these two categories combined may produce synergistic effects (Judde et al., 2003; Marinova et al., 2008; Rossetto et al., 2002).

Exogenous antioxidants can hypothetically counteract the oxidation problems negatively impacting cryopreservation processes. The only reports on exogenous antioxidant use during plant cryopreservation involved glutathione (Wang and Deng, 2004) and PVP (Wang et al. 2005). Adding glutathione at all four steps of the PVS2 vitrification, pretreatment, loading, cryoprotectant and regrowth medium, doubled shoot recovery of cryopreserved *Citrus* shoot tips (Wang and Deng, 2004). There is no experimental evidence that shows the amount of oxidative damage that occurs during cryopreservation or studies that show how oxidative damage directly impacts regrowth and the role that antioxidants play as mediators in the process.

Problems with cryopreservation protocols

The standard cryopreservation protocols; controlled rate cooling, encapsulation-dehydration and vitrification are successfully applied to a range of *Rubus* germplasm (Chang and Reed, 1999; Gupta and Reed, 2006; Reed, 1993b; Vysotskaya et al., 1999; Wang et al., 2005). All of these studies show that recovery is dependent on genotype, and varies from poor to excellent. Poor recoveries are caused by stresses imposed on cells and tissues during cryopreservation protocols. Volk (2006) examined chemical components of the PVS2 vitrification on shoot tips of mint and found the mixture to be highly damaging to the tissues. Cryopreservation procedures begin with excision of samples from donor plants. Studies on embryonic axes of Chestnut seeds showed that ‘excision and dessication’ led to oxidative burst or transient superoxide production

(Roach et al., 2008). Superoxide is readily converted to hydrogen peroxide and then in the presence of free metal ions, like iron or copper to hydroxyl radical (Halliwell and Gutteridge, 1999; Quan et al., 2008). Cryopreservation processes also induced transitory changes in DNA methylation and affected transcriptional activities in *Ribes* (Johnson et al., 2009). Improvements in cryopreservation protocols are needed in order to decrease regrowth variation among genotypes or allow for more efficient storage of diverse germplasm collections.

The studies presented in following chapters explore the efficacy of the standard cryopreservation protocols on mint species and cultivars of blueberry, cranberry and blackberry and add to knowledge of their cryopreservation response. These studies establish that sensitivity to water deficit and oxidative stress are important factors causing the death of cryopreserved plants and that antioxidant vitamins, non-vitamin and anti-stress compounds are effective for improving plant cryopreservation in liquid nitrogen.

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CHAPTER 2

A COMPARATIVE STUDY OF THREE CRYOPRESERVATION PROTOCOLS FOR EFFECTIVE STORAGE OF *IN VITRO*-GROWN MINT (*MENTHA SPP.*)

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CryoLetters 29 (3) 181-188, 2008

Abstract

This study was designed to determine the response of diverse mint genotypes to three commonly used cryopreservation techniques. Four mints [*Mentha* x *piperita* nothosubsp. *citrata* (Ehrh.) Briq., *M. canadensis* L., *M. australis* R. Br, and *M. cunninghamii* Benth] were cryopreserved using three protocols: controlled rate cooling (CC), encapsulation dehydration (ED) and PVS2 vitrification (VIT). Regrowth of mint species following controlled rate cooling (93%) was significantly ($P<0.0001$) better than encapsulation dehydration (71%) and vitrification (73%). All four genotypes responded well to the controlled rate cooling protocol but there was some variability with the other two protocols. Genotype specific response to the individual protocols showed that there were significant differences in the recovery of *Mentha* x *piperita* nothosubsp. *citrata* and *M. australis* with CC > VIT > ED. There were also significant differences in the recovery of *M. cunninghamii* and *M. canadensis*, with CC and ED significantly better than VIT. Regrowth of the shoot tips of these mints ranged from 60% to 95% for all but one treatment. The overall results of this study compare favorably to other techniques. These improved results may be due to a combination of favorable growth conditions, cold acclimation and recovery medium. Controlled rate cooling was the most successful technique for the storage of these diverse mint genotypes; however recovery of shoot tips from VIT and ED was high and these techniques could also be used for cryogenic storage of mint germplasm.

Keywords: cold acclimation, controlled rate cooling, encapsulation dehydration, *in vitro*, vitrification

Introduction

Cryopreservation has developed greatly in the last 10 years and is now a reliable option for the long-term storage of clonal selections (Engelmann, 2000; Reed, 2008). Diverse genotypes can be cryopreserved, but several techniques may be required to store large collections due to differential genotypic response (Staats et al., 2006).

Optimal results from cryopreservation require a combination of good plant growth conditions, adequate conditioning of plant materials, properly performed protocols, and good regrowth conditions. Cryopreservation protocols that are effective for diverse species or cultivars are needed for conservation programs and genebanking, and sometimes available techniques can be directly applied to new species (Reed et al., 2003).

Mint (*Mentha* spp.) belongs to the family Lamiaceae which includes over 2500 species and varieties (Banthope, 1996). Seeds can be used to preserve some types of mint, but sterile species and clonal selections require an alternative option to retain the unique characteristics of the genotype and guard against loss. Cryopreservation protocols that achieve high recovery are available for many individual *Mentha* species. Towill (1988) found 29 to 100% survival of mint shoot tips following a controlled rate cooling procedure with a cooling rate of 0.25 °C/min to -35°C followed by plunging in liquid nitrogen (LN). Towill (1990) also provided the first report on vitrification of *Mentha* with a mean recovery of 44% (*M. aquatica* L. x *M. spicata* L.). Towill and Bonnart (2003) examined shoot tips of mint species (*M. aquatica, M. arvensis*, *M. piperita and M. spicata*) following extensive physical cracking of the external glass within semen straws during vitrification and found that this condition did not adversely affect survival. Hirai and Sakai (1999) achieved 90% recovery of *Mentha spicata* (spearmint) using encapsulation vitrification. This compared to 50% recovery with encapsulation dehydration in the same study and with 51.7% recovery reported by Sakai et al. (2000). Senula et al. (2007) recovered up to 89% of *Mentha* x *piperita* L., M. x *villosa* Huds and *M. spicata* L using droplet vitrification. Forty-six core accessions, representing over 20 *Mentha* species were screened for cryopreservation at the National Center for Genetic Resources Preservation, Fort Collins, CO (Staats et al., 2006). Thirty-one accessions (67%) of these were successfully cryopreserved following a sucrose pretreatment and PVS2 vitrification on aluminum foil. The remaining accessions were cold acclimated for 6 weeks, and 8 accessions were successfully cryopreserved using PVS2 vitrification in tubes and the five accessions remaining were successfully cryopreserved using encapsulation dehydration. These

results are based on a standard of 40% minimum viability that was shown to provide adequate recovery from stored samples (Reed, 2001).

Although there seem to be many successful cryopreservation programs already in place for mint storage, often results are not effective for all species. There are also no well controlled comparisons of these commonly used protocols. The objective of this study was to directly compare three well-tested techniques, controlled rate cooling, PVS2 vitrification and encapsulation dehydration, and to determine their efficiency as storage protocols for diverse mint genotypes.

Materials and Methods

Plant material

Micropropagated *Mentha* spp. [*Mentha* x *piperita* nothosubsp. *citrata* (Local # 124.001; PI 557993); *M. canadensis*, (571.001; PI 557613); *M. australis* (690.001;PI 617498) and *M. cunninghamii* (666.001; PI 617481)] from the *in vitro* collections of the USDA-ARS National Clonal Germplasm Repository, Corvallis, OR, were multiplied on NCGR-Mentha (MEN) medium containing MS mineral salts and vitamins (Murashige and Skoog, 1962), with 0.5 $mg \cdot l^{-1}$ N^6benzyladenine (BA), 0.1 $mg \cdot l^{-1}$ indole-3-butyric acid (IBA), 3.0 g/l agar (Difco, Detroit, MI), 1.25 g/l gelrite (PhytoTechnology Lab., Shawnee Mission, KS), and 30 g/l sucrose at pH 5.7, dispensed in Magenta GA_7 boxes (Magenta Corp., Chicago, IL). The plantlets were subcultured every 3 weeks and grown at 25 °C under a 16 h light and 8 h dark photoperiod (40 $\mu E \bullet m^{-2} \bullet s^{-1}$).

Cold acclimation (CA)

Plantlets three weeks after the last subculture were cold acclimated for 2 weeks with alternating cold and warm temperatures [22 °C 8-h light (10 $\mu E \bullet m^{-2} \bullet s^{-1}$) and -1 °C 16-h dark]. Shoot-tips (approx. 1 mm) were excised from cold-acclimated plantlets.

Encapsulation-dehydration procedure (ED)

The protocol of Dereuddre (Dereuddre et al., 1991; Dereuddre et al., 1990), as described by Chang et al. (1999) was used for this study. Two-week CA shoot tips were excised and placed in 3% (w/v) low viscosity alginic acid (Sigma, USA) in liquid MS without calcium at pH 5.7. Beads were formed by suspending shoot tips in alginate and dripping them into a saturated calcium chloride solution (MS medium with 100 mM $CaCl_2$ and 0.4M sucrose). Beads were allowed to polymerize for 20 min. Encapsulated shoot tips were preconditioned in liquid MS with 0.75 M sucrose on a rotary shaker (50 rpm) for 18 h. Beads were blotted dry on sterile filter paper, transferred to an open glass Petri dish and dehydrated for 5.5 hrs (approx. 36% bead residual moisture content) in a laminar-flow hood at 0.6 m/sec air flow with ambient temperature of ~22 °C and 35±2 % relative humidity. Dehydrated beads (20 per treatment) were placed in 1.2 ml cryovials (10 beads per cryovial) and plunged into liquid nitrogen (LN). The cryovials were rewarmed in 45 °C water for 1 min and in 25 °C for 1 min. The beads were rehydrated in liquid MS medium for 5 min before transfer onto MEN regrowth medium (0.5 $mg \cdot l^{-1}$ BA, 2.5 g/l agar, 1 g/l gelrite, and no IBA) in 24-cell tissue culture plates (Costar, Cambridge, MA). Pretreatment controls were set to determine that shoot tips remained viable after each critical step by plating 5 beads each after: 1) 18 h preconditioning in liquid MS medium with 0.75 M sucrose and 2) following air dehydration under the laminar flow hood.

Water content determination

Four sets of 10 empty dehydrated beads were used to determine the moisture content [(Fresh Wt - Dry Wt)/Fresh Wt x 100]. Fresh weight is the weight of beads after dehydration under the flow hood. Dry weight is the oven dried (~103 °C, 12 h) weight of the same beads.

Vitrification procedure (VIT)

The PVS2 vitrification procedure described by Yamada et al. (Yamada et al., 1991) and modified by Luo and Reed (1997) was used. Twenty-five shoot-tip explants from 2-wk CA *Mentha* plantlets were pretreated for 48 h on MS agar plates containing 5%

(v/v) dimethyl sulfoxide [DMSO, Sigma-Aldrich Co., St Louis, MO] with 3.5 g agar and 1.75 g/l gelrite under CA conditions. Shoot tips were transferred into 1.2 ml cryovials and treated with 1 ml loading solution (LS) [2 M glycerol in 0.4 M sucrose MS medium (v/v), pH 5.8] for 20 min at 25 °C. LS was removed and 1 ml PVS2 cryoprotectant solution (30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS medium with 0.4 M sucrose) (Sakai et al., 1990) was added at 25 °C and held for 20 min. Five shoots of each accession were rinsed in liquid MS medium containing 1.2 M sucrose and planted in regrowth medium as described above to determine sensitivity to PVS2. Twenty shoots of each accession in PVS2 solution were plunged into LN for each replication. Rewarming was done as described above for encapsulation dehydration. Shoot tips were plated on MEN regrowth medium.

Controlled rate cooling (CC)

The controlled rate cooling protocol used was described by Reed (Reed, 1990; Reed, 2008). Shoot-tips from 2 wk CA *Mentha* plantlets were excised and placed on 5% DMSO agar plates as above for 48 h in CA conditions. Shoot tips were transferred to 1.2 ml plastic cryovials on ice containing 2 drops of liquid MS medium. Two drops of the PGD cryoprotectant [10% each polyethylene glycol (MW 8000), glucose, and DMSO in liquid MS medium] were added to cryovials every 2 min for 6 min then 4 drops every 2 min over a 30 min period. Cryovials were equilibrated for 30 min on ice. Excess cryoprotectant was removed to 1 ml before loading samples into a programmable freezer (Cryomed, Leona, MI), (0.1 °C/min to -9 °C for seeding, to -40 °C). At the end of the programmed run, the samples were plunged in LN. Samples were rewarmed as above, rinsed in liquid MS medium and plated on MEN regrowth medium. The unfrozen controls were left on ice until the cooled samples exhibited an exotherm, after which control shoot tips were rinsed in liquid MS and plated on MEN regrowth medium and the cooled samples continued cooling in the freezer.

Experimental design and data analysis

Regrowth assessment was done 6 weeks after rewarming. Greening, shoot production and leaf expansion were all required for samples to qualify as fully recovered from cryopreservation. Each experiment included 20 cryopreserved and 5 to 10 unfrozen shoot tips per treatment and was replicated three times. Data were analyzed by analysis of variance (ANOVA). The data are presented as percentages with means separation using Duncan's Multiple Range Test (Alpha = 0.05) (SAS, 2003).

Results

All of the mint accessions cryopreserved with the three protocols produced moderate to high regrowth after liquid nitrogen exposure. Shoots developed without intermediate callus formation. ANOVA showed a significant (P<0.0001) interaction of genotype and technique (Table 2.1).

Table 2.1. Factorial ANOVA of Genotype by Treatment Interaction

	DF	Mean square	Pr > F
Genotype	3	37.2615	< 0.0001
Technique	2	67.2314	< 0.0001
Genotype * Technique	6	33.5407	< 0.0001
Error	24	1.05555	

Comparison by technique

When data for all four accessions were pooled, the regrowth following the CC protocol (93%) was significantly better (P< 0.0001) than recovery with the other two techniques. Mean regrowth of shoot tips following encapsulation dehydration (71%) and vitrification (73%) did not differ significantly.

Response of genotypes to cryopreservation protocols

For *Mentha* x *piperita* nothosubsp. *citrata*, each technique produced significantly different recovery percentages (P< 0.0001). The best recovery was with CC and yielded 95% regrowth, followed by VIT with 60% and ED with about 40% (Fig. 2.1A). *M. australis* recovery also varied significantly among the protocols, but all were above 76% (Fig. 2.1B). *M. cunninghamii* had good recovery with CC and ED (> 80%); however, recovery was significantly less with VIT (61%) (Fig. 2.1C). *M. canadensis* recovery was not significantly different between CC and ED (> 90%) but regrowth following the VIT protocol was significantly less, at 78% (Fig. 2.1D).

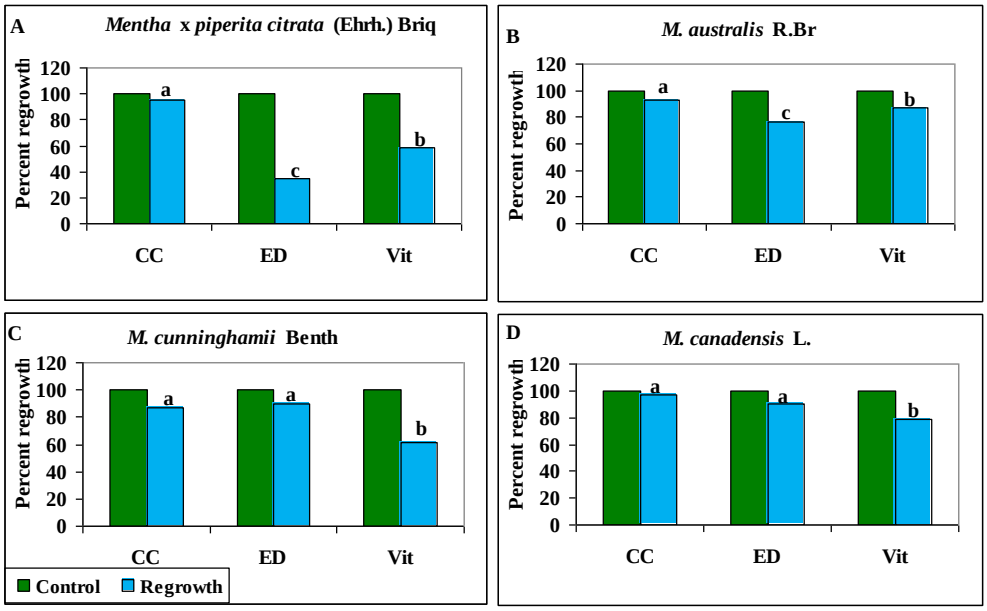

Fig. 2.1: Effect of cryopreservation protocols on the regrowth of cryopreserved *Mentha* species. Shoot tips were cryopreserved with Controlled rate cooling (CC), PVS2 vitrification (VIT) or Encapsulation dehydration (ED). Means with the same letter are not significantly different at alpha = 0.05.

Discussion

Developing a new cryopreservation method for every plant accession is difficult, time consuming, and makes backing up large germplasm collections very difficult. With the

continued development of cryopreservation techniques, it is now possible to choose a technique and quickly adapt it to a new species (Reed, 2008). Application of tested methods to related plant types can considerably speed up storage of plant collections (Reed et al., 2003). In the case of mint, all three of the standard NCGR cryopreservation techniques produced good to excellent regrowth following LN exposure.

Cold acclimation and CC is a new method for mint cryopreservation and it produced the best overall recovery for all genotypes (Fig 2.1). Comparison of the three protocols showed significantly better recovery ($P < 0.0001$) using the CC protocol compared to the other two techniques. Regrowth of the four mint species following controlled rate cooling (93%) was significantly ($P < 0.0001$) better than encapsulation dehydration (71%) and vitrification (73%). Our results are similar to studies of other temperate plants cryopreserved by CC following cold acclimation (Reed, 1990; Reed et al., 2003; Reed and Yu, 1995). These results show that the CC protocol would be useful for the cryopreservation of difficult-to-cryopreserve mint accessions as reported by Staats et al. (Staats et al., 2006). The ED and VIT techniques were more variable, but produced moderate to good results for all genotypes.

M. piperita nothosubsp. *citrata* had 95% recovery from controlled rate cooling (Fig. 2.1A) compared to the 71% regrowth reported by Towill (1988). Differences in plant culture conditions, pretreatments, cooling rates and intermediate cooling temperatures can affect recovery and may account for the observed difference. Towill used 6 to 8 wk culture without CA pretreatment and a 0.25 °C/min cooling rate to a pre-plunge temperature of -35 °C while we used CA plants with 0.1 °C/min to -40 °C (1988).

The PVS2 vitrification protocol is routinely used for successful cryopreservation of both temperate and tropical crop species (Charoensub et al., 1999; Niino et al., 1992; Sakai, 1997; Takagi et al., 1997). However, most plants are sensitive to the highly concentrated PVS2 solution, even for a short duration (Makowska et al., 1999; Nishizawa et al., 1993; Wu et al., 1999). Thus there is a need to carefully regulate the

exposure time for each accession. This requirement for critical timing can make it difficult to handle many samples at the same time. Several VIT protocols are successfully used to cryopreserve mint. Towill (1990) found recovery of 44 % from mint shoot tips (*M. aquatica* x *M. spicata*) using a vitrification solution of 35% ethylene glycol, 1 M DMSO, 10% PEG (MW 8000). Staats et al. (2006) used two VIT techniques for storing a group of mint accessions. The PVS2 droplet technique with sucrose pretreatment was successful for 31 of the 46 mint accessions tested, including three included in our study. Eight that did not respond to droplet vitrification did recover well from CA and standard PVS2 vitrification. Senula et al. (2007) tested cryopreservation of six mint accessions using PVS2 droplets on aluminum foil. Recovery following LN exposure improved significantly when source plants had 2 to 6 wks alternating temperature CA of 25 °C/-1 °C, similar to the CA protocol used at NCGR. They found that with alternating temperature CA *M. spicata* recovery significantly improved from 68 to 98%, *M.* x *villosa* from 60 to 70% and *M.* x *piperita* from 57 to 80%.

Recovery of shoot tips following ED was not significantly different from VIT in our study. Three of the four mint accessions in our study had good recovery following ED (> 75%), with only *M.* x *piperita* nothosubsp. *citrata* having low regrowth (~40%) (Fig. 2.1A). Sakai et al. (2000) and Hirai and Sakai (Hirai and Sakai, 1999) found that apices of *M. spicata* cv. spearmint recovered at 51.7% when cryopreserved with the conventional encapsulation-dehydration protocol but regrowth increased to 87% with encapsulation-vitrification protocol. However, for Staats et al. ED was successful for five of eight accessions that did not respond well to two VIT protocols (Staats et al., 2006).

Recovery of plants following cryopreservation is dependent on a variety of factors including the age of the mother plants, pretreatments, the length and type of CA, the technique used and the recovery process. Variation in results can arise from any of these factors. Callus formation in plants is often promoted in the presence of auxin (Irvine et al., 1983). Injury which occurs during cryopreservation promotes callus

formation, but removing IBA from the recovery medium significantly decreased callus proliferation in cryopreserved blackberries (Chang and Reed, 1999). The stock plant materials used for this study were grown on medium containing plant growth regulators (BA and IBA), but we excluded IBA (auxin) from the cryopreservation recovery medium. Towill (Towill, 1988) observed callus formation on mint shoot tips cryopreserved by slow cooling while the mints in our study did not form callus.

Differential results can also occur from subtle changes in the growth of the mother plants or in the composition of the recovery medium. Optimal results from cryopreservation require a combination of good plant growth conditions, adequate conditioning of plant materials, properly performed protocols, and good regrowth conditions. Differences in the standard growth protocols in the mint studies cited above may have important implications in the recovery of plants using any of the standard techniques. For example our standard protocol for mint includes PGRs in the growth medium but removes auxin from the cryopreservation recovery medium, while Staats et al. (2006) used PGR-free medium for the mother plants and applied PGRs for regrowth. Standardization of these culture variables could be an important key to the variability noted between laboratories using the same techniques and plant materials (Reed et al., 2004).

This comparison of three cryopreservation protocols found that all the procedures were more than adequate for storage of mint collections. Recovery of shoot tips was 60% to 95% for the four genotypes, much better than the 40% minimum required for secure long-term storage of germplasm collections (Reed, 2001; Reed et al., 2003). These results indicate that the choice of technique may be less important than the care of the plant material and the careful execution of the protocol. The overall results of this study compare favorably to other studies using a variety of techniques.

The high regrowth noted in our study is likely due to the combination of favorable growth conditions for the mother plants and suitable cold acclimation before cryopreservation, as well as regrowth on an optimal recovery medium. Overall, for

cryopreservation of mint germplasm we recommend cold acclimation of healthy *in vitro*-grown shoot cultures, followed by carefully executed standard cryopreservation techniques, and recovery on medium without auxins.

Acknowledgements

Many thanks to Jeanine DeNoma for technical assistance. This work was funded by USDA-ARS CRIS project 5358-21000-033D. Esther E. Uchendu was supported by funds from the Ford Foundation International Fellowship Program.

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CHAPTER 3

DESICCATION TOLERANCE AND CRYOPRESERVATION OF *IN-VITRO* GROWN BLUEBERRY AND CRANBERRY SHOOT TIPS

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Acta Horticulturae 810: 567-574, 2009

Abstract

In-vitro grown shoot tips of two cranberry (*Vaccinium macrocarpon*) cultivars, Wilcox (PI 614079) and Franklin (PI 554998) and three blueberry (*Vaccinium corymbosum*) cultivars, Berkeley (PI 554883), O'Neal (PI 554944) and Brigitta (PI 618166) from the tissue culture collections of the USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis, were tested for recovery from desiccation and following cryopreservation using three protocols. Cold acclimated, encapsulated and sucrose-pretreated blueberry cultivars were tolerant of desiccation under laminar flow of up to 7 h while cranberry cultivars were very sensitive to drying by 3 h. Cryopreservation screening followed 2 wk of alternating-temperature cold acclimation. The three blueberry cultivars cryopreserved with the encapsulation-dehydration technique (ED) produced 83% to 92% regrowth. PVS2 vitrification (VIT) (33% to 87%) and controlled rate cooling (CC) (50% to 67%) were also successful for some blueberries. The cranberry cultivars had poor (5% to 37%) recovery with all three techniques and will require further study to improve recovery after desiccation and cryopreservation.

Keywords: cold acclimation, controlled rate cooling, encapsulation dehydration, liquid nitrogen, PVS2 vitrification, *Vaccinium*, desiccation

Introduction

The genus *Vaccinium* L. is diverse and includes a broad range of small fruit crops (blueberry, lingonberry, cranberry) which are high in antioxidant compounds (Zheng and Wang, 2003). The United States Department of Agriculture (USDA), Agricultural Research Service (ARS), National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, preserves about 200 genotypes of cranberries and more than 1500 accessions of blueberries, from about 80 taxa including subspecies and varieties that originate from 34 countries (Boches et al., 2006).

Cryopreservation, the storage of living materials in liquid nitrogen, is now widely available as a solution for the long-term storage of vegetatively propagated plants (Engelmann, 2004; Reed et al., 2005). Specific cryopreservation techniques are available for a wide range of crop species (Razdan and Cocking, 1997; Reed, 2008), but there is very little information available for *V. corymbosum* L. (blueberry) and none for *V. macrocarpon* Aiton (cranberry). Earlier studies in our laboratory determined freezing rate and cold acclimation requirements for controlled rate cooling of *V. corymbosum, V. uliginosum* L. and *V. ovatum* Pursh. (Reed, 1989). Cold acclimation for 5 weeks increased *V. corymbosum* shoot tip recovery to 58% compared to 26% for the control. Recovery of *V. corymbosum* was significantly better with a 0.1 °C/min cooling rate after 0 to 7 weeks cold acclimation than with more rapid cooling. However, the other species were less successful. The only other report of *Vaccinium* cryopreservation is with cell cultures of *Vaccinium pahalae* Skottsberg that were successfully cryopreserved with the encapsulation-dehydration technique (Shibli et al., 1999).

Desiccation/dehydration tolerance is a key factor for success in most cryopreservation protocols. Cold acclimation can increase both cold tolerance and desiccation tolerance. Non-acclimated *Zoysia* Willd. and *Lolium* L. shoot tips encapsulated and air desiccated to 20% moisture content all died; cold acclimated *Lolium* shoot tips could be dehydrated to 20% moisture content without losing viability while *Zoysia* viability declined to 60% at that moisture content with 60% recovery following cryopreservation (Chang et al., 2000). Cold acclimation is also effective for conditioning other temperate plants (Scottez et al., 1992). Desiccation can also be accomplished by osmotic stress. A pretreatment on medium with a high sucrose concentration promotes intracellular water reduction by osmosis and may also contribute to protection of plasma membrane integrity (Plessis et al., 1993).

In many cases scientists begin cryopreservation studies by testing all of the parameters for a cryopreservation protocol. This is a time consuming process that may not result in a useable protocol. Several recent studies show that many times existing techniques

can be directly applied to additional plant species with few, if any, modifications (Reed et al., 2003; Reed et al., 2006; Uchendu and Reed, 2008). Many new techniques, and improvements of old techniques, were developed since the first study of *Vaccinium* cryopreservation. By initially utilizing well established protocols, it may be possible to determine a technique that shows promise and then optimize it for the plant of interest. This strategy could greatly reduce the time required to optimize a method for safe storage of important plant materials in liquid nitrogen (Reed, 2001).

The objectives of this study were to evaluate the desiccation tolerance of blueberry and cranberry cultivars and to determine if one of the three most commonly used cryopreservation techniques was suitable for storage of these cultivars.

Materials and Methods

Plant material

Plantlets from three *in vitro* grown blueberry (*V. corymbosum*) northern highbush cultivars, Berkeley (VAC 849.001, PI 554883), and Brigitta (VAC 1312.001, PI 618166), and a southern highbush blueberry, O'Neal (VAC 312.001, PI 554944) and two cranberry (*V. macrocarpon*) cultivars, Wilcox (VAC 1299.001, PI 614079) and Franklin (VAC 743.001, PI 554998), were multiplied on woody plant medium (WPM) (Lloyd and McCown, 1980), with 2 $mg \cdot L^{-1}$ zeatin, 30 $g \cdot L^{-1}$ sucrose, 3 $g \cdot L^{-1}$ agar (Difco, Detroit, MI), 1.25 $g \cdot L^{-1}$ Gelrite (Phyto Technology Lab., Shawnee Mission, KS) at pH 5.2. Standard growth room conditions were 25 °C with a 16 h light / 8 h dark photoperiod (40 $\mu E \bullet m^{-2} \bullet s^{-1}$)(Reed and Abdelnour-Esquivel, 1991).

Cold acclimation (CA)

Micropropagated plantlets of all cultivars were incubated under 22 °C with 8 h low light (10 $\mu E \bullet m^{-2}$)/-1 °C 16 h dark CA conditions for two weeks (Reed, 1990). Shoot tips (0.8 -1 mm) with attached leaf primordia were excised from cold-acclimated plantlets and further exposed to CA conditions for 48 hours on pretreatment medium with 5% dimethyl sulfoxide (DMSO) and 0.8% agar before cryopreservation.

Desiccation procedure

The ability of shoot tips of blueberry and cranberry cultivars to retain viability under water stress conditions was tested. Shoot tips were excised from 2-week cold acclimated, *in vitro* grown plantlets and encapsulated in alginate beads. The samples were precultured in 0.75 M sucrose for 19 hrs. Samples were further exposed to laminar air flow (0.6 $m \bullet sec^{-1}$ air flow at ~22 °C and 35±2% relative humidity) and allowed to air-dry from 3 to 7 h. Five beads of each cultivar were plated as controls before air-drying the samples. Beads were rehydrated in liquid medium for 10 min after each selected drying period and transferred to culture medium (WPM) in standard growth room conditions. Regrowth assessment was done after 6 wk of culture.

Fresh weight (Fw)/Dry weight (Dw)

Four samples of 20 dried empty beads were used to determine the percentage moisture content of beads after dehydration. This was calculated as (Fresh Wt - Dry Wt)/Fresh Wt x 100. Fresh weight is the weight of beads after pretreatments and before dehydration under the flow hood. Dry weight is the oven dried weight of beads (102 °C for 24 h).

Cryopreservation techniques

Shoot tips were plunged into liquid nitrogen (-196 °C) for cryopreservation following encapsulation dehydration (ED), PVS2-vitrification (VIT) and controlled rate cooling (CC). Sample vials were rapidly rewarmed in 45 °C water for 1 min and in 25 °C water for an additional minute before the samples were rinsed and plated on recovery medium.

Encapsulation-dehydration procedure (ED)

The protocol described for pear shoot tips was used (Dereuddre et al., 1990). Shoot tips were dissected from plantlets, temporarily cultured on an agar plate and immediately encapsulated in beads of 3% (w/v) low viscosity alginic acid (Sigma chemical Co, USA) in liquid MS medium (Murashige and Skoog, 1962) without calcium at pH 5.7. The beads with shoot tips were polymerized in MS medium with 100 mM $CaCl_2$ and

0.4 M sucrose for 20 min. Encapsulated shoot tips were preconditioned in liquid MS with 0.75 M sucrose on a rotary shaker (50 rpm) for 18 h. Beads were blotted dry on sterile filter paper, transferred to an empty sterile glass Petri dish and dehydrated for 6 h (~22% bead moisture content) in a laminar-flow hood. Twenty dehydrated beads were placed in 1.2 ml cryovials (10 beads per cryovial) and plunged into liquid nitrogen (LN). The alginate beads were rehydrated in liquid MS for 10 min after rewarming as described above and before being transferred onto regrowth medium (WPM) in 24-cell tissue culture plates (Costar, Cambridge, Mass.). The controls were prepared by rehydrating and planting 5 beads for each cultivar after 18 h of preconditioning in liquid MS with 0.75 M sucrose and after 6 h of dehydration under the laminar flow hood.

Vitrification procedure (VIT)

The PVS2 vitrification procedure (Sakai et al., 1990) was followed. Shoot tips excised from cold acclimated plantlets were pretreated for 48 h on MS agar plates containing 5% (v/v) dimethyl sulfoxide [DMSO (Sigma-Aldrich Co., St Louis, MO)] with 3.5 g agar and 1.75 g/L Gelrite under CA conditions. Shoot tips were transferred into 1.2 ml cryovials and treated with 1 ml loading solution (LS) (2 M glycerol in 0.4 M sucrose solution (v/v), pH 5.8) for 20 min at 25 °C. LS was removed and 1 ml PVS2 cryoprotectant solution [30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS with 0.4 M sucrose (v/v)] was added to cryovials for 20 min at 25 °C, then vials were plunged into LN. The shoot tips of each cultivar were rinsed in 1.2 M sucrose liquid MS and planted on recovery medium as controls after pretreatment with PVS2. Rewarming was done as described above. The PVS2 solution was diluted with liquid MS medium containing 1.2 M sucrose and shoot tips were drained on filter paper and planted on the recovery medium.

Controlled rate cooling (CC)

Controlled rate cooling was done following the protocol of Reed (1990). Shoot tips were excised and placed on MS agar plates containing 5% (v/v) dimethyl sulfoxide [DMSO (Sigma-Aldrich Co., St Louis, MO)] with 1.75 $g \cdot L^{-1}$ Gelrite and 0.8% agar for

48 h. The shoot tips were transferred to 1.2 ml plastic cryovials containing 2 drops of liquid MS medium on ice. Two drops of the PGD cryoprotectant [polyethylene glycol (10% w/v PGD (MW 8000), glucose, and DMSO in liquid MS medium] (Finkle and Ulrich, 1979) was added to the cryovials every 2 min for 6 min then 4 drops every 2 min over a 30 min period. Cryovials were equilibrated for 30 min on ice. Excess cryoprotectant was decanted to 1 ml before loading samples into the programmable freezer (0.1/min to -9 °C for exotherm; 0.1/min to -40 °C). The unfrozen controls were left on ice until the exotherm, after which the PGD was removed, samples were rinsed in liquid MS and the shoot tips were planted on recovery medium. The frozen samples in cryovials were plunged in LN at the end of the programmed run. Samples were thawed as above, rinsed in liquid MS medium and planted on recovery medium.

Statistics

Assessments of the recovery of shoot tips were made 6 wk after rewarming. Shoot survival, proliferation, leaf expansion and greening were considered in order to determine successful recovery from cryopreservation. Each experiment was replicated three times. Each experiment included 20 cryopreserved shoots (n = 60 shoots for each treatment) and 5 unfrozen but cryoprotected shoot tips per treatment (n = 15 shoots for each treatment). The data is presented in graphs as percentages. ANOVA and Duncan's Multiple Range Test ($P \leq 0.05$) using SAS version 9.1 was applied to data analysis (SAS, 2003).

Results and Discussion

Effect of *in vitro* desiccation

There were significant differences between the encapsulated blueberry and the cranberry shoot tips in response to desiccation under laminar flow ($P < 0.0001$). All the blueberry cultivars recovered at a high rate and significantly better than the cranberries. Blueberry cultivars retained > 80% viability with up to 7 h desiccation (Fig. 3.1A). The regrowth of cranberry cultivars declined sharply with 3 or more hours of desiccation, indicating that they are highly desiccation sensitive (Fig. 3.1B). Water

content of the beads declined to 20% from the initial 90% after 7 h dehydration (Fig. 3.1).

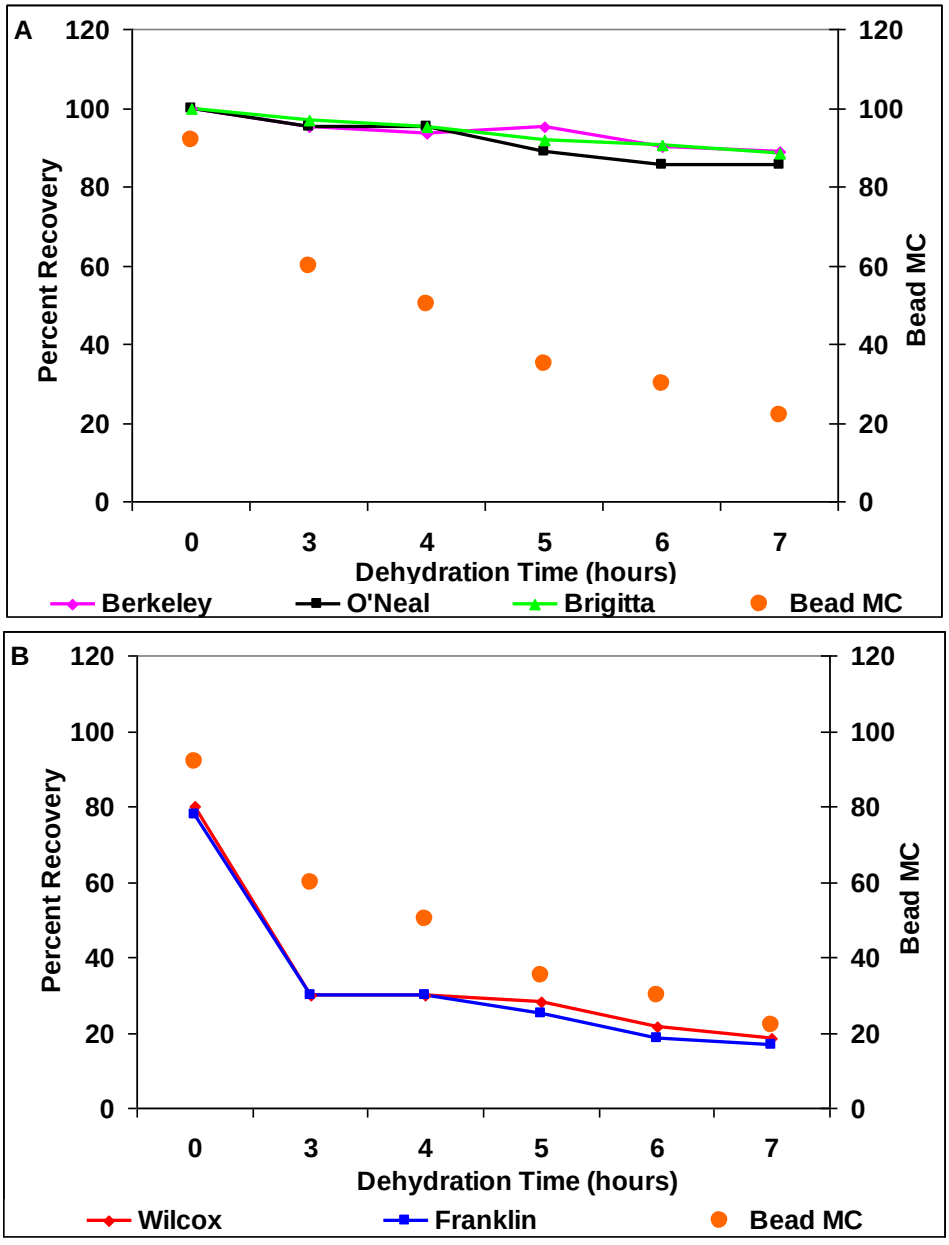

Fig. 3.1: Percent recovery and moisture content (MC) of blueberry cvs. (A) and cranberry cvs. (B) 6 weeks after encapsulation in alginate beads, 18 h in 0.75 M sucrose and 0 to 7 h desiccation under laminar flow without exposure to liquid nitrogen. Beads were rehydrated and plated on recovery medium after each time period.

Effect of cryopreservation protocols on the recovery of blueberry cultivars

The blueberry cultivars tested had significant differences in recovery with each protocol, but in most cases produced moderate to high regrowth (Fig. 3.2). The ANOVA showed an interaction of genotype by technique ($P < 0.0001$). For 'Brigitta' all three protocols produced good regrowth but ED and VIT were significantly better than CC. For 'Berkeley' ED was significantly better than VIT and CC but VIT and CC were not significantly different. 'O'Neal' regrowth was also best with ED, lower with CC and lowest with VIT.

The only other study of *Vaccinium* shoot tip cryopreservation studied CC. In that study, 5 wk cold acclimation prior to controlled rate cooling at 0.1 °C/min produced some regrowth for the three species tested. One *V. corymbosum* accession had 58% recovery, but only 10 to 40% recovery was reported for shoot tips of *V. ovatum*, *V. uliginosum* and another *V. corymbosum* cultivar in that study (Reed, 1989). Our current CC results with *V. corymbosum* cultivars CA for 2 weeks were in the same range (51-67%) as *V. corymbosum* in the earlier study.

The northern and southern highbush blueberries all responded well (> 80% regrowth) to cryopreservation with the ED technique which relies on both osmotic and air drying to condition the shoot tips (Fig. 3.2). The northern highbush blueberries are naturally adapted to areas with cold winters while southern highbush blueberries are adapted to hot summers (Trehane, 2004). The natural adaptation of these plants to desiccating conditions allows us to manipulate them in culture and take advantage of their natural desiccation tolerance. Additional preculture steps before cryopreservation using VIT or CC would likely improve blueberry regrowth. The high recovery of encapsulated blueberry shoot tips following desiccation in laminar flow is attributable to the combination of cold acclimation and desiccation in 0.75 M sucrose applied in this study (Fig. 3.1A). This CA-sucrose desiccation combination is also the prelude to ED cryopreservation that resulted in very good regrowth (Fig. 3.2).

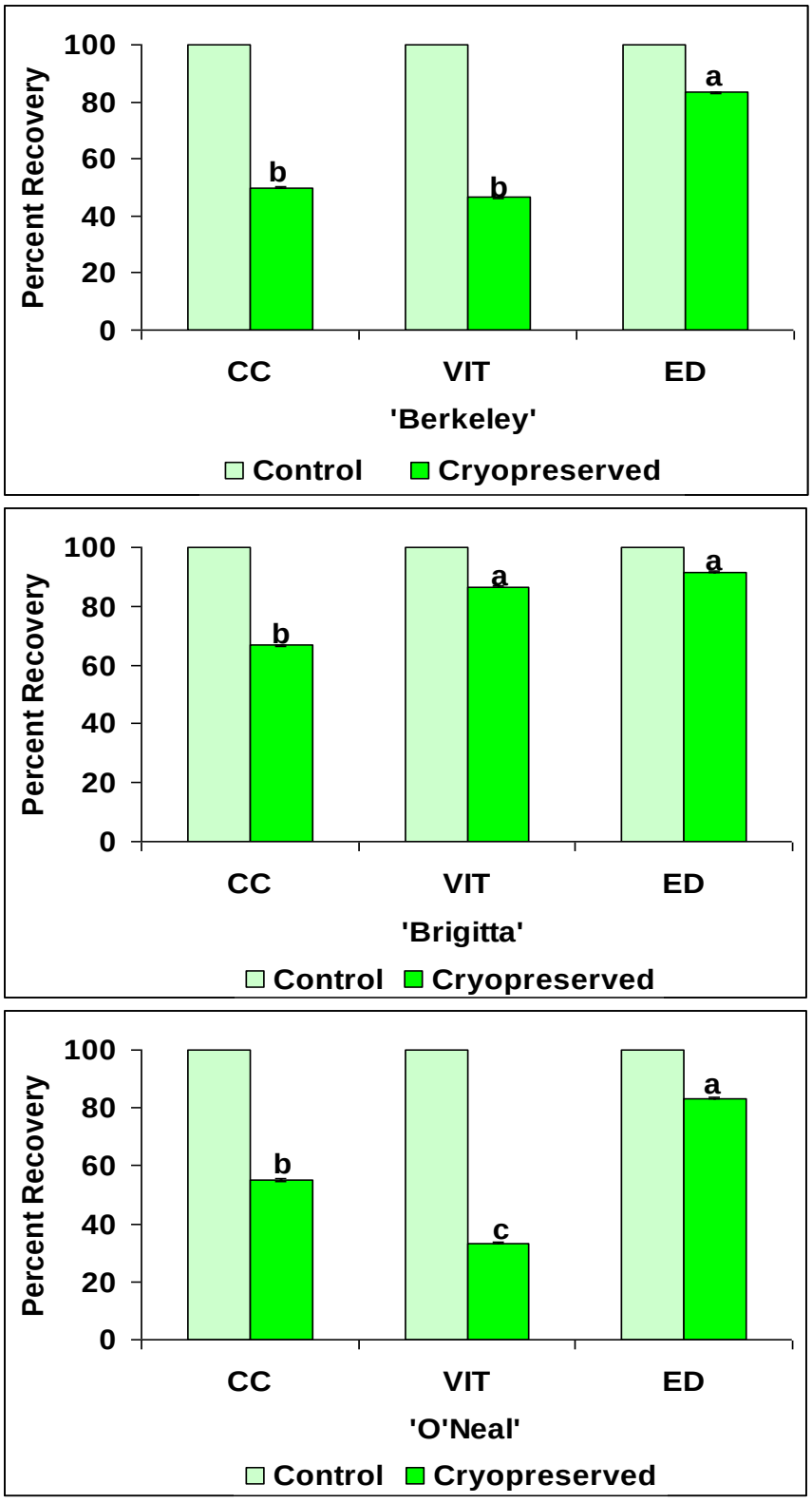

Fig. 3.2: Percentage recovery of blueberry cultivars following controlled rate cooling (CC), vitrification (VIT) and encapsulation dehydration (ED). Means with different letters are significantly different at $p \leq 0.05$.

The VIT and CC procedures for ‘Berkeley’ and ‘O’Neal’ might show improved results with increased CA or a sucrose pretreatment step prior to cryopreservation. Cryobiologists often apply cold acclimation as a pretreatment strategy to improve tolerance to desiccation and increase survival following cryopreservation (Chang and Reed, 2000; Dereuddre et al., 1990; Reed, 1990). Cold acclimation is known to enhance accumulation of polyamines and special proteins including dehydrins, and to up-regulate the expression of low temperature genes in *Vaccinium* (Naik et al., 2007). Cold acclimation may also promote stability of membrane structure through the action of dissolved solutes which may form hydrogen bonds with hydrophilic molecules present in the cells (Taylor, 1987).

Effect of cryopreservation protocols on the recovery of cranberry cultivars

Both cranberry cultivars had significantly less regrowth than the blueberry cultivars ($P < 0.0001$). Recovery ranged from 8% to 37% following the three cryopreservation techniques (Fig. 3.3). ‘Franklin’ had the best recovery (37%) with the VIT procedure, but recovery from CC and ED was significantly lower. ‘Wilcox’ regrowth with ED and VIT was not significantly different. VIT and CC were not significantly different and all recovery of Wilcox was < 20%. Neither cranberry cultivar reached the 40% recovery recommended for germplasm storage (Reed, 2001; Reed et al., 2005).

The key role of desiccation tolerance in cryopreservation is clearly illustrated by the differential recovery of these two *Vaccinium* species to desiccation and cryopreservation. Blueberry cultivars were tolerant to desiccation even at 20% moisture content whereas the cranberry cultivars were not (Fig. 3.1). Consequently, blueberry cultivars had good regrowth with all three cryopreservation techniques (Fig. 3.2) while cranberry cultivars performed poorly (Fig. 3.3). This result demonstrates that a reduction in freezable intracellular water is one of the key requirements for the cryopreservation of *Vaccinium* species and cultivars. This parallels the results obtained with many other plants including *Cichorium intybus* where reducing residual water to about 20% significantly improved recovery following cryopreservation

(Vandenbussche et al., 1993). Paul et al. (2000) found that 6 h dehydration to a 21% water content resulted in 83.7% recovery of ED cryopreserved shoot tips of apple.

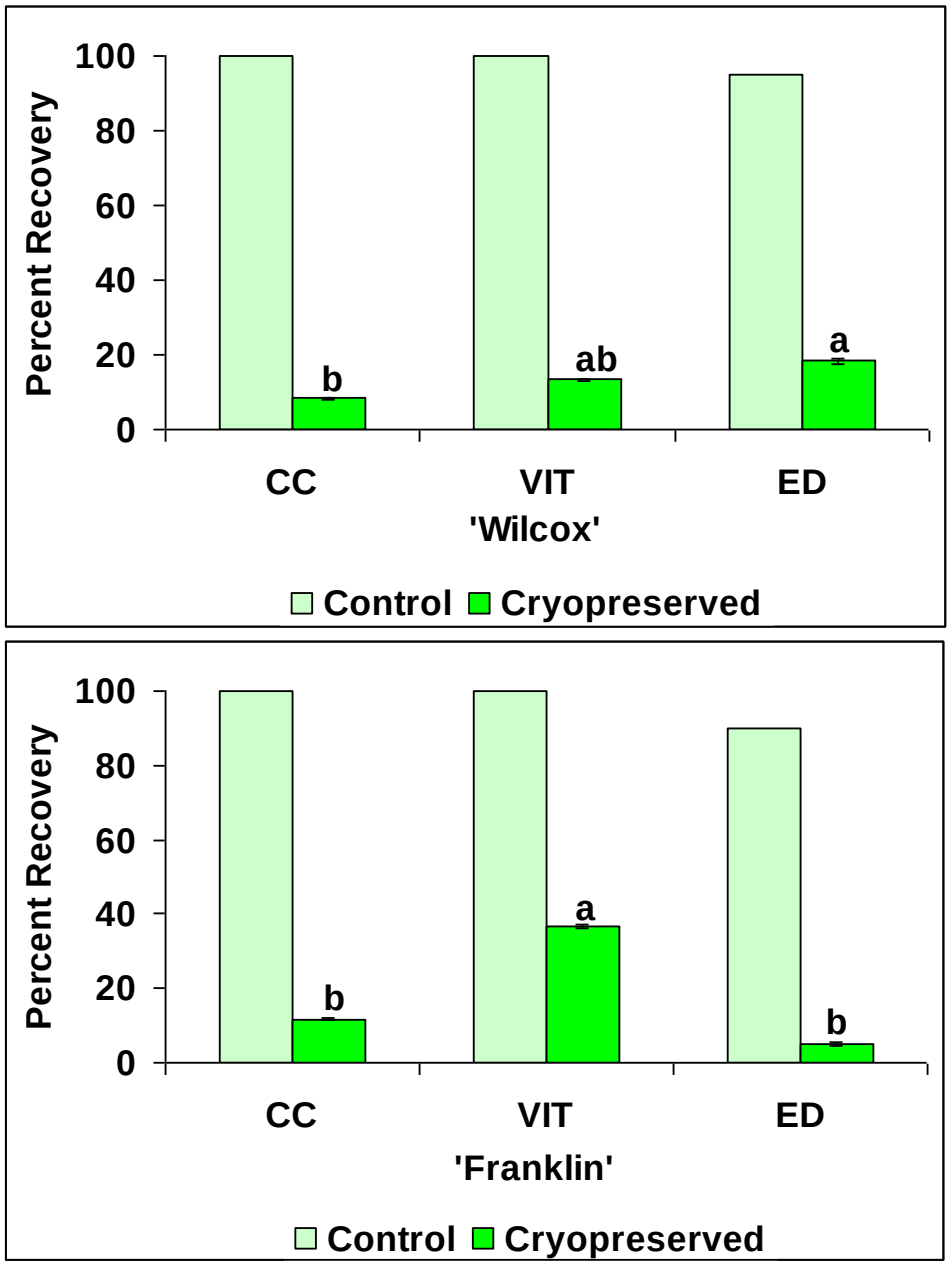

Fig. 3.3: Percentage recovery of cranberry cultivars following controlled rate cooling (CC), vitrification (VIT) and encapsulation dehydration (ED). Means with different letters are significantly different at $p \leq 0.05$.

Cranberries are a wetland-adapted crop species and can thrive even in flooded soils (Eck, 1990). This natural wet adaptation indicates that the plants may require high water content for growth and would require a slower dehydration procedure, perhaps in a stepwise manner, in order for them to tolerate desiccation and cryopreservation. To improve cranberry desiccation tolerance we propose a preculture in sucrose or abscisic acid (ABA). ABA is known to play a significant role in plant water balance and in the adaptation of plants to environmental stresses (Bravo et al., 1998). Preculture in sucrose and ABA medium enhanced desiccation and freezing tolerance in *Gentiana scabra* Bunge var. *buergeri* Maxim. axillary buds (Suzuki et al., 2006). In addition, pretreatment with increasing concentrations of sucrose prior to PVS2 exposure could be explored as a dehydration technique for cranberries (Sakai et al., 1990).

Conclusions

This study provides additional information on the cryopreservation of *Vaccinium* spp. We determined that *in vitro* grown blueberry cultivars responded well to the stresses imposed by desiccation and produced high recovery when cryopreserved; whereas the cranberry cultivars were desiccation sensitive and will require additional manipulation to reduce cellular water content. Blueberry cultivars that were cold acclimated for 2 wk could be cryopreserved by any of the three techniques, but ED was excellent for all three cultivars tested. Cranberry cultivar recovery was very low following cryopreservation by each of the three techniques, and the protocols will require modification before cranberries can be stored for long-term preservation.

Acknowledgements

This project was supported by the U.S. Department of Agriculture, Agricultural Research Service CRIS project (5358 150 2100 033 00D). Esther E. Uchendu was supported by the American Association of University Women (AAUW) scholarship program.

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CHAPTER 4

VITAMINS C AND E IMPROVE REGROWTH AND REDUCE LIPID PEROXIDATION OF BLACKBERRY SHOOT TIPS FOLLOWING CRYOPRESERVATION

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Submitted to Plant Cell Reports

Abstract

Oxidative processes involved in cryopreservation protocols may be responsible for the reduced viability of tissues after liquid nitrogen (LN) exposure. Antioxidants that counteract these reactions should improve recovery. This study focused on oxidative lipid injury and the effects of exogenous vitamin E (tocopherol, Vit E) and vitamin C (ascorbic acid, Vit C) treatments on regrowth at four critical steps of the PVS2 vitrification cryopreservation technique; pretreatment, loading, rinsing, and regrowth. Initial experiments showed that Vit E at 11-15 mM significantly increased regrowth ($P<0.001$) at all 4 steps. There was significantly more malondialdehyde (MDA), a lipid peroxidation product, at each of the steps than in fresh untreated shoot tips. Vit E uptake was assayed at each step and showed significantly more α- and γ-tocopherols in treated shoots than those without Vit E. Vit E added at each step significantly reduced MDA formation and improved shoot regrowth. Vit C (0.14-0.58 mM) also significantly improved regrowth of shoot tips at each step compared to the controls. A combination of Vit E (11 mM) and Vit C (0.14 mM) produced significantly higher regrowth than the control or Vit E alone. Vit C alone was not significantly different from the combination, suggesting that the benefit was mostly due to Vit C. We recommend adding Vit C (0.28 mM) to the pretreatment medium, the loading solution or the rinse solution in the PVS2 vitrification protocol. This is the first report of the application of vitamins for improving cryopreservation of plant tissues by minimizing oxidative damage.

Keywords: Ascorbic acid, Malondialdehyde, Oxidative stress, *Rubus*, Tocopherol

Introduction

Cryopreservation is the use of ultra-low temperature, usually liquid nitrogen (LN) (-196 °C) to store biological materials such that they remain capable of regrowth upon rewarming. It provides an option for cost effective long-term preservation of vegetatively propagated plants. Cryopreservation is applicable for long-term storage of

many plant species, animal cells and microbes but regrowth variation among diverse genotypes is still a problem. Techniques used for cryopreservation impose stresses on cells and tissues that can cause damage, resulting in reduced growth or death upon rewarming. The stresses associated with vitrification-based techniques arise from excision, osmotic injury, desiccation and changes in temperature. These factors lead to the production of reactive oxygen species (ROS) that cause oxidative damage. Antioxidants are compounds that arrest ROS before oxidative damage occurs (Asada, 1992). When ROS production exceeds the capacity of antioxidants in cell systems, it results in physiological decline. ROS-induced stress is thought to be a fundamental cause of cell death in cryopreserved samples (Benson, 1990). ROS, including superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen, are formed as a result of partial reductions of ground-state oxygen. They are the primary reactive oxygen species associated with oxidative damage in plant systems (Halliwell, 2006). Damage caused by ROS includes lipid peroxidation, protein denaturation, alterations in nucleic acids, membrane disruption, severe cellular disorder (Halliwell, 2006; Halliwell and Whiteman, 2004) and premature senescence (Thompson et al., 1987). Damage to the cellular membrane is critical to cell survival. Damage resulting from phospholipid degradation was observed in membranes of poplar (*Populus euramenricana cv gelrica*) cortical tissues subjected to subzero temperatures during early cryobiological studies (Heber, 1968; Yoshida and Sakai, 1974).

Lipids are the major class of biomolecules targeted by ROS in a membrane. Lipid peroxidation is of concern because it affects the integrity of membrane structure and alters its functions, leading to cell death. The main lipids targeted by ROS are the polyunsaturated fatty acids (PUFA) (Esterbauer et al., 1991). PUFA make up 50% to 90% of membrane lipids (Douce et al., 1973). When PUFA are oxidized, reactive aldehydes are formed as by-products, some of which cause damage. Malondialdehyde (MDA) is a breakdown product of PUFA (Davey et al., 2005; Yamauchi et al., 2008). MDA is a highly reactive and toxic molecule. Its attachment to nucleic acids and proteins causes modifications that disrupt biological functions (Del Rio et al., 2005; Esterbauer et al., 1991; Yamauchi et al., 2008). The degree of lipid peroxidation can

be estimated by the amount of MDA present in tissues (Davey et al., 2005; Esterbauer and Cheeseman, 1990). High levels of intracellular antioxidants were detected in *Arabidopsis thaliana* tissues during studies on oxidative stress induced by low temperature (8 °C) (Havaux et al., 2005). Johnson *et al.* (2007) found that an increased ratio of total phenolics to ROS in shoot cultures of *R. nigrum* correlated with significantly improved regrowth following cryopreservation.

Vitamin E (Vit E) is a common term used for tocopherols and tocotrienols. These antioxidants are directly involved in scavenging oxygen free radicals and quenching lipid peroxidation chain reactions that occur during oxidation reactions with PUFA (Sattler et al., 2004). Vit E reactions result in the formation of tocopheroxyl radicals that react with other antioxidants to regenerate the active molecule (Carelli et al., 2005; Liebler, 1993). Vitamin C (ascorbic acid, Vit C) is a well-known water soluble antioxidant that works to regenerate Vit E from its radical form (Kamal-Eldin and Appelqvist, 1996; Leung et al., 1981). Vit C also has direct reactivity with hydrogen peroxide, superoxide, hydroxyl radical and lipid hydroperoxides (Shao et al., 2008). Vit C can be regenerated from its radical form via the Ascorbate-GSH cycle that uses NADPH generated from the pentose phosphate pathway as a reducing agent (Sgherri and Navari-Izzo, 1995).

Several laboratories have successfully cryopreserved *Rubus* genotypes using a variety of cryopreservation techniques (Popov et al., 2006; Reed, 2008). Recovery of shoot tips cryopreserved using these techniques on diverse genotypes varies from 15% to 100% and often is genotype dependent. Plant vitrification solution number 2 (PVS2) (Sakai et al., 1990) has been modified for *Rubus* (Gupta and Reed, 2006). The steps of the PVS2 vitrification technique include: excision, pretreatment, loading solution, cryoprotectant, cooling, rewarming, rinsing and regrowth. Each of these steps presents the possibility of oxidative stress because physical damage and osmotic stress are often involved. For example, oxidative burst and ROS production were observed during embryonic axis excision and reduction of cellular water content in *Castanea sativa* (Roach et al., 2008). The only report of antioxidant addition during cryopreservation involved glutathione. Adding glutathione at four steps; pretreatment,

loading, cryoprotectant and regrowth medium during the PVS2 vitrification technique doubled shoot recovery of cryopreserved *Citrus* shoot tips (Wang and Deng, 2004). There is no experimental evidence available to the best of our knowledge that directly shows the effect of antioxidant vitamins such as Vit C and Vit E on recovery from cryopreservation or the relationship between oxidative damage caused by ROS and the role of Vit C or Vit E in reducing oxidative damage and improving regrowth of shoot tips following cryopreservation.

The goals of this study were [1] to evaluate if exogenous Vit C and Vit E added at critical steps of the PVS2 vitrification technique can significantly improve regrowth following cryopreservation; [2] to quantify malondialdehyde, a lipid peroxidation product formed during oxidative stress, at each step and [3] to determine the relationship between lipid peroxidation and shoot-tip regrowth following cryopreservation.

Materials and Methods

Plant propagation

Plantlets of blackberry cultivars Chehalem and Hull Thornless were selected from the *in vitro* collections of the USDA-ARS, NCGR, Corvallis, OR, USA. The plantlets were propagated for 3 weeks on RUB medium (Reed, 1990), consisting of MS (Murashige and Skoog, 1962) mineral salts, vitamins with doubled iron, 1 mg•l^{-1} N^6 - benzyladenine (BA), 0.1 mg•l^{-1} gibberellic Acid (GA_3) (Sigma-Aldrich Co., St Louis, MO), 0.1 mg•1^{-1} indole-3-butyric acid (IBA) (Sigma-Aldrich Co., St Louis, MO), 3.5 g•l^{-1} agar (Difco, Detroit, MI), 1.45 g•l^{-1} Gelrite (Phyto Technology Lab., Shawnee Mission, KS) and 30 g•l^{-1} sucrose at pH 5.7, dispensed into Magenta GA7 boxes (Magenta Corp., Chicago, IL). The plantlets were grown at 25 °C with 16h light/ 8 h dark photoperiod (80 µE•m^{-2}•s^{-1}) and a 3 week subculture interval. Regrowth medium following cryopreservation was RUB medium but without IBA (Chang and Reed, 1997) and on reduced agar (3.0 g•l^{-1}) and Gelrite (1.2 g•l^{-1}), under low light (26-30 µE). For the final Vit C experiment iron was eliminated from the regrowth medium.

Cold acclimation (CA)

After 3 weeks in the growth room, plantlets were transferred to a cold-acclimation chamber for 2 weeks to induce cold tolerance. The CA conditions were 22 °C with 8 h light (10 $\mu E \bullet m^{-2} \bullet s^{-1}$) and -1 °C with 16 h dark (Reed, 1988).

PVS2 vitrification

The vitrification procedure for blackberry shoot tips was previously described (Gupta and Reed, 2006). Shoot tips (0.8 -1 mm) from 2-wk CA plantlets were pretreated for 48 h on MS agar plates containing 5% (v/v) dimethyl sulfoxide (Sigma-Aldrich Co., St Louis, MO), with 3.5 g agar and 1.75 g•l-1 Gelrite, under CA conditions. Shoot tips were transferred into 1.0 ml vials (Nunc, Roskilde, Denmark) and treated with 1 ml loading solution [2 M glycerol in 0.4 M sucrose solution, pH 5.8] for 20 min at 25 °C. The loading solution was removed and 1 ml PVS2 cryoprotectant solution [30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS medium with 0.4 M sucrose (v/v) (Sakai et al., 1990)] was added and held for 20 min at 25 °C. Twenty shoot tips of each cultivar in 1 ml PVS2 solution were plunged into LN and held for 30-45 min, rewarmed in 45 °C water for 1 min and 25 °C water for 1 min, rinsed and planted on regrowth medium in 24 well plates. Five control shoot tips of each cultivar used for testing the pre-vitrification treatments were rinsed three times in liquid MS medium containing 1.2 M sucrose and planted on regrowth medium.

Antioxidants

The Vit E used was AQUA-E^{TM} (Yasoo Health Inc. Morrisville, NC), a water soluble mixture containing tocopherols, tocotrienols and α-tocopheryl polyethylene glycol 1000 succinate (TPGS), provided as a gift from Dr. Andreas M. Papas. AQUA-E was added at 0, 5, 10, 15, and 20 mM. Vit E analysis found these concentrations to be approximately 0, 4, 7, 11 and 15 mM in our samples. Ascorbic acid (Sigma Chemical Co., St Louis, MO) was tested at 0, 0.14, 0.28, 0.43 and 0.58 mM. Cryopreservation testing involved separate treatments with exogenous Vit E or Vit C added at one of the following steps at a time: the pretreatment medium (48 hrs), loading solution (20 min), rinse solution (~5 min) and regrowth medium (6 weeks).

Application

Vitamin E

In the pretreatment medium Vit E was dissolved in 5% DMSO and added after autoclaving. Vit E was added in the loading and rinse solutions during solution preparation and before adjusting pH (5.7), and then filter sterilized (0.45µm Supor® membrane, PAL Corporation, Cornwall, UK). Vit E was filter sterilized and added to regrowth medium after autoclaving. Vit E was added one step at a time (pretreatment, loading, rinsing or regrowth) and in one experiment to both the pretreatment and rinsing steps.

Vitamin C

Vit C was added into the culture medium and solutions before the pH were adjusted to 5.7. The pretreatment and regrowth media were autoclaved while the loading and rinsing solutions were filter sterilized using membrane filters (0.45µm, 150-mL analytical filter unit, NALGENE, Rochester, NY). Vit C could not be added to media after autoclaving because it greatly altered the medium pH. Vit C (0.14-0.58 mM) was initially added at all 4 steps in standard medium with iron. Then iron was excluded from the regrowth medium in a separate experiment. In a separate experiment, Vit C was added at all 4 steps with exclusion of iron from the media and solutions. Shoot tips from experiments without iron in the media and solutions were transferred into regrowth medium with iron but no Vit C after 14 days.

Antioxidant combination

At each step, Vit E (11 mM) and Vit C (0.14 mM) were added separately and in combination (Vit E + Vit C) to test the antioxidants' synergistic effects on regrowth. For the combined antioxidants study, we did not exclude iron from the regrowth medium or solutions.

Relationship between lipid peroxidation, Vit E and regrowth

Shoot tips treated with and without added Vit E (15 mM) at the pretreatment, loading and rinsing steps were cryopreserved (20 shoot tips per treatment) and planted on RUB regrowth medium. Five shoot tips per treatment (treated with all the solutions but not cryopreserved) were also planted on the regrowth medium. Ten shoot tips per treatment were assayed for Vit E and MDA. Vit E was added one step at a time.

Quantification of MDA in shoot tips

Ten shoot tips (10-30 mg) of 'Chehalem' were collected from fresh plantlets in the growth room (control) and from those treated with and without added Vit E at each step of cryopreservation. These were surface cleaned thoroughly with distilled water and blotted dry with filter papers. The shoot tips were placed in 1 ml cryotubes separated by treatment and transported in LN. MDA was measured by the method of Davey et al. (2005). Briefly, shoot tips were added to 100 µL of 1% butylated hydroxytoluene (BHT) stabilized with 5% (w/v) meta phosphoric acid (MPA) (Sigma, St Louis, MO) in a 2 mL microcentrifuge tube. To this solution were added 50 µL each of 1% thiobarbituric acid (TBA) in 50 mM NaOH and 25% (v/v) HCl at pH 1.0. The reaction mixture was heated to 95 °C for 60 min. The MDA $(TBA)_2$ adduct was partitioned out of the mixture with 150 µL of n-butanol. Following centrifugation, 10 µL of the supernatant was injected onto a Shimadzu HPLC reverse phase system including an autosampler and a Beckman 5µ ODS, 4.6 x 250 mm column. The MDA $(TBA)_2$ adduct was eluted using an isocratic mobile phase consisting of 50% methanol and 50% of 25 mM phosphate buffer at pH 6.5 and at a flow rate of 1.5 mL/min. MDA was detected by fluorescence at excitation 532 nm and emission 553 nm. Quantitation was done using an external standard of 1, 1, 3, 3-tetraethoxypropane (Sigma, St Louis, MO) prepared using the same method as the samples.

Quantification of shoot-tip tocopherol content

Ten shoot tips each (~30 mg) of 'Chehalem', from both untreated (control) and those with and without added Vit E (15 mM), at the pretreatment, loading and rinsing steps of the PVS2 vitrification technique were prepared for analysis. Also analyzed were shoot tips of field grown plants of 'Chehalem'. For the assay of α- and γ-tocopherols,

a modification of the method by Podda *et al.* (1996) was used. Briefly, the shoot tips were saponified with alcoholic KOH, extracted with hexane, dried under nitrogen, resuspended in 1:1 ethanol: methanol, then injected into a Shimadzu HPLC system (Columbia, MD). The HPLC system consisted of a Shimadzu LC-10ADvp controller, and a SIL-10ADvp auto injector with a 50µl sample loop. Tocopherols were detected using an LC-4B amperometric electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.) with a glassy carbon working electrode, and a silver chloride reference electrode. The column used was a Waters Spherosorb ODS2 C-18 column, 100 × 4.6 mm, 3 µm particle size with a Waters Spherisorb ODS precolumn, 10 × 4.6 mm, 5 µm. An isocratic mobile phase delivery system was used, with a total run time of 6 minutes. The mobile phase used was 99:1 (v:v) methanol:water containing 0.1% (w:v) lithium perchlorate. The electrochemical detector was in the oxidizing mode, potential 600 mV, full recorder scale at 500 nA. Peak areas were integrated using the Shimadzu EZStart 7.2 software package, and tocopherols were quantified using authentic standards.

Statistics

Analysis of variance (ANOVA) was performed using SAS and the data presented as means or percentage of means. Means grouping was with Duncan's Multiple Range Test (Alpha = 0.05). Experiments were run individually for each step of the protocol. Each experiment was run three times. Twenty shoot tips were cryopreserved in LN after each antioxidant treatment (n=60). Twenty control shoot tips were also cryopreserved without any added antioxidants (n=60). Five additional shoot tips per treatment were planted without exposure to LN (n=15). Regrowth data were taken at 6 weeks following reculture. Recovery from cryopreservation required shoot growth and leaf emergence from the original shoot tip.

Results

Shoot tocopherol assay

In-vitro grown shoot tips without added Vit E had very low α- and γ-tocopherol concentrations (<20 nmol/shoot-tip). Exogenous application of Vit E significantly increased the α- and γ-tocopherols in the shoot tips at each step ($P<0.001$). The tocopherol concentrations were higher at the loading than at the pretreatment and rinsing steps for both isomers. The γ-tocopherol was lower than the α-tocopherol concentrations in all the shoot tips (Fig. 4.1). The amounts taken up at each step were equally effective in improving shoot tip regrowth at these steps (Fig. 4.2B). The tocopherol content of *in vitro* shoot tips was also compared to shoot tips from field-grown plants. Field-grown plants had three to six times the amount of tocopherol as the *in-vitro* grown shoots but amounts present were much less than shoots with added Vit E.

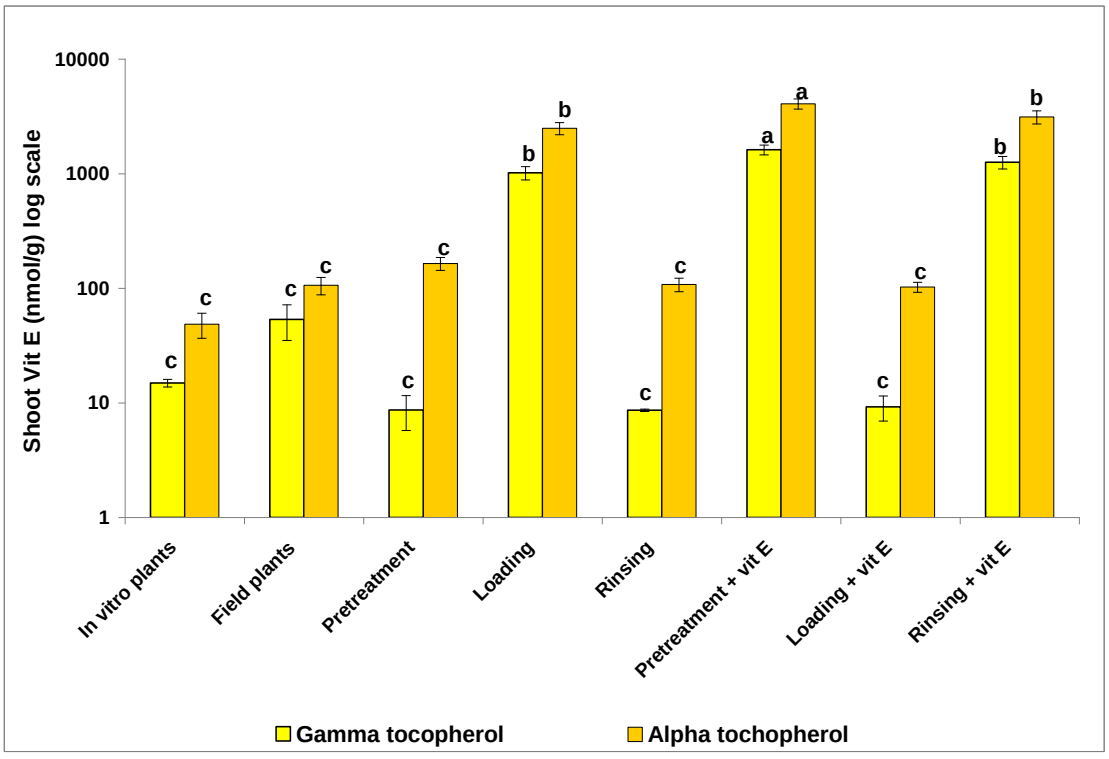

Fig. 4.1: Quantitation of tocopherol isomers in shoot tips of 'Chehalem' from fresh *in vitro* and field grown plants and shoot tips treated with and without 15 mM Vit E during the PVS2 vitrification procedure (log scale) (means ± SD). Means with the same letter are not significantly different. $P<0.001$ (n =60).

Malondialdehyde detection

Significantly more MDA was produced in shoot tips during the three cryopreservation steps than in the control ($P<0.001$). MDA formation was significantly higher after the loading treatment compared to the pretreatment and rinsing steps (Fig. 4.2A). We observed a reduction in MDA formation at each of the critical steps with added Vit E, compared to that of the controls (untreated shoot tips at the same steps). Shoot tips treated with all solutions with and without added Vit E but not exposed to LN, all grew (100%) for each treatment. Regrowth of shoot tips after LN exposure was low (mean of 45%) when MDA was high and high (mean of 71%) when MDA was low in presence of added Vit E ($p=0.002$) (Fig. 4.2). The high regrowth percentages from treatments with added Vit E were not significantly different in these steps ($p=0.002$) (Fig. 4.2B).

Vitamin E

Exogenous Vit E added at each step significantly improved shoot regrowth following LN exposure ($p<0.001$). Shoot tips treated with solutions including Vit E but not exposed to LN, had high regrowth (96%). Regrowth of shoot tips treated with Vit E at 11 and 15 mM and exposed to LN was significantly higher than the controls at each step. These two concentrations were both effective for regrowth of each cultivar at each step (Fig. 4.3). Post-LN regrowth of ‘Chehalem’ shoot tips treated with Vit E (7 mM) at pretreatment and rinsing steps was also significantly better than the control (Fig. 4.3A).

Vitamin E added at two steps

Vit E (11-15 mM) added at both the pretreatment and rinsing steps improved regrowth significantly ($P<0.001$) compared to controls (data not shown). Regrowth was not significantly different from when Vit E was added one step at a time (Fig. 4.3). Some cryopreserved shoot tips initially formed callus before shoot emergence, thus no further testing was done with Vit E in multiple steps.

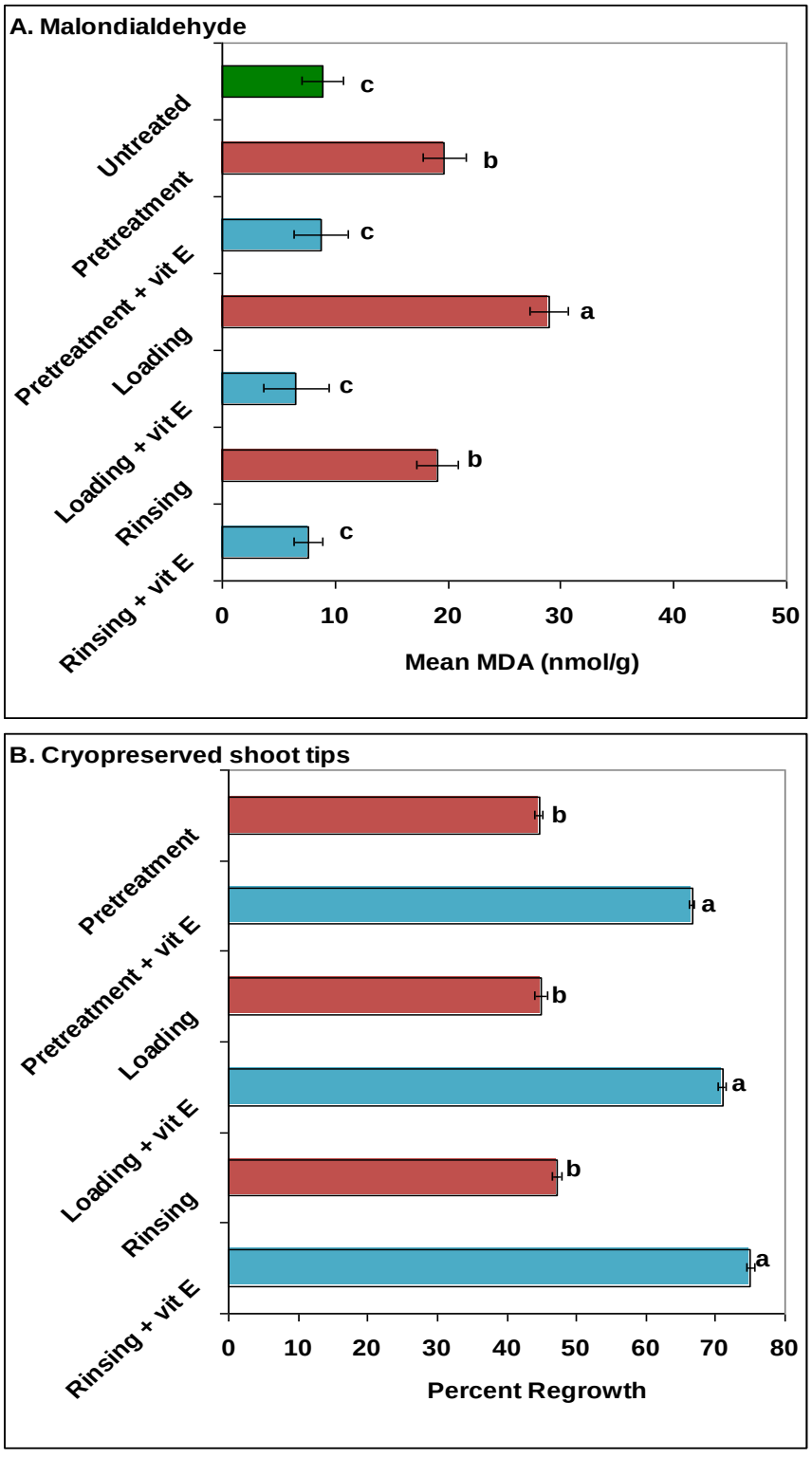

Fig. 4.2: A. Malondialdehyde (MDA) formation in untreated 'Chehalem' shoot tips (control) and PVS2 treated shoot tips with and without added Vit E (15 mM). Means ± SD. B. Regrowth with and without added Vit E (14.72 mM) after exposure to liquid nitrogen. Means with the same letter are not significantly different. $P<0.05$ (n = 60).

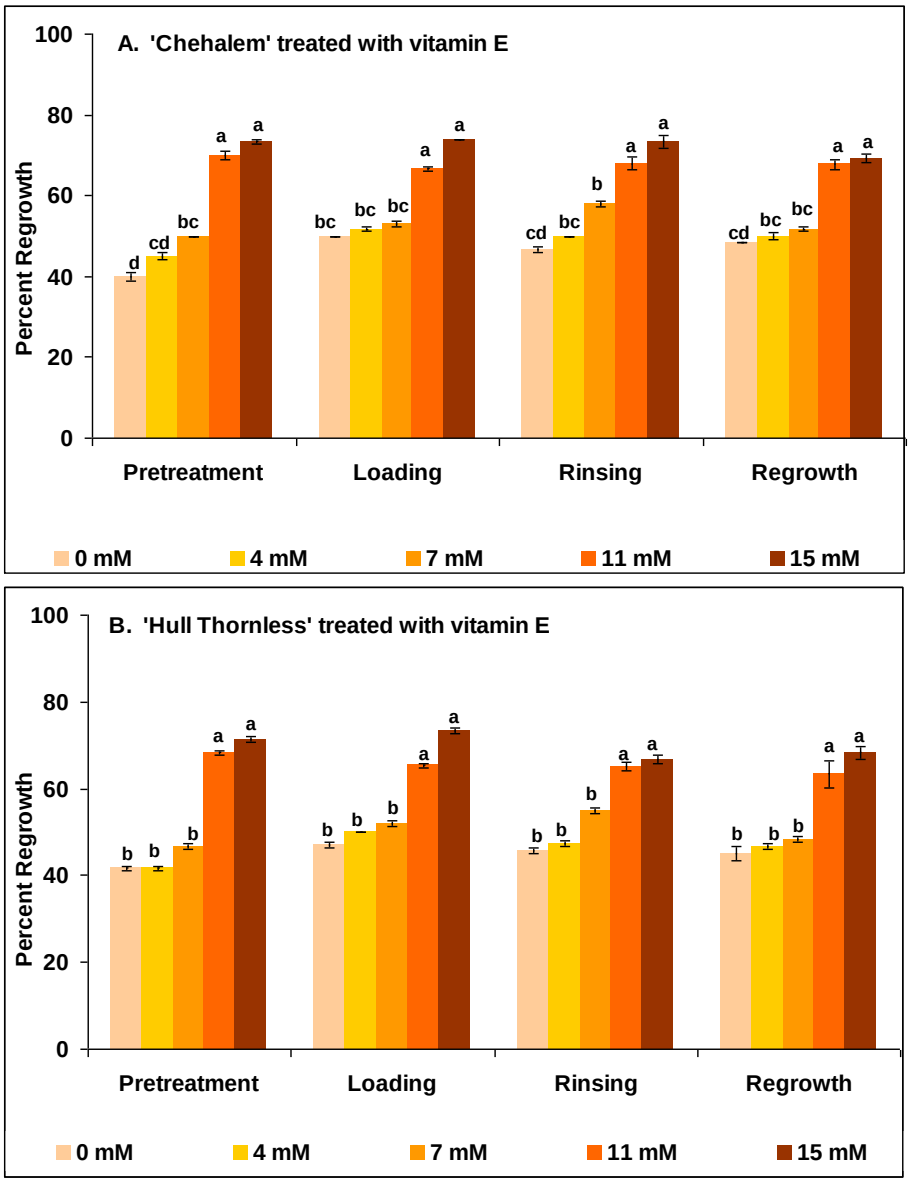

Fig. 4.3: Regrowth of A. 'Chehalem' and B. 'Hull Thornless' after liquid nitrogen exposure of shoot tips treated with and without vitamin E at 4 critical steps of the PVS2 vitrification technique: pretreatment, loading, rinsing and regrowth. Means ± SD. Means with the same letter are not significantly different. $P<0.05$ ($n = 60$).

Vitamin C

Shoot tips with added Vit C at the four critical steps and exposed to LN had significantly higher regrowth compared to control for both cultivars (Fig. 4.4). Increased regrowth always occurred at the pretreatment, loading and rinsing steps. Regrowth on RUB medium with Vit C was significantly lower (10-18%) than the controls (42-45%) for both cultivars during initial experiments. When iron was removed from the regrowth medium in later experiments, the Vit C treated shoot tips improved at the same high percentages as the other three steps. Regrowth of shoot tips on iron-free medium was followed by a transfer to RUB medium with iron (no Vit C) after 2 weeks. Shoot tips treated with Vit C and not exposed to LN had mean regrowth $\geq$ 98%. In an attempt to further improve recovery, iron was excluded from all the solutions and Vit C was applied at all 4 steps. Some callus formation was observed and shoots did not recover (data not shown).

Combined Vit C and Vit E treatments

Vit C (0.14 mM) combined with Vit E (11 mM) at each of the critical steps resulted in regrowth that was significantly higher than controls ($P<0.001$). Regrowth with Vit C alone was not significantly different from combined treatment (Vit E + Vit C). Vit E alone produced significantly less regrowth from combined treatment but the regrowth was significantly better than the control. This trend held for all four steps and both cultivars (Fig. 4.5). The control shoot tips that were not exposed to LN at each treatment had 100% regrowth (Fig. 4.6).

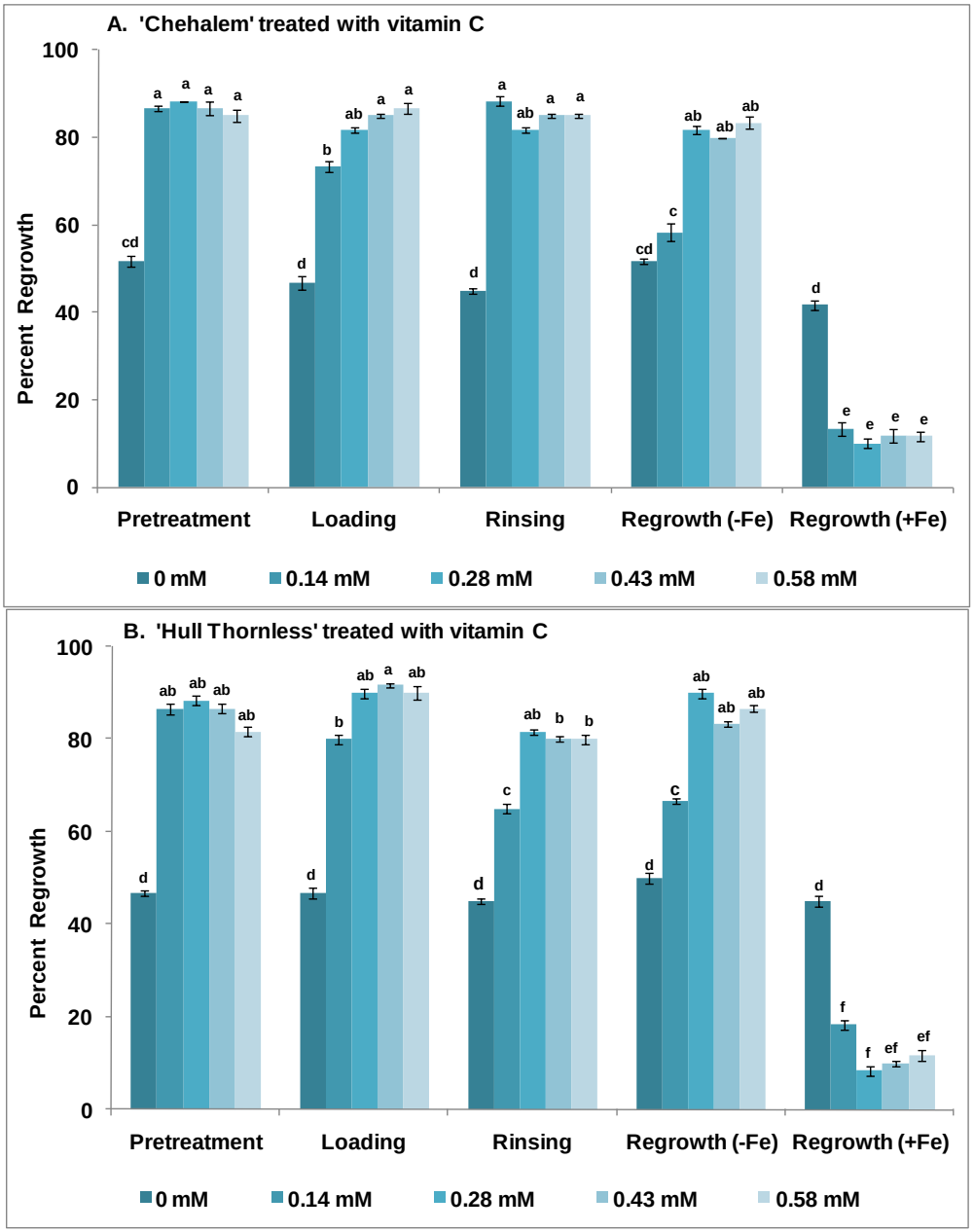

Fig. 4.4: Regrowth of A. 'Chehalem' and B. 'Hull Thornless' shoot tips with and without Vit C addition at 4 critical steps of the PVS2 vitrification technique: pretreatment, loading , rinsing and regrowth. Means ± SD. Means with the same letter are not significantly different. $P \leq 0.05$ (n = 60).

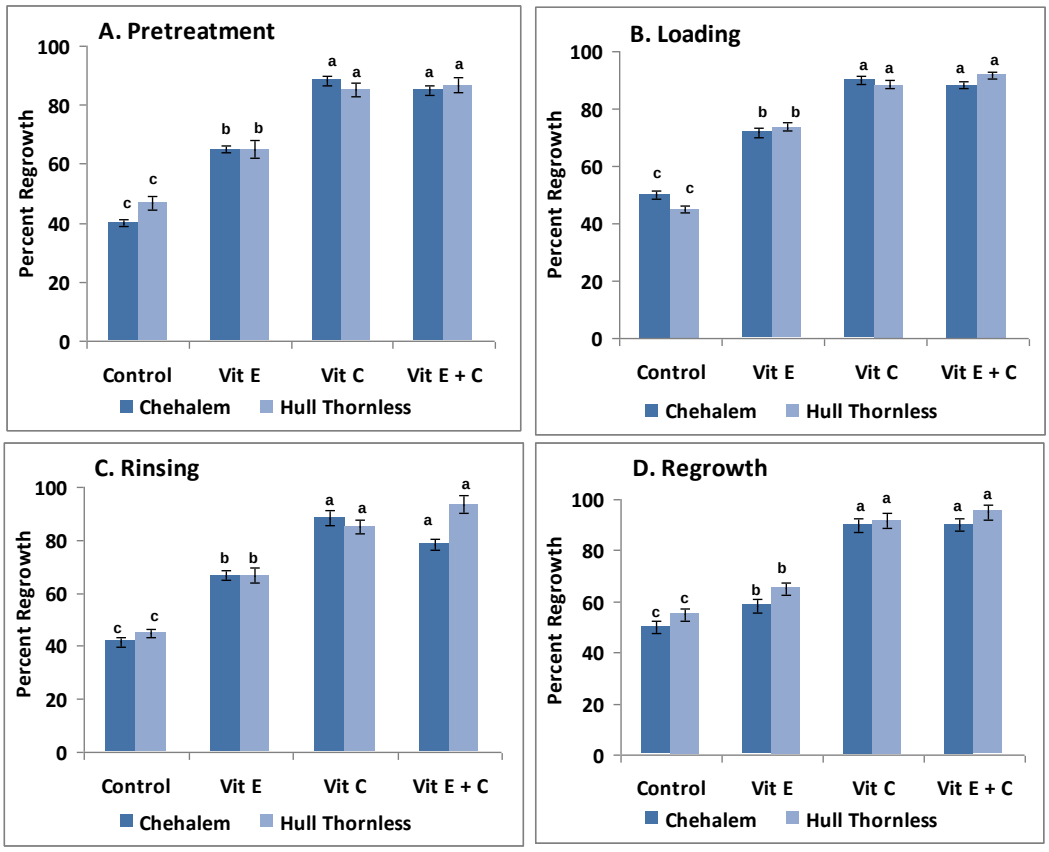

Fig. 4.5: Effect of antioxidant treatments: Vit E (11 mM), Vit C (0.14 mM) and combined on regrowth of cryopreserved shoot tips of blackberry cultivars at 4 critical steps of the PVS2 vitrification technique: A. pretreatment, B. loading, C. rinsing and D. regrowth. Means ± SD. Means with the same letter are not significantly different. $P \leq 0.05$ (n = 60).

Discussion

Exogenous Vit E or Vit C added at critical steps of the PVS2 vitrification technique significantly increased regrowth of both cultivars. This finding suggests that oxidative stress is an important factor in the death of plant tissues following cryopreservation. 'Chehalem' and 'Hull Thornless' were chosen for this study because they had stable but moderate mean regrowth of 40-50% following cryopreservation by PVS2 vitrification. This allowed for improvement with the antioxidant treatments. Vit E and Vit C were chosen because of their free radical scavenging capabilities. This study

determined the mean regrowth at each treatment and the amount of lipid peroxidation occurring at each step, and then provided a solution for reducing the oxidative damage. We quantified MDA, a lipid peroxidation product, at each of the critical steps of the PVS2 vitrification and found a significant rise in the amount of MDA produced at each of these steps compared to fresh shoot tips or the untreated (controls) (Fig. 4.2A). Roach et al., (2008) characterized ROS formation during cryopreservation treatments (specifically 'wounding and desiccation') on embryonic axes of chestnut seeds (*Castanea sativa*). They found that excision led to a transient burst of superoxide in 5 min and the stress imposed by a combination of excision and dehydration from 60% to 30% water content doubled the initial rates of superoxide production.

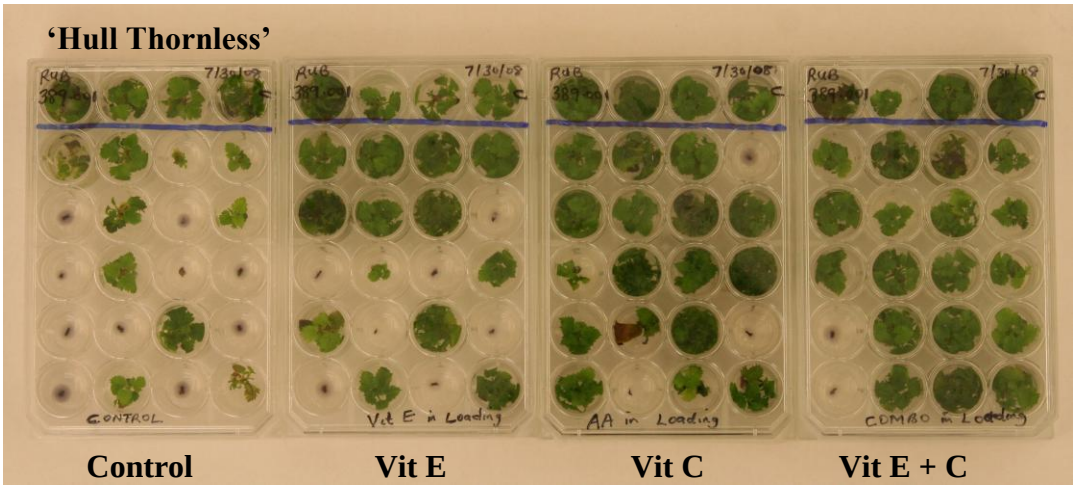

Fig. 4.6: Six wks regrowth of *Rubus* hybrid cv. Hull Thornless following antioxidant treatments in the loading solution. The top row of each plate contains plantlets that had all the treatments but were not exposed to LN. The control was cryopreserved with the standard technique but without added antioxidants, Vit E = 11 mM, Vit C = 0.14 mM.

Superoxide is known to spontaneously produce H_2O_2, a precursor for the formation of the highly reactive hydroxyl radicals (Halliwell and Gutteridge, 1999). Our data showed that shoot tips with high MDA had reduced regrowth after cryopreservation compared to those with low MDA (Fig. 4.2). This agrees with a study which showed that accumulation of 4-hydroxy-2-nonenal and malondialdehyde in *Daucus carota* L. *in vitro* plant cells correlated with loss of regeneration potential (Adams et al., 1999).

We observed that significantly higher lipid peroxidation occurred at the loading than at the pretreatment and rinsing steps (Fig. 4.3A). This result may be due to the osmotic effect of the loading solution on shoot tips. The high sucrose concentration and glycerol in the loading solution may increase stress as a result of desiccation of the cells. These results may also be a result of the antioxidant effect of DMSO in the solutions at the other steps. DMSO is a known antioxidant and free radical scavenger (Yu and Quinn, 1994) and thus it may be involved in reducing oxidative damage at these steps.

Exogenously applied Vit E significantly increased the quantities of α- and γ-tocopherols in blackberry shoot-tip cultures (Fig. 4.1). Adding Vit E during the steps of the cryopreservation protocol provides a simple and cost effective method of improving a plant's tocopherol status. Alpha tocopherol was increased in cell cultures of sunflower by the addition of exogenous homogentisic acid and phytol (Caretto et al., 2004). The elevation of antioxidant status using exogenous glutathione was demonstrated with tobacco cell cultures (Schneider et al., 1992) and canola plants (Lappartient and Touraine, 1997). Initial experiments to determine what concentration of Vit E could significantly increase regrowth following LN exposure showed that 11 mM and 15 mM significantly improved regrowth of shoot tips compared to controls at each of these steps (Fig. 4.3). Regrowth following exogenous Vit E addition at both pretreatment and post cryopreservation steps was not significantly better than when Vit E was added one step at a time. Intermediate callus was formed before shoots emerged suggesting that some risk might be associated with the application of Vit E at two or more steps at once. Vit E, in addition to its antioxidant effects has been shown to exert pro-oxidative effects which may be deleterious. For example, an *in vitro* experiment with micellar cell cultures showed that excessive doses of Vit E caused pro-oxidative effects due to partial reduction of Vit E radical (Mukai, 1993). A study on rat erythrocytes showed that excessive doses of Vit E lowers the activities of antioxidant enzymes in vivo (Eder et al., 2002). Vit E decreased MDA formation (Fig. 4.2A) and significantly improved shoot regrowth after cryopreservation (Fig. 4.2B), most likely due to the reduction in MDA formation. These data indicate that Vit E is effective in preventing or repairing damage caused by lipid peroxidation. Sattler et al.

(2004) also showed that tocopherols are vital in preventing lipid peroxidation in seeds of *Arabidopsis thaliana* during germination. Vit E scavenges ROS, reacts with by-products of PUFA and modulates signal transduction (Halliwell and Gutteridge, 1999; Noctor, 2006). We observed a correlation between shoot tocopherol uptake and MDA formation. The amounts of tocopherols and the MDA produced in Vit E treated shoot tips (Fig. 4.1, 4.2A), were highest at the loading step compared to the pretreatment and rinsing steps. These data suggest that as the level of oxidative stress increases in *Rubus* shoot cultures so does antioxidant status. Our analysis found that field-grown blackberry plants exposed to the stresses of the outdoor environment had three to six times the amount of tocopherol found in the *in-vitro* blackberry plants grown in a controlled environment (Fig. 4.1). In *Ribes* shoot cultures a high phenolics to ROS ratio was observed in a cold-tolerant genotype compared to one considered cold sensitive (Johnston et al., 2007). Antioxidants thus may modulate the effects of ROS.

Vit C is a water soluble molecule and is taken up by plants after exogenous application (Arrigoni et al., 1997; Paciolla et al., 2001). Vit C reacts with ROS including hydroxyl radicals and hydrogen peroxide and forms a stable free radical (monodehydroascorbate). This radical has a very short life and is readily converted into dehydroascorbate and Vit C, leading to the termination of the chain reactions of ROS (Halliwell, 2006). Vit C (0.14–0.58 mM) significantly improved regrowth of cryopreserved blackberry shoot tips (Fig. 4.4). Vit C (0.1 mM) in tissue-culture medium greatly improved *in vitro* shoot and root growth of somatic embryos of white spruce (Stasolla and Yeung, 1999). The initial low regrowth of cryopreserved *Rubus* shoot tips in this study may be due to the presence of higher than normal concentrations of iron in the medium; iron and Vit C participate in the Fenton reaction, leading to the production of hydroxyl radicals (Halliwell, 2006; Halliwell and Gutteridge, 1984). Thus, the combination of doubled concentration of iron in the standard propagation medium (RUB) and Vit C were damaging. A similar growth decline caused by iron-induced reactions was observed during regeneration of *Arachis hypogaea* L explants (Zheng et al., 2005). Removal of iron from the regrowth medium resulted in high regrowth of our cryopreserved *Rubus* shoot tips (Fig. 4.4).

Vit C was combined with Vit E to determine if the combination was better than single treatments for cryopreservation. Vit C recycles Vit E, keeping Vit E in its active form (Scarpa et al., 1984). Regrowth of shoot tips when Vit E and Vit C were combined was not significantly better than Vit C alone in any of the steps, suggesting that the benefit was mostly due to Vit C rather than Vit E (Fig. 4.5). These data suggest that antioxidant protection by exogenous Vit C is likely more efficient compared to Vit E. It is also possible that improvement would be seen if the plants tested had even lower initial recovery. Improving *Rubus* regrowth from 90% with Vit C to significantly greater than 90% would be less likely than improving recovery of a cultivar with only 30% regrowth. A study of shoot organogenesis found that exogenous Vit C (0.1mM) increased the number of shoots and the percentage of organogenesis in cultured leaf segments of *Gladiolus* more than α-tocopherol of equal concentration. At a higher concentration (0.5 mM) of both, Vit E was better (Gupta and Datta, 2003). We did not observe decreased regrowth of shoot tips when Vit C was added in a regrowth medium with both iron and Vit E (Fig. 4.5D). Possibly Vit E quenched any deleterious Fenton reactions and prevented damage to the tissues. Also, we did not observe a growth decline with Vit C and the regular concentration of MS iron when both occurred in the presence of DMSO (Fig. 4.4, 4.5). This may be due to the fact that MS iron is not doubled in these solutions as it is in our regrowth medium or that DMSO may have a beneficial antioxidant protection against ROS. We did not test the antioxidant effect of Vit C on reducing lipid peroxidation of shoot tips, but exogenous Vit C is known to reduce lipid peroxidation by ROS in tomato seedlings (Shalata and Neumann, 2001), and in sorghum and sunflower seedlings (Zhang and Kirkham, 1996). Vit C provides antioxidant protection against the hypersensitivity of a Vit C mutant of *Arabidopsis thaliana* to oxidative damage and Vit C is also an important cofactor for enzymatic reactions that promote cell division, rapid cell expansion and growth (Smirnoff, 2000). Exogenous Vit C, tocopherol and glutathione significantly improved the regeneration frequency and number of shoot tips of a *Gladiolus* cultivar during organogenesis (Gupta and Datta, 2003).

Conclusions

Vit E or Vit C added at critical steps during PVS2 vitrification significantly improved regrowth of shoot tips following LN exposure. MDA formation significantly increased at each step of the PVS2 vitrification protocol. Adding exogenous Vit E significantly increased the α- and γ-tocopherol contents of the *in vitro* blackberry shoot tips and significantly decreased lipid peroxidation at each of the steps. These findings enhance our understanding of the stresses associated with cryopreservation procedures that limit regrowth and provide a solution for reducing damage to plants during these processes. This study to the best of our knowledge is the first report on the successful use of antioxidant vitamins to substantially increase regrowth of cryopreserved plants. Our studies with Vit E demonstrated that ROS are involved in lethal damage to plants via lipid peroxidation during the cryopreservation process. Although Vit E and Vit C additions greatly improve regrowth of these *Rubus* cultivars, further research may expand this technique to a broader range of genotypes, particularly 'difficult to cryopreserve species'.

Acknowledgements

Research was funded by the United States Department of Agriculture (USDA), Agricultural Research Service CRIS project 5358-21000-038-00D. Vitamin E and malondialdehyde analysis was done by the Linus Pauling Institute. Thanks are extended to Jeanine DeNoma for her technical advice and to the Ford Foundation International Fellowship Program, and the University Club of Portland Inc. for fellowships to Esther Uchendu.

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CHAPTER 5

ANTIOXIDANT AND ANTI-STRESS COMPOUNDS IMPROVE REGROWTH OF CRYOPRESERVED *RUBUS* SHOOT TIPS

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Submitted to In Vitro Cellular and Developmental Biology – Plant

Abstract

Regrowth of plants after cryopreservation varies and resulting regrowth ranges from poor to excellent. Oxidative stress is a potential cause of damage in plant tissues. Antioxidants and anti-stress compounds may improve regrowth by preventing or repairing the damage. Lipoic acid (LA), glutathione (GSH), glycine betaine (GB) and polyvinylpyrrolidone (PVP) were tested on cryopreservation of shoot tips using the plant vitrification solution 2 (PVS2) protocol. Two *in vitro* grown blackberry cultivars were cold acclimated, and then cryopreserved in liquid nitrogen (LN). The antioxidant and anti-stress compounds were added at 4 critical steps of the protocol: pretreatment, loading, rinsing, and regrowth. Three out of the 4 compounds significantly improved regrowth of cryopreserved shoot tips. Regrowth ranged from 40-50% for controls to >80% for treated shoot tips. LA (4-8 mM) produced high regrowth at pretreatment, loading and rinsing for 'Chehalem' and at all steps for 'Hull Thornless'. Recovery improved at all steps with GSH (0.16 mM) and GB (10 mM). PVP had a neutral or negative impact on regrowth. Overall addition of LA, GSH and GB improved regrowth by ~25% over the shoot tips cryopreserved using the regular PVS2 protocol (control). This study shows that adding antioxidants and anti-stress compounds during the PVS2-vitrification protocol improves regrowth of shoot cultures following cryopreservation.

Keywords: blackberry; lipoic acid; germplasm; glutathione; glycine betaine; long-term storage; oxidative stress; polyvinylpyrrolidone

Introduction

The development of efficient cryopreservation protocols for vegetatively propagated species is of key importance to the long-term conservation of clonally propagated germplasm collections (Reed, 2008). Cryopreservation provides stable, long term, low cost storage of plants safe from diseases or environmental damage. Storing plants at low temperatures does have some deleterious effects. Chilling and freezing injury

promote many sub-lethal changes such as metabolic uncoupling, which can then lead to increased production of free radical species (Day et al., 2000). Reactive oxygen species (ROS) include superoxide radicals ($O_2^{\bullet}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Asada, 2006). ROS have unpaired electrons that can readily cause damage by extracting electrons from biological molecules such as lipids, proteins, enzymes and nucleic acids, resulting in their modification or permanent alteration (Møller et al., 2007; Takahama, 2004). Free radicals attack lipids in membranes and this result in the formation of lipid hydroperoxides, which decompose to form volatile hydrocarbons and aldehydes (Valenzuela, 1991). These secondary or breakdown products form cross-links with macromolecules such as DNA and proteins (Esterbauer et al., 1991).

ROS formation occurs under normal physiological conditions in the mitochondria, peroxisomes and chloroplasts as byproducts of cellular respiration and photosynthesis (Mittler et al., 2004). ROS reactions causes damage including lipid peroxidation which alters membrane integrity, protein denaturation and disruption of nucleic acids (Batkova et al., 2008). These changes can hamper physiological processes and cause downstream effects that lead to permanent damage, thus the need to control ROS production in order to preserve physiological functions and viability. Plants have evolved natural antioxidant defense mechanisms to combat the danger posed by the presence of ROS during physiological stress. These include ROS scavenging mechanisms involving antioxidant enzymes such as superoxide dismutase, peroxidases, mono- and dehydroascorbate reductase, glutathione reductase and catalase (Elstner and Osswald, 1994; Wu et al., 2009). Abiotic stresses resulting from low temperature, water deficit or nutrient deprivation can further increase ROS formation to toxic levels in cellular systems (Mittler, 2002). Uncontrolled free radical production occurs when the antioxidant capacity of the cell is exceeded (Benson, 2000). Plants respond to abiotic stress by accumulating compatible solutes such as sugars (Valluru and Van den Ende, 2008) and antioxidants compounds like flavonoids (Kytridis and Manetas, 2006; Takahama, 2004), however the response varies with genotype. These natural adaptations to stress are utilized by researchers to protect

plants from the damage associated with oxidation. Recent studies showed that an increase in galactinol and raffinose in *Arabidopsis thaliana* transgenic cultures protected against oxidative damage by quenching hydroxyl radicals (Nishizawa et al., 2008). Enhancement of plants' cellular antioxidants increases stress tolerance. Exogenous vitamin C was applied in growth medium and reduced lipid peroxidation caused by salt stress in tomato seedlings (Shalata and Neumann, 2001). Vitamin C also reduced polyethylene glycol-induced stress in sorghum and sunflower seedlings (Zhang and Kirkham, 1996). Antioxidants form stable complex compounds when reacting with oxygenic byproducts resulting from mechanical injury or environmental stress (Tang et al., 2004).

The steps in cryopreservation protocols can impose a series of stresses on plants, particularly oxidative stress mediated by ROS. A recent study found that tissue injury inflicted during cutting of embryo axes or dehydration of tissues led to superoxide production (Roach et al., 2008). Superoxide readily reacts to form hydrogen peroxide, a precursor for the formation of the highly reactive hydroxyl radical (Halliwell, 2006). Transitory changes were observed in DNA methylation and transcriptional activities in *Ribes* meristems during a cryopreservation procedure (encapsulation dehydration) (Johnson et al., 2009).

There are many antioxidant and anti-stress compounds available in cellular systems that alleviate oxidative stress or damage. Lipoic acid (LA) is an antioxidant compound that readily permeates cell membrane and cellular organelles and has strong antioxidant properties (Kagan et al., 1992). LA exhibits its biological activity in the cell membrane (Busse et al., 1992) and cytoplasm (Packer et al., 1996). LA is known to regenerate both hydrophilic and hydrophobic antioxidants including GSH and vitamins E and C (Biewenga et al., 1997; Kagan et al., 1992). LA provides antioxidant protection by scavenging ROS (Navari-Izzo et al., 2002) and inhibiting lipid peroxidation (Bast and Haenen, 1988). LA included in plant tissue culture media significantly lowered tissue browning and improved transformation efficiency of wheat, soybean and cotton (Dan et al., 2009).

Glutathione (GSH) is one of the most abundant low molecular weight thiols naturally found in plants. It controls ROS formation (Foyer and Noctor, 2001), protects membrane lipids against peroxidation (Jocelyn, 1972) and promotes shoot organogenesis (Gupta and Datta, 2003). GSH content increases with the accumulation of frost hardiness of *Picea abies* (Esterbauer and Grill, 1978). An increase of GSH was observed after osmotic stress in wheat (Lascano et al., 2001), suggesting that GSH aids in controlling the damage associated with osmotic injury. Wang and Deng (2004) found that GSH added during cryopreservation significantly increased regrowth of *Citrus* shoot tips. They reported 55% regrowth with GSH (20 mg•l^{-1}) added in the regrowth medium and >80% regrowth for GSH (40 mg•l^{-1}) in pretreatment, loading and cryoprotectant solutions compared to 40% for the control.

Glycine betaine (GB) is found in a variety of organisms including plants, animals (Rhodes and Hanson, 1993) and microorganisms (Ishitani et al., 1993). Plants synthesize and accumulate GB in response to abiotic stress (Allard et al., 1998). GB accumulation increases as plant stress increases (Rhodes and Hanson, 1993). There is very little information available concerning the GB mode of action. Park et al. (2006) showed that intracellular GB content of shoot apices treated with exogenously applied GB (20 mM), increased from 0 to 2 µmol g^{-1} fresh weight after 3 days of application and resulted in reduced H_2O_2 and increased catalase activity in treated plants compared to controls. GB maintains the integrity of plant membranes, enzymes and proteins (Jolivet et al., 1982; Papageorgiou and Murata, 1995).

Polyvinylpyrrolidone (PVP) is commonly used to reduce injury and browning in tissue cultured plantlets (Saxena and Gill, 1986; Tang et al., 2004). PVP prevents oxidative browning of wound-induced polyphenolics via a hydrogen bonding mechanism (Figueiredo et al., 2001). PVP (2%) was added to preculture medium to decrease browning of raspberry shoot tips. PVP treatment resulted in 10% improvement in survival compared to the control (Wang et al., 2005).

Cryopreservation protocols including controlled rate cooling, encapsulation-dehydration, encapsulation-vitrification and PVS2 vitrification are successfully applied to a range of *Rubus* germplasm (Chang and Reed, 1999; Gupta and Reed, 2006; Reed, 1993; Vysotskaya et al., 1999; Wang et al., 2005). Results from these studies vary from poor to excellent indicating that recovery is dependent on genotype. Modifications to existing techniques can be used to decrease genotype variation and allow for more efficient storage of diverse germplasm collections (Reed, 2008).

The goals of this study were to evaluate the influence of antioxidant and anti-stress compounds, added at critical steps of the PVS2 protocol, on regrowth of shoot tips and to determine the range of concentrations of LA, GSH, GB and PVP beneficial for shoot tip regrowth of two blackberry cultivars.

Materials and Methods

Plant material

Micropropagated plantlets of *Rubus* hybrid cvs. Chehalem (PI 55327, local 761.001) and Hull Thornless (PI 553299, local 389.001) obtained from the collections of the USDA-ARS NCGR, Corvallis, OR, USA were used for these studies. *In vitro* plantlets were multiplied on NCGR-*Rubus* (RUB) medium which contains Murashige and Skoog (1962) (MS) mineral salts and vitamins with double Fe-EDTA, 2 $mg \cdot l^{-1}$ N^6-benzyladenine (BA), 0.1 $mg \cdot l^{-1}$ indole-3-butyric acid (IBA), 0.1 $mg \cdot l^{-1}$ gibberellic acid (GA_3) (Sigma-Aldrich Co., St. Louis, MO), 3.5 $g \cdot l^{-1}$ agar (Difco, Detroit, MI), 1.45 $g \cdot l^{-1}$ Gelrite (Phyto Technology Lab, Shawnee Mission, KS) and 30 $g \cdot l^{-1}$ sucrose at pH 5.7 in Magenta GA_7 boxes (Magenta Corp., Chicago, IL). These plantlets were subcultured every 3 weeks and grown at 25 °C (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) with a 16 h light/8 h dark photoperiod.

Pretreatment

Plantlets, 3 weeks after the last subculture, were cold acclimated (CA) for 2 weeks under alternating temperatures [22 °C with 8 h light (10 $\mu E \bullet m^{-2} \bullet s^{-1}$) / -1 °C 16 h dark] (Reed, 1988). Shoot tips with 2 or 3 pairs of leaf primordia (~0.8 mm) were excised from CA plantlets. The dissected shoot tips were grown for 48 h on MS medium containing 5% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co., St. Louis, MO) (v/v) with 3.5 $g \bullet 1^{-1}$ agar and 1.75 $g \bullet 1^{-1}$ Gelrite under the standard CA conditions.

Vitrification procedure

The PVS2-vitrification protocol modified for blackberry (Gupta and Reed, 2006) was used for this study. Shoot tips were transferred into 1 ml cryovials (Nunc, Roskilde-Denmark) and treated with 1 ml loading solution (LS) (2 M glycerol and 0.4 M sucrose in MS medium) for 20 min. LS was removed and the cryoprotectant PVS2 [30% glycerol, 15% DMSO and 15% ethylene glycol and 0.4 M sucrose in liquid MS medium at pH 5.8 (Sakai et al., 1991)] was added to the vials, held for 20 min, then plunged in liquid nitrogen (LN) and held for 30 min. The vials were rewarmed in 45 °C water for 1 min, then in 25 °C water for 1 min. The shoot tips were immediately rinsed twice in liquid 1.2 M sucrose MS medium. Shoot tips were planted on regrowth medium (RUB with no IBA), one shoot tip per cell in 24-cell plates (Costar, Cambridge, Mass). PVP was tested in the pretreatment and regrowth media while the other compounds were tested at pretreatment, loading, rinsing and regrowth. The time of antioxidant exposure was 48 h (pretreatment), 20 min (loading), 5 min (rinsing) and 6 wks (regrowth).

Effect of antioxidants on regrowth from cryopreservation

We evaluated the effects of LA (T-1395, Sigma-Aldrich Co., St. Louis, MO) at 0, 2, 4, 6, 8 and 10 mM, GSH (G-6013, Sigma Chemical Co., St Louis, MO) at 0, 0.08, 0.16, 0.24 and 0.33 mM, GB (B-2629, Sigma-Aldrich Co., St. Louis, MO) at 0, 5, 10, 15 and 20 mM, and PVP [10,000 MW (Sigma-Aldrich Co., St. Louis, MO)] at 0, 1, 2.5, 5

and 10 mM. These compounds were added into regrowth medium and solutions before the pH (5.7) were adjusted. The pretreatment and regrowth media were autoclaved and the cryopreservation solutions (loading and rinsing) were filter sterilized using membrane filters (0.45µm, 150-ml analytical filter unit, NALGENE, Rochester, NY). LA was dissolved in 2-3 drops of ethanol before adding into solutions and media. The compounds were added to only one step at a time. Experiments were run individually three times for each step.

Regrowth assessment and data analysis

Each experiment included 20 cryopreserved shoot tips for each concentration of antioxidant or anti-stress compound and 20 non-antioxidant treated controls. Each treatment also included 5 non-cryopreserved controls. Each experiment was done three times. Regrowth of shoot tips was analyzed at 4 weeks for the PVP study and at 6 weeks for the other compounds. Analysis of variance (ANOVA) was done with SAS (2007). Duncan's multiple range test was applied for mean grouping at the 5% level of probability. Regrowth data were graphed using means ± SD. Plantlets were considered fully recovered from cryopreservation when new shoots and leaves were observed.

Results

Lipoic Acid

LA significantly promoted shoot-tip regrowth in both cultivars ($p<0.001$). In most cases, the percent regrowth was increased from a control of ~45% to >70%. LA added to regrowth medium did not significantly improve 'Chehalem' shoot tips compared to the controls but 6 mM significantly improved regrowth of 'Hull Thornless' compared to the controls (Fig. 5.1). The beneficial effect of LA declined at higher concentrations in many experiments.

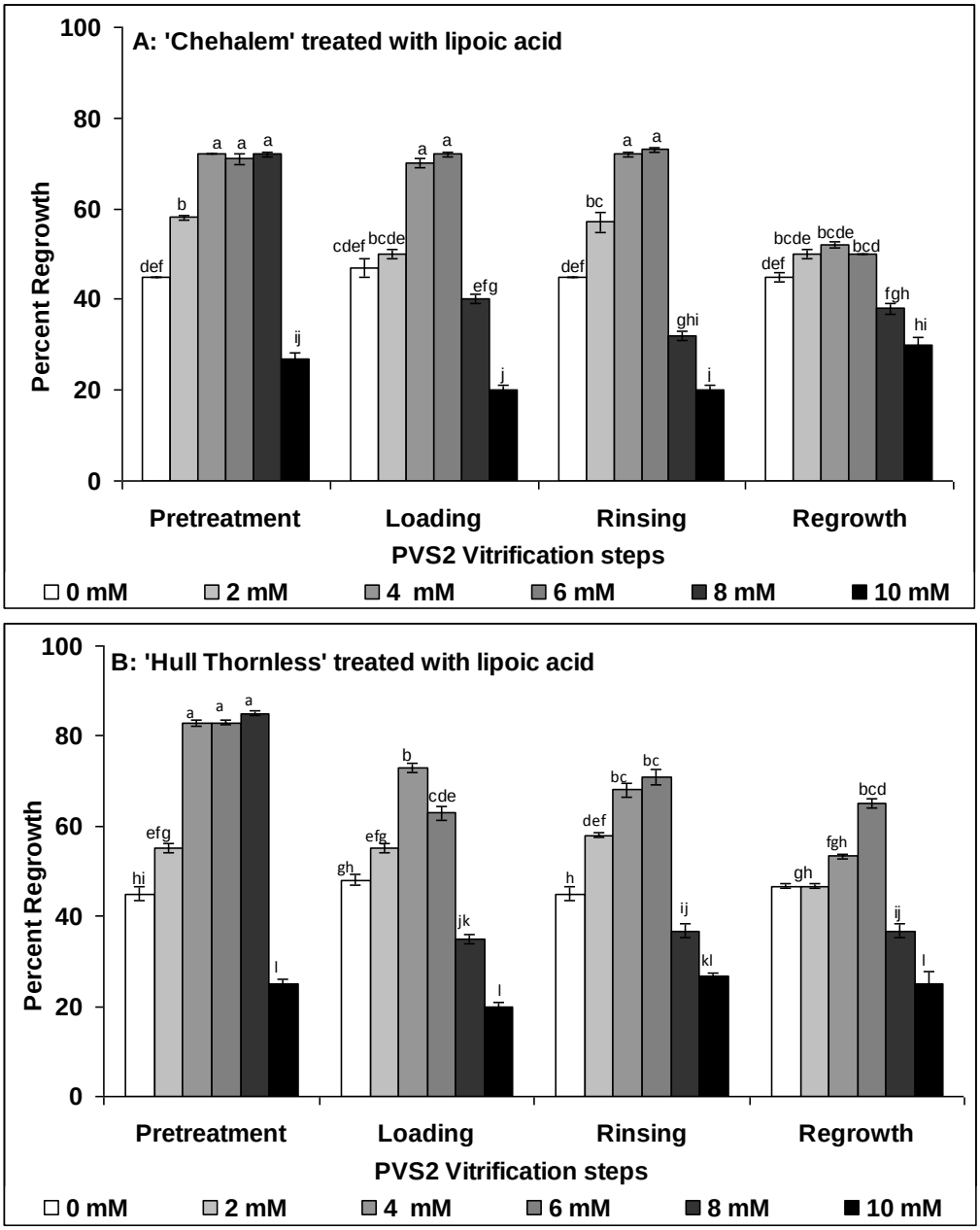

Fig. 5.1: Regrowth of 'Chehalem' (A) and 'Hull Thornless' (B) shoot tips at 6 weeks, treated with and without lipoic acid at four steps of the PVS2-vitrification technique (pretreatment, loading, rinsing, regrowth). Means with the same letter are not significantly different, $P \leq 0.001$ (n=60).

Glutathione

GSH significantly increased regrowth compared to the controls with slight differences in the effective concentrations for both cultivars. ‘Chehalem’ regrowth was significantly improved at all 4 steps and ‘Hull Thornless’ at 3 steps ($p<0.001$). At loading, GSH (0.16 mM) significantly increased regrowth of ‘Chehalem’ (Fig. 5.2A) but did not significantly increase regrowth for ‘Hull Thornless’ (Fig. 5.2B). All concentrations of GSH produced good regrowth when added at pretreatment for ‘Chehalem’ and rinsing or regrowth steps for ‘Hull Thornless’ (Fig. 5.2).

Glycine betaine

GB significantly improved regrowth for both cultivars at all steps ($p<0.0001$). The best regrowth was observed at 10-15 mM for ‘Chehalem’ at each step (Fig. 5.3A) and 10 mM for ‘Hull Thornless’ at each step (Fig. 5.3B).

Polyvinylpyrrolidone

PVP was detrimental for cryopreservation of both cultivars at the concentrations tested (Fig. 5.4). PVP at higher concentrations (5-10 mM) added at pretreatment significantly reduced regrowth of both cultivars ($p<0.05$). At lower concentrations PVP did not significantly change regrowth for either cultivar. Regrowth of shoot tips markedly declined with PVP at all concentrations when added at regrowth compared to the controls. Shoot-tip regrowth values were below 20% for each cultivar when PVP was added post cryopreservation.

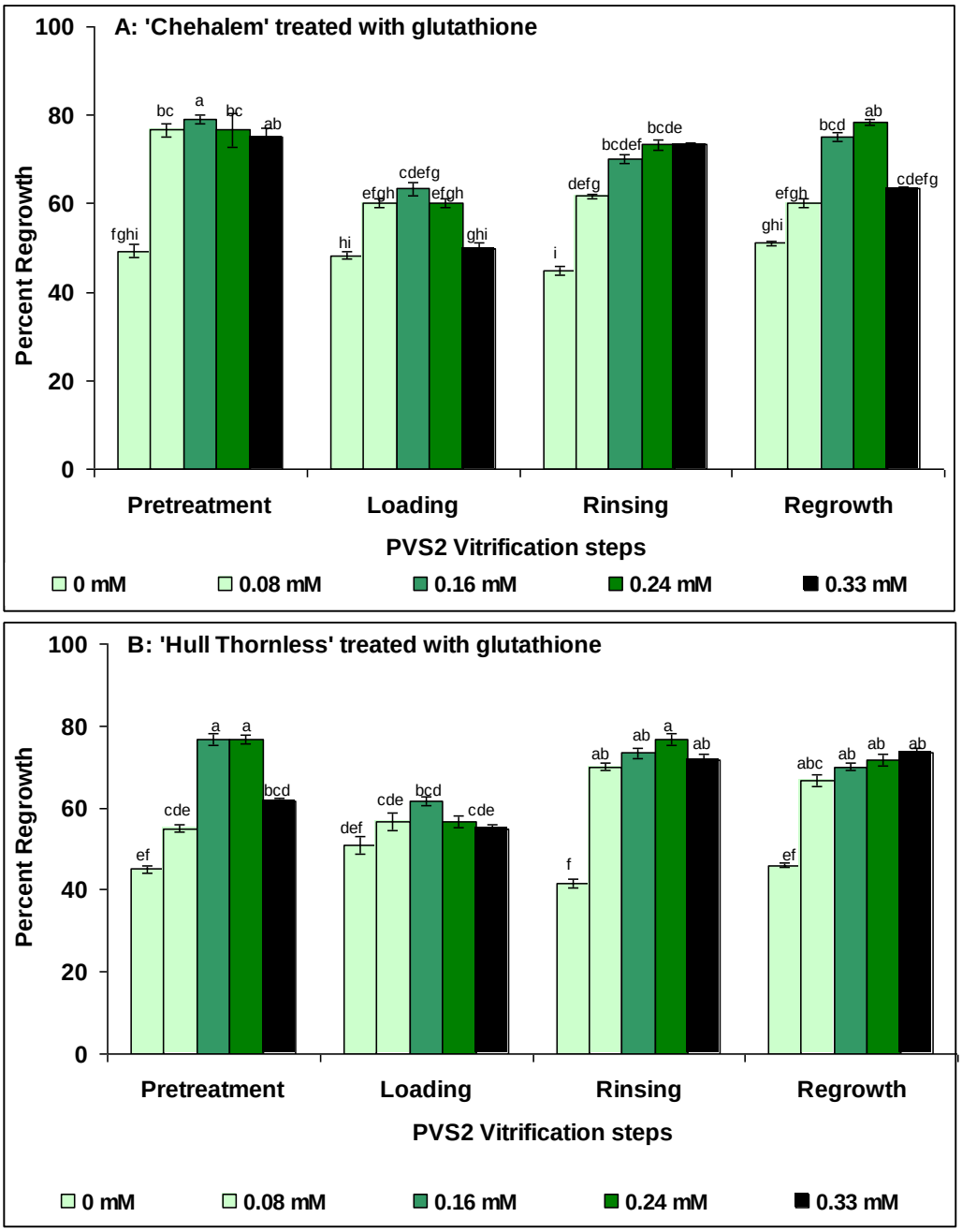

Fig. 5.2: Regrowth of 'Chehalem' (A) and 'Hull Thornless' (B) shoot tips at 6 weeks, treated with and without glutathione at four steps of the PVS2 vitrification technique (pretreatment, loading, rinsing, regrowth). Means with the same letter are not significantly different, $P \leq 0.001$ (n=60).

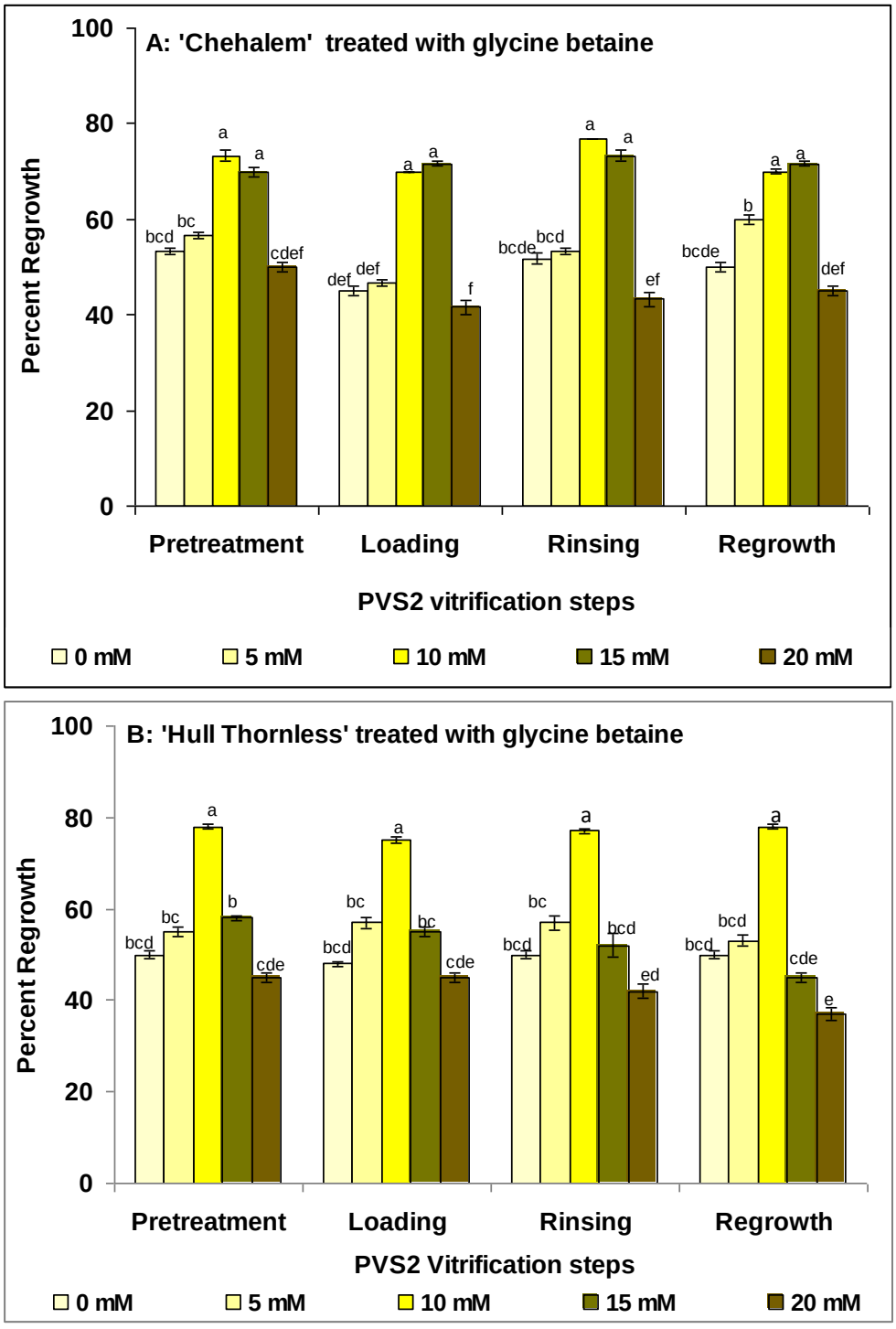

Fig. 5.3: Regrowth of 'Chehalem' (A) and 'Hull Thornless' (B) shoot tip cultures at 6 weeks, treated with and without glycine betaine at four steps of the PVS2-vitrification technique (pretreatment, loading, rinsing, regrowth). Means with the same letter are not significantly different, P=0.0001 (n=60).

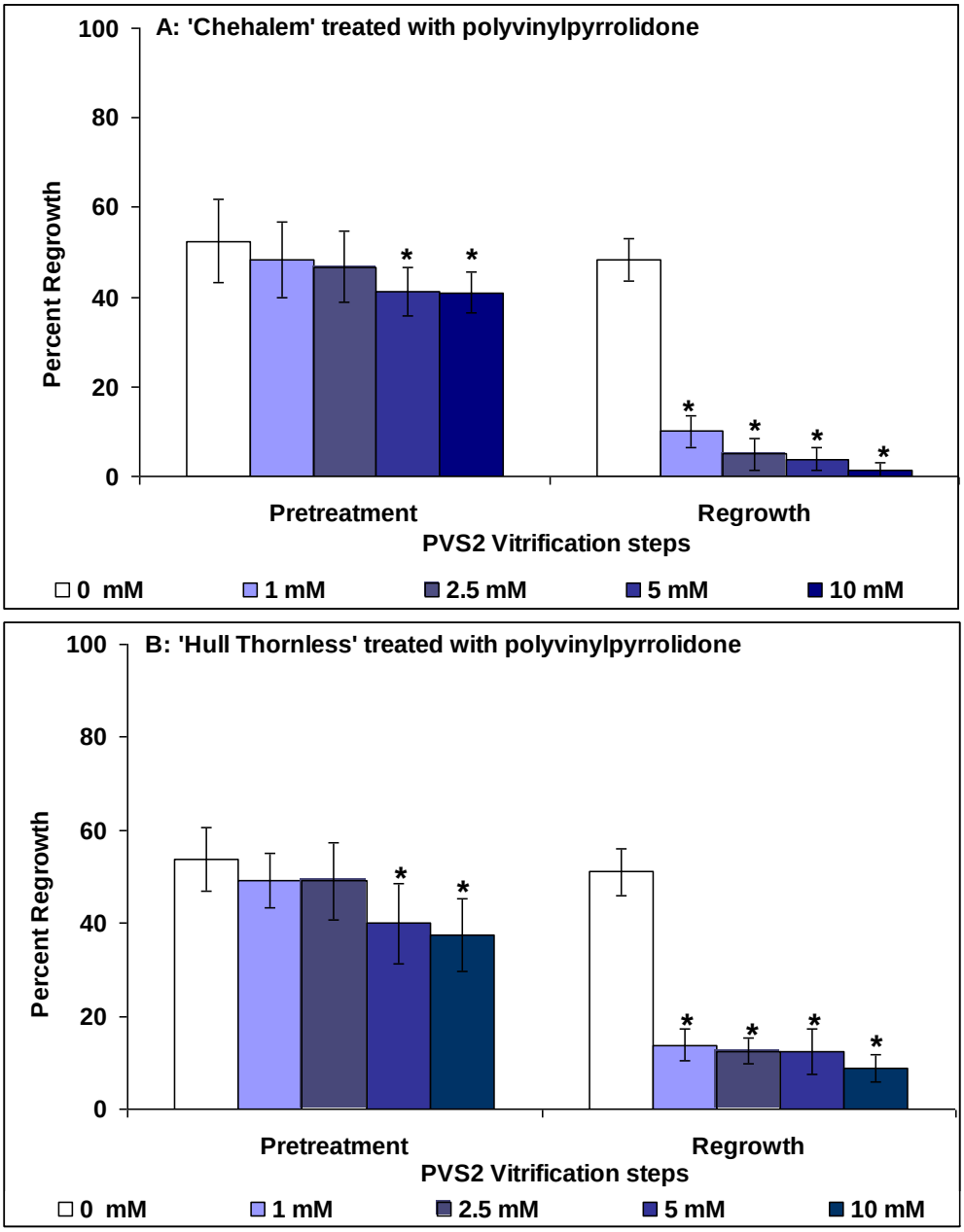

Fig. 5.4: Regrowth of cryopreserved 'Chehalem' (A) and 'Hull Thornless' (B) shoot tips at 4 weeks with and without polyvinylpyrrolidone added at pretreatment and regrowth steps. *Significantly different from the controls, $P \leq 0.05$ (n=60).

Discussion

Improving the regrowth of genetically diverse plants after exposure to LN and improving the efficiency of existing cryopreservation methods would allow storage of a greater range of plant germplasm, improve quality of stored germplasm and guarantee its security over unlimited period of time. In this study, we tested the effects of antioxidant and anti-stress compounds added at four steps in the PVS2 protocol to determine their impact on improving regrowth after exposure to LN. Three of the four compounds significantly increased regrowth of shoot tips after exposure to LN. Our earlier study found that antioxidant vitamins C and E greatly decreased lipid peroxidation in shoot tips and greatly increased regrowth following cryopreservation (Uchendu et al., 2009). The results of the present study suggest that oxidative damage is also ameliorated by LA, GSH, and GB resulting in improved shoot regrowth.

LA protects many cell types against ROS-mediated damage and is readily available because it is very permeable to cellular systems (Packer and Tritschler, 1996). LA quenches ROS and is useful in recycling other antioxidants including ascorbate and glutathione, back into an active form (Packer et al., 1995). A recent plant transformation study showed that LA (5-10 µM) added to the culture medium at the

selection step improved transformation compared to control; and LA (500 µM) was effective during co-culture with agrobacterium (Dan et al., 2009). To the best of our knowledge, there have been no studies of LA with plant cryopreservation. Our data indicate that LA is very effective for improving the regrowth of *Rubus* shoot tips following cryopreservation. Shoot-tip regrowth for both cultivars was best following pretreatment with 4-8 mM LA (~77% average), and significant improvement was also seen at the other steps with 4-6 mM LA (60-73%). The addition of LA (4-8 mM) at the pretreatment step produced the most consistent results for both cultivars (Fig. 5.1). This result may be due to the fact that shoot tips were exposed to pretreatment medium containing LA immediately after excision. A recent study found that during tissue excision ROS production occurs (Roach et al., 2008), thus the immediate availability

of LA at pretreatment might be the reason that a wide range of concentrations are effective for preventing oxidative damage. LA at 10 mM caused a decline in shoot recovery at all steps indicating that this higher concentration was toxic and in some way damaging to the tissues.

Our findings with glutathione confirm that GSH improves plant regrowth following cryopreservation using PVS2-vitrification protocol. Exogenous GSH substantially improved shoot-tip regrowth for both *Rubus* cultivars at pretreatment and also at rinsing and regrowth (Fig. 5.2). Cryopreservation of *Citrus* shoot-tips with GSH by Wang and Deng (2004) showed that regrowth significantly improved when GSH (0.13 mM) was added to the preculture medium and (0.03 mM) in the regrowth medium. The final protocol established was to preculture for 3 days on 40 $mg \bullet l^{-1}$ (0.13 mM) GSH, then add 40 $mg \bullet l^{-1}$ GSH to the loading and PVS2 solutions and plant on 10 $mg \bullet l^{-1}$ (0.03 mM) GSH for regrowth. In the case of *Citrus,* regrowth doubled with the use of GSH in the preculture, loading, cryoprotectant and recovery medium together (average of 78%). For *Rubus* we found that a single exposure to GSH (0.16-0.24) at the pretreatment, rinsing, or regrowth step nearly doubled regrowth (~75-80% average). We did not test GSH on *Rubus* at multiple steps due to the initial excellent recovery percentages at slightly higher concentrations than those used with *Citrus*. Nomura et al. (1998) noted that exposure of shoot tips collected from greenhouse-grown apple root stocks to GSH (0.1 mM) before transfer of shoot tips to *in vitro* culture medium produced 100% regrowth whereas double exposure of shoot tips to GSH (0.1 mM) at pretreatment and culture medium reduced the regrowth from 100 to 70% after 30 days, but this was better than the 60% growth for the control (no GSH). After 120 days, the apple shoot regrowth remained at 100% with a single exposure to GSH but was reduced to 50% with double exposure and was only 40% for the control shoots. Their study suggests that oxidative stress is a major factor affecting regrowth of *in vitro* shoot tips (not exposed to LN) and that single step exposure to GSH was more beneficial for shoot development than exposure at two steps. Our study found that exposure to GSH in a single step significantly increased regrowth of LN-treated

shoot tips. It is likely that the beneficial effect of GSH is due to the protection it provides against oxidative damage (Foyer et al., 1997).

Regrowth increased from ~50% for controls to 75-80% with GB treatments. GB significantly increased regrowth of cryopreserved shoot tips with the addition of 10-15 mM for 'Chehalem' and 10 mM for 'Hull Thornless' at any step (Fig. 5.3). These results indicate that GB effectively protects shoot cultures against abiotic stresses, as was reported by Chen and Murata (2008). Treatments with GB (1 mM) solution on greenhouse grown tomato plants significantly reduced ROS production and improved plant tolerance to chilling stress. Catalase activity was higher in the GB-treated tomato plants compared to the controls and the authors concluded that GB induction of chilling tolerance was possibly due to an enhanced antioxidant response (Park et al., 2006). *In vivo* application of GB (25 mM) to barley plants also significantly improved survival following an osmotic stress imposed by polyethylene glycol (Itai and Paleg, 1982).

PVP had neutral or detrimental effects on regrowth of shoot cultures after cryopreservation. It was especially toxic in the regrowth medium. We observed a dramatic reduction in regrowth with all PVP concentrations compared to controls for both cultivars (Fig. 5.4). These results differ from the results of Wang et al. (2005) who tested the impact of PVP on browning and survival of shoot tips of a raspberry clone cryopreserved by encapsulation-dehydration and encapsulation-vitrification methods. In this study shoot tips in alginate beads were pregrown on medium with PVP (0.01 mM) and produced a 10% increase in survival and a 10% decrease in browning compared to the control. The differences in survival are likely due to the direct contact of shoot tips with PVP medium in our study as well as the higher concentration of PVP in the medium. In the study of Wang et al. (2005) shoot tips were encapsulated in alginate beads and not directly exposed to PVP. PVP was used successfully in several *in vitro* studies to control oxidation of phenolics and improve regeneration of explants (Abdelwahd et al., 2008; Tyagi et al., 1981) but PVP had no positive effect in our experiments, similar to the observation of Prajapati et al. (2003)

who tested PVP (0.3%) on *Curculigo orchioides* Gaertn regeneration and found no improvement with PVP. Lower concentrations of PVP (<1 mM) should be tested to determine if they would be more effective.

The three effective chemicals in this study improved regrowth at all the steps in a similar range (~25%) and are all relatively stable compounds. GSH is the most cost effective of these and is therefore recommended to be added in the pretreatment medium.

Conclusions

This study is the first to use LA and GB in the cryopreservation of plant tissues. In addition we confirmed the efficacy of GSH for shoot tip cryopreservation and determined that PVP was not effective at 1 mM or higher concentrations. Our data defines the effective concentrations of these compounds for shoot tip cryopreservation. Regrowth differences at each step show suitable concentrations and the best places to add antioxidant or anti-stress compounds in order to achieve a substantial increase in regrowth. *Rubus* is a very diverse genus so the responses of the two cultivars tested may not be representative of the entire genus; however it is likely that this range of concentrations would be effective for many species. This study shows that applying antioxidant and anti-stress compounds during the cryopreservation protocol can significantly increase the regrowth of shoot tips. GSH, LA and GB showed great potential for improving recovery by ameliorating oxidative damage incurred during the PVS2 cryopreservation protocol. Future research to screen a wide range of genotypes with the effective ranges of these compounds should make these improvements even more valuable for germplasm storage.

Acknowledgements

This study was funded by CRIS project 5358-21000-03300D USDA-ARS. Thanks to Drs. Tony Chen and Ralph Reed for very useful advice and to the Ford Foundation International Fellowship Program and the University Club of Portland Inc. for awards

to Esther Uchendu. Dr. Magfrat Muminova acknowledges the International Union of Biochemistry and Molecular Biology (IUBMB) for awarding a Wood-Whelan fellowship.

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CHAPTER 6

CONCLUSIONS

Cryopreservation in liquid nitrogen (-320˚F) is an important strategy for conserving plant genetic resources, maintaining large populations of diverse origin or saving plant life over an unlimited period of time. Plants respond differently to cryopreservation protocols depending on a number of factors including the ability to withstand stress conditions. The standard cryopreservation protocols (controlled rate cooling, encapsulation dehydration, and vitrification) can be applied for storing many plant types and groups of related species with little if any modification. Several mint species, blueberry and cranberry cultivars were tested with the three techniques. Mint had high regrowth with all three protocols. The recoveries mostly exceeded the 40% recommended for storage of clonally propagated species. Controlled rate cooling was the most effective for storing the *Mentha* species. Blueberry cultivars could also be stored with any of these protocols. The recovery rate of blueberry shoot tips following each of these protocols was suitable for germplasm storage; however, encapsulation dehydration was the best overall. Cranberry cultivars had poor recovery (< 38 %) with each of these protocols. Cranberry cultivars required additional treatments before they could be cryopreserved with any technique. The reduction of intracellular freezable water is critical to cell survival. A major factor that determines survival of species following cryopreservation procedures appear to be desiccation tolerance. We determined that *in vitro* grown blueberry cultivars were desiccation tolerant, and thus, survived with high rates of regrowth, whereas cranberry cultivars were sensitive to desiccation and had poor survival with each technique. Water deficit imposes stress on plant materials.

Oxidative stress occurs in cells when the antioxidant capacity is sub-optimal. It is likely that multiple stresses occur during cryopreservation procedure such as those caused by water deficit, excision and the activities of reactive species. These stresses and the resulting oxidative damage may be responsible for the reduced viability of tissues after exposure to liquid nitrogen and rewarming. We quantified the degree of

oxidative stress by measuring malondialdehyde (MDA) formation during cryopreservation of blackberry cultivars using the PVS2 vitrification protocol. MDA is a reliable marker for lipid peroxidation. MDA production was 10-20 % higher in cryopreserved shoot tips than in fresh shoot tips unexposed to the cryopreservation procedure. The most MDA was produced at the loading step. This data shows that the most popular cryopreservation protocol (PVS2 vitrification) induces uncontrolled oxidative damage. There are many cryopreservation protocols currently available but none with available data that explores in detail the specific damage imposed on plant cells or tissues by these protocols after they are invented. Vitamin E treatments had a significant effect in elevating shoot tocopherols from their original status and reducing MDA formation to levels similar to the controls. This data provides evidence for the role of antioxidants preventing or repairing oxidative damage in cellular systems under stress conditions. Regrowth following liquid nitrogen exposure was ~30% higher when MDA production was low. Adding antioxidant and anti-stress compounds (vitamin E, vitamin C, glutathione, lipoic acid and glycine betaine) greatly increased regrowth of blackberry cultivars from ~45% for the standard PVS2 vitrification protocol to as high as 92% with vitamin C. Regrowth following liquid nitrogen exposure was best with vitamin C added at any of the steps of the protocol. Vitamin C improved regrowth of blackberry cultivars by ~40% and a cranberry cultivar; ‘Franklin’, by 20-30%. Polyvinylpyrrolidone did not improve regrowth of cryopreserved shoot tips at the concentrations tested although other sources have shown that it useful for increasing regeneration and reducing browning during tissue culturing.

These studies show that stresses resulting from desiccation and oxidative damage are significant problems affecting the recovery of plant germplasm following cryopreservation. Adding antioxidant and anti-stress compounds improves the efficacy of the PVS2 vitrification protocol. Vitamin C was especially effective for improving regrowth of several species. These findings will be useful for improving *in vitro* storage of diverse plant collections as well as making cryopreservation procedures less stressful and more successful for all types of plants.

Further research

Studies that explore new cryoprotective antioxidants, their specific intracellular location after absorption and the mechanism(s) of antioxidant biochemical or physiochemical healing process in plants is needed to expand the body of evidence surrounding the use of exogenous antioxidants during cryopreservation. We observed the effects of exogenous vitamin E on lipid degradation using *Rubus* shoot tips as experimental material. Additional information on exogenous antioxidant effects on other macro molecules including protein and nucleic acids would make our findings even more meaningful. It would also be important to study the effects of antioxidant vitamins and non-vitamins on improving the physiological status i.e. metabolism and morphology of donor plants at the pre-growth stage. Health of parent plants is crucial to survival. It is known that during cold stress, plant synthesize and accumulate antioxidant or anti-stress compounds. There are no reports that show whether feeding the donor plants with antioxidants at the pre-growth stage speeds acclimation to cold tolerance and growth and what, if any, phytochemical relationship or network exists between the natural antioxidants and those exogenously applied. Exogenous anti-stress hormones, for example abscisic acid (ABA), improve cold acclimation of *in vitro* plantlets resulting in increased freezing tolerance; however the effects are genotype dependent. Some studies have shown that oxidative stress occurs during cold acclimation as a result of the formation and activities of reactive oxygen species. The use of antioxidants or other anti-stress compounds during cold acclimation may also produce a good response for improving cryopreservation and may reduce the length of time required for cold acclimation of plants.

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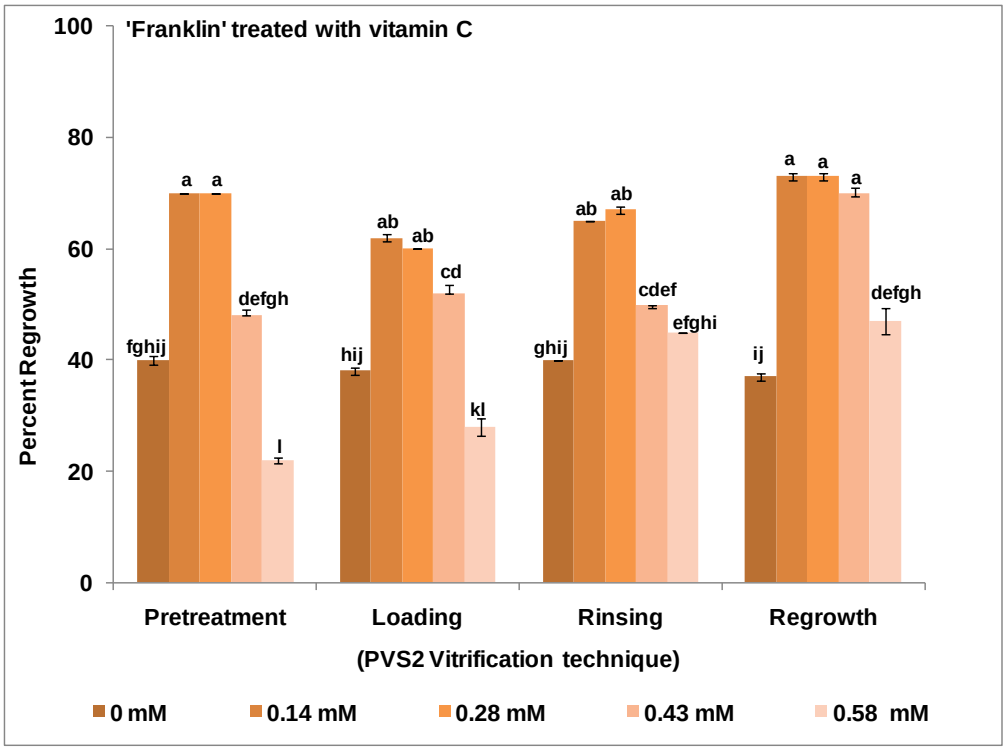
Appendix: Shoot tips of a cranberry cultivar treated with vitamin C



Regrowth of cryopreserved shoot-tips of cranberry cultivar Franklin 6 weeks after LN exposure and rewarming. Treatments are with and without vitamin C at four critical steps of the PVS2 vitrification technique (pretreatment, loading, rinsing, regrowth). Means with the same letter are not significantly different, $P \leq 0.05$ (n=60).