JEAN CARLOS BETTONI

CRIOPRESERVAÇÃO PARA FORMAÇÃO DE BANCO DE SEGURANÇA EM VIDEIRA E CRIOTERAPIA PARA ERRADICAÇÃO DE VÍRUS EM MACIEIRA

Tese apresentada ao Programa de Pós-Graduação em Produção Vegetal do Centro de Ciências Agroveterinárias, da Universidade do Estado de Santa Catarina, como requisito parcial para obtenção do grau de Doutor em Produção Vegetal.

Orientadora: Dra. Aike Anneliese Kretzschmar
Coorientadora: Dra. Gayle Marie Volk

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Lages, 06 de setembro de 2018.

Aos meus pais, Carlos e Ivanir, e a minha companheira, Juliana, por todo amor, compreensão e apoio ao longo dessa jornada.

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“Não vemos as coisas como elas são, mas
como nós somos.”
(**Anaïs Nin**)

RESUMO

BETTONI, Jean Carlos. **Criopreservação para formação de banco de segurança em videira e crioterapia para erradicação de vírus em macieira.** 2018. 195 p. Tese (Doutorado em Produção Vegetal) – Centro de Ciências Agroveterinárias, Universidade do Estado de Santa Catarina. Lages, 2018.

A criopreservação é a preservação de materiais biológicos em temperaturas ultrabaixas (-196 °C); é atualmente o único método seguro para conservação a longo prazo de material vegetal, permite que os recursos vegetais sejam preservados em um espaço reduzido e com mínima manutenção. Técnicas de criopreservação estão disponíveis para diversas espécies de plantas de importância econômica e, cada vez mais, são aplicadas rotineiramente em bancos de germoplasma em todo o mundo. Recentemente, foi observado que a criopreservação iria além da conservação em si, pois tecidos *in vitro* inicialmente infectados por vírus, quando criopreservados, geraram plantas livres de vírus. Desse modo, surgiu à denominação crioterapia, que fornece alta frequência de plantas regenerantes livres de vírus em curto espaço de tempo. A cultura da videira e macieira representam as fruteiras com maior expressão econômica em nível mundial e as doenças de etiologia viral constituem um importante obstáculo para o desenvolvimento e produção dessas espécies frutíferas. Este estudo teve como objetivo estabelecer protocolos práticos e eficientes para a criopreservação de espécies de videira utilizando as técnicas de vitrificação em gotas e V crio-placa e avaliar a efetividade da crioterapia por encapsulamento-desidratação e vitrificação em gotas, baseadas em protocolos de criopreservação, na eliminação de vírus em acessos de macieira. Nos capítulos I e II são apresentados os métodos de criopreservação de vitrificação em gotas e V crio-placa para espécies de videira, utilizando como fonte de explantes meristemas apicais excisados de culturas *in vitro* e, ainda com o método de vitrificação em gotas, no capítulo III é apresentado um protocolo de criopreservação de videira utilizando como fonte de explantes meristemas apicais excisados de plantas mantidas em câmara de crescimento. Os capítulos IV e V demonstram a efetividade das técnicas de encapsulamento-desidratação e vitrificação em gotas para eliminação de espécies virais em macieira. Os resultados encontrados para a criopreservação de espécies de videira demonstram uma ampla aplicabilidade da técnica de vitrificação em gotas com níveis de regeneração de pelo menos 43% para 12 espécies de *Vitis* e em 3 genótipos de videira utilizando meristemas apicais excisados de culturas *in vitro* e plantas *ex vitro*, respectivamente. Além disso, a técnica de criopreservação V crio-placa descrita em dois genótipos de videira foi facilmente executada e resultou em altos níveis de regeneração (≥70%). Nos ensaios de crioterapia, meristemas axilares do porta-enxerto de macieira 'Marubakaido' e da cv.'Monalisa' expostos ao nitrogênio líquido, regenerados e cultivados em estufa por pelo menos 6 meses foram submetidos à análises de PCR. Todas as plantas de ‘Marubakaido’ criopreservadas por encapsulação-desidratação e avaliadas estavam livres de *Apple chlorotic leaf spot virus* (ACLSV) e *Apple stem pitting virus* (ASPV), mas 10% das plantas recuperadas ainda estavam infectadas com *Apple stem grooving virus* (ASGV). Todas as plantas de ‘Monalisa’ criopreservadas por vitrificação em gotas e avaliadas foram livres de ASPV, 95% livres de ACLSV e 35% livres de ASGV. As tecnologias geradas nesse trabalho podem ser utilizadas por instituições especializadas na manutenção e recuperação de materiais com potencial agronômico, beneficiando a cadeia produtiva

da videira e macieira, por meio da conservação de recursos genéticos vegetais e a geração de fontes propagativas de alta qualidade.

Palavras-chave: Conservação de recursos genéticos. Erradicação de vírus. Vitrificação em gotas. V crio-placa. Encapsulamento-desidratação.

ABSTRACT

BETTONI, Jean Carlos. **Cryopreservation for the formation of backup in grapevine and cryotherapy for eradication of virus from apple tree.** 2018. 195 p. Thesis (Doctorate in Plant Production). Center of Agroveterinaries Sciences. Santa Catarina State University. Lages, 2018.

Cryopreservation is the storage of biological materials at ultralow temperatures (-196 ° C), is currently the only safe method for the long-term storage of plant material, allows plant resources to be preserved in a small space and with minimal maintenance. Cryopreservation techniques are available for many plant species of economic importance and are increasingly being routinely applied in genebanks around the world. Recently, cryopreservation was observed beyond of the conservation, since in vitro plant tissues initially virus-infected when cryopreserved, generated virus-free plants. Thus, cryotherapy denomination was created, which provides a high frequency of virus-free plants among regenerants within a short time frame. Grapevine and apple represent the most economically important fruit crops in the world and diseases of viral etiology are major obstacles to the highly cost-effective development and production of these fruit species. This study aimed at establishing of practical and efficient grapevine species cryopreservation protocols using droplet-vitrification and V cryo-plate techniques and to evaluate the effectiveness of cryotherapy by encapsulation-dehydration and droplet-vitrification, based on cryopreservation protocols, in the virus elimination in apple tree accesses. Chapters I and II show droplet-vitrification and V cryo-plate methods for grapevine species cryopreservation, using apical shoot tips excised from *in vitro* cultures as the explants source and, also to droplet-vitrification method, in chapter III, a cryopreservation protocol for grapevine is presented using apical shoot tips excised from growth chamber source plants. Chapters IV and V demonstrate the effectiveness of encapsulation-dehydration and droplet-vitrification techniques to eradicate viral species in apple trees. The results found for grapevine species cryopreservation demonstrate a wide applicability of the droplet-vitrification technique with regrowth levels at least of 43 % in 12 Vitis species and 3 genotypes of grapevine using apical shoot tips derived from *in vitro* and *ex vitro* sources plants, respectively. In addition, the V cryo-plate cryopreservation technique described to two genotypes of grapevine was easily executed and results in high regrowth levels (≥70%). In the cryotherapy studies, axillary shoot tips from apple rootstock ‘Marubakaido’ and the cv. 'Monalisa' were exposed to liquid nitrogen, regrowth, and growth in the greenhouse for at least 6 months were submitted to the PCR analysis. All of the evaluated cryopreserved Marubakaido plants by encapsulation-dehydration were free of *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem pitting virus* (ASPV), but 10% recovered plants were still infected with *Apple stem grooving virus* (ASGV). All of the evaluated cryopreserved Monalisa plants by droplet-vitrification that were tested were free of ASPV, 95% were free of ACLSV and 35% were free of ASGV. The technologies generated in this thesis can be used by institutions specialized in the maintenance and recovery of plant materials with agronomic potential, benefiting grapevine and apple production chain, by the conservation of plant genetic resources and to generate the high-quality plant material.

Keywords: Genetic resources conservation.Viruses eradication. Droplet-vitrification. V cryo-plate. Encapsulation-dehydration.

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LISTA DE ABREVIATURAS E SIGLAS

ACLSV	*Apple chlorotic leaf spot virus*
AIA	Indol-3-acético
ApMV	*Apple mosaic virus*
ASGV	*Apple stem grooving virus*
ASPV	*Apple stem pitting virus*
BA	6-Benzilaminopurina
BM	Meio basal
$CaCl_2$	Cloreto de cálcio
cv.	Cultivar
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra acético
EG	Etileno glicol
HCl	Ácido clorídrico
-LN	Não exposto em nitrogênio líquido
+LN	Tratado em nitrogênio líquido
MS	Meio de cultura Murashige e Skoog (1962)
NH_4	Amônio
NL	Nitrogênio líquido
PCR	Reação em cadeia da polimerase
pH	Potencial higrogeniônico
PPM	*Plant Preservative Mixture*
PVS2	Solução de vitrificação de plantas número 2
PVS3	Solução de vitrificação de plantas número 3
RNA	Ácido ribonucleico
USDA-ARS	Agricultural Research Service of the United States Department of Agriculture
VNL	Vapor de nitrogênio líquido

LISTA DE SÍMBOLOS

%	Porcentagem
ºC	Graus Celsius
±	Margem de erro
≥	Maior ou igual
≤	Menor ou igual
1/2X	Metade de algo
° Brix	Graus brix
h	Hora
min	Minuto
L	Litro
cm	Centímetro
mm	Milímetro
ºC dia^{-1}	Graus por dia
ºC h^{-1}	Graus por hora
mL	Mililitro
mg L^{-1}	Miligrama por litro
µL	Microlitro
U/µL	Unidade por microlitro
µm	Micrometro
g L^{-1}	Grama por litro
h light day^{-1}	Horas de luz por dia
M	Molar
mM	Milimolar
µM	Micromol
µM m^{-2} s^{-1}	Micromol por metro quadrado por segundo
v/v	Volume por volume
w/v	Peso por volume

SUMÁRIO

1 INTRODUÇÃO GERAL

As cadeias produtivas da videira e da macieira possuem inserção destacada no cenário mundial da fruticultura e estão entre as mais importantes fruteiras de clima temperado cultivadas e consumidas no mundo (OIV, 2017; VOLK et al., 2015). Ainda que o cultivo comercial se concentre em algumas espécies de *Vitis* e *Malus*, a manutenção das demais espécies e materiais selvagens é essencial para fornecer a variabilidade genética necessária aos programas de melhoramento focados no desenvolvimento de novos cultivares e porta-enxertos tolerantes/resistentes a pragas e doenças e adaptados às diversas e variáveis condições ambientais (WANG et al., 2018a; PERTOT et al., 2017; SMITH et al., 2016). Portanto, a disponibilidade e o fácil acesso a um determinado conjunto de recursos genéticos de videira e macieira é determinante para o sucesso de futuros requerimentos de programas de melhoramento que ainda são desconhecidos.

Tradicionalmente as coleções de germoplasma de *Vitis* e *Malus* são mantidas em repositórios a campo. Entretanto, a manutenção de plantas no campo é dispendiosa, requer extensiva área plantada, além da vulnerabilidade a estresses bióticos e abióticos (PATHIRANA et al., 2016). Duplicatas de segurança *in vitro* representam uma alternativa para a conservação de germoplasma vegetal a médio prazo (MAIA et al., 2015; DOBRÁNSZKI; DA SILVA, 2010). A manutenção de bancos *in vitro* requer frequentes manuseios de culturas, o que resulta em uma elevação de custos, risco de perda do acesso vegetal e principalmente apresenta potencial para gerar variação somaclonal (MATHEW et al., 2018; SOUZA et al., 2015).

A criopreservação de plantas que consiste no armazenamento de material biológico em nitrogênio líquido (-196 °C) ou vapor de nitrogênio líquido (-160 ºC para -196 ºC), é uma alternativa ou um método complementar para os tradicionais métodos de conservação de germoplasma a campo e *in vitro* e permite que os recursos vegetais sejam preservados de forma segura, em um espaço reduzido e com mínima manutenção (SOUZA et al., 2018; POPOVA et al., 2015; ENGELMANN, 2011; BENSON, 2008). Nas últimas décadas estudos de criopreservação têm evoluído em vários gêneros de plantas e, atualmente, representa o único método seguro para conservação a longo prazo de material vegetal, principalmente pela manutenção da integridade genética de materiais criopreservados (WANG et al., 2018a; BI et al., 2017; REED et al., 2017).

Recentemente foi comprovado que a criopreservação iria além da conservação de material biológico, já que, tecidos *in vitro* inicialmente infectados por vírus, quando criopreservados, regeneraram plantas sadias. Desse modo, surgiu a denominação crioterapia, como uma estratégia que pode fornecer em curto espaço de tempo uma alta frequência de plantas ivres de vírus (BETTONI et al., 2016; VIEIRA et al., 2015). A erradicação de vírus é baseada na distribuição desigual de vírus nas plantas. Após a exposição do material biológico ao nitrogênio líquido, ocorre a sobrevivência de grupos de células localizados na região do domo meristemático e na base dos primórdios foliares (WANG; VALKONEN, 2009a). Os vírus, em teoria, não conseguem infectar essas regiões sobreviventes, pois se movimentam na planta através do sistema vascular; por outro lado, as outras células e tecidos mais distantes do meristema, nos quais os vírus podem estar presentes, são mortas pelo efeito letal da temperatura ultrabaixa (WANG et al. 2009; LI, HARTUNG; LEVY, 2006).

Infecções virais interferem no funcionamento celular comprometendo rotas bioquímicas e fisiológicas da célula vegetal. Os vírus utilizam constituintes químicos das células do hospedeiro para replicação e movimento, resultando em distintas desordens na célula vegetal, como alterações na expressão gênica e acúmulo de proteínas, alterações hormonais e na atividade fotossintética (HULL, 2001). Fruteiras de clima temperado, tais como a macieira e a videira, são afetadas por uma série de espécies virais. Em virtude das interferências celulares apontadas acima, as fruteiras infectadas por vírus apresentam menor vigor, redução da produtividade e qualidade dos frutos, bem como uma maior suscetibilidade a outros agentes fitopatogênicos (KUMAR et al., 2014; GUERRA et al., 2012; HADIDI; BARBA, 2011; CIEŚLIŃSKA; RUTKOWSKI, 2008; NICKEL et al., 2001). Uma vez que o plantio seja realizado com material propagativo contaminado é impossível tomar medidas de controle.

O desenvolvimento de metodologias práticas e confiáveis de criopreservação que resultem em altos níveis de viabilidade (≥40%) após a exposição ao nitrogênio líquido, é a chave para o desenvolvimento de coleções de plantas criopreservadas (VOLK et al., 2016).

O acesso e a transferência de métodos confiáveis de criopreservação entre laboratórios é importante para o uso generalizado dessas ferramentas biotecnológicas (REED et al., 2004). Isso torna o procedimento de criopreservação um candidato para uso em pesquisas de crioterapia, na qual os patógenos são eliminados de materiais vegetais clonais infectados como resultado da exposição ao nitrogênio líquido

(BETTONI et al., 2018b; BETTONI et al., 2016; WANG et al., 2014). A implementação do protocolo de criopreservação aplicável a material vegetal com uma ampla diversidade genética pode facilitar o uso de métodos de crioterapia na erradicação de vírus (BETTONI et al., 2018b; BETTONI et al., 2016; PATHIRANA et al., 2015; BAYATI, SHAMS-BAKHSH; MOIENI, 2011; WANG; VALKONEN, 2009a).

A disponibilidade de materiais livres de agentes patogênicos é um fator limitante para a sustentabilidade e a longevidade na atividade agrícola, principalmente em espécies de plantas propagadas vegetativamente, como a videira e a macieira. O agravante é que a disseminação e o acúmulo viral são beneficiados pelo emprego de material propagativo infectado, que contribui para dispersão e contaminação em novas áreas. Portanto, materiais vegetais para formação de jardins clonais e plantas matrizes, que são fornecedoras de explantes, devem estar isentos de microorganismos patogênicos, especialmente os de etiologia viral (WANG et al., 2014).

A baixa oferta de mudas de alta qualidade fitossanitária contribui de forma direta para agravar ainda mais essa situação, já que por vezes, se constata materiais sendo comercializados sem garantia de qualidade e procedência. Diante desse preocupante cenário, torna-se necessário identificar e implementar métodos funcionais e robustos, a fim de eliminar microorganismos, usando protocolos eficientes, capazes de gerar materiais em quantidade e qualidade para o fomento dos sistemas agrícolas (PATHIRANA et al., 2015, 2013). Metodologias adequadas podem facilitar a adoção de medidas regulamentadoras rigorosas a viveiros em relação à manutenção, propagação e comercialização de materiais vegetais.

Nesse sentido, a crioterapia é um novo método que tem se mostrado eficiente na erradicação de vírus em diversas espécies de importância econômica; possui potencial para se tornar uma técnica emergente devido à ampla gama de protocolos de criopreservação existentes, associado com o baixo custo operacional e o desenvolvimento de plantas livres de vírus de forma rápida e com uma taxa elevada. Em adicional, a criopreservação é atualmente o único método seguro para conservação a longo prazo de material vegetal, principalmente pela manutenção da integridade genética de materiais criopreservados. Tais características tornam os métodos muito atraentes para as instituições que visam recuperação, manutenção e multiplicação de plantas base de alta qualidade fitossanitária (BETTONI et al., 2016).

Mesmo com a aplicabilidade da criopreservação em diversas espécies de importância econômica e resultados positivos da crioterapia para eliminação de espécies virais, desafios ainda permanecem, principalmente os relacionados à repetibilidade dos resultados de protocolos publicados. Diante dessas limitações que ainda perduram, é importante aprimorar os conhecimentos de aspectos relacionados à criopreservação/crioterapia de espécies e cultivares de plantas; isso significa o mapeamento de cada fase que envolve os protocolos.

Com base neste contexto, objetivou-se estabelecer protocolos práticos e eficientes para a criopreservação de espécies de videira, utilizando as técnicas de vitrificação em gotas e V crio-placa, assim como, avaliar o potencial de técnicas de encapsulamento-desidratação e vitrificação em gotas, para eliminação de vírus em acessos de macieira.

Este trabalho de tese foi estruturado no formato de capítulos, composto de uma revisão bibliográfica atualizada apoiada em pesquisas realizadas até o presente, seguido por cinco capítulos que abordam técnicas para a criopreservação e crioterapia de videira e macieira, respectivamente, que serão descritos a seguir.

O capítulo I apresenta um protocolo de criopreservação de videira baseado na técnica de vitrificação em gotas utilizando meristemas apicais excisados de plantas *in* vitro. Os ensaios resultaram em um protocolo aplicável para 12 espécies de videira e replicado com sucesso por outro técnico.

No capítulo II foi determinado o efeito do tamanho do explante para o protocolo de criopreservação de videira, baseado na técnica de vitrificação em gotas, utilizando meristemas apicais excisados de plantas *in vitro*. Ainda neste capítulo, é apresentado resultado inédito do uso do método V crio-placa para a criopreservação da videira.

O capítulo III apresenta um novo protocolo de criopreservação de videira baseado na técnica de vitrificação em gotas utilizando meristemas apicais isolados de plantas mantidas em câmara de crescimento.

No capítulo IV é demonstrado a efetividade da técnica de encapsulamento-desidratação para a erradicação de espécies de vírus em macieira, a partir de meristemas axilares excisados de culturas *in vitro* de porta-enxerto de macieira 'Marubakaido'. Esse estudo é o primeiro relato no Brasil da aplicação da técnica de crioterapia a partir do método de criopreservação por encapsulamento-desidratação para erradicação de vírus em macieira.

No capítulo V é demonstrado a efetividade da técnica de vitrificação em gotas para a erradicação de espécies de vírus em macieira, a partir de meristemas axilares excisados de culturas *in vitro* de cultivar de macieira 'Monalisa'. Esse estudo é o primeiro relato no Brasil da aplicação da técnica de crioterapia por meio do método de criopreservação por vitrificação em gotas para erradicação de vírus em macieira.

2 REVISÃO DA LITERATURA

2.1 CONSERVAÇÃO DE RECURSOS GENÉTICOS VEGETAIS

Existem duas estratégias complementares para a conservação de recursos genéticos vegetais, nomeadas como conservação *in situ* e conservação *ex situ* (DULLOO, HUNTER; BORELLI, 2010). A conservação *in situ* consiste na manutenção do recurso vegetal no ambiente natural de origem, preservando a organização do ecossistema. Por outro lado, a conservação *ex situ* é a manutenção do recurso genético fora do ambiente natural e geralmente é utilizada para proteger coleções de plantas de uma potencial ameaça de perda para futura utilização (BOROKINI, 2013; RAO, 2004).

Nas últimas décadas esforços consideráveis foram realizados em todo o mundo para implementação de bancos de germoplasma (*ex situ*) com o objetivo principal de conservar recursos genéticos fundamentais para a segurança alimentar e tentar minimizar os efeitos da antropização e da erosão genética (HALEWOOD et al., 2018; VOLK et al., 2015).

Tradicionalmente, os bancos de germoplasma de espécies propagadas vegetativamente, incluindo a videira e a macieira, são conservados *ex situ* como plantas mantidas em coleções de campo. Esses acessos estão disponíveis para fins de avaliação, caracterização, melhoramento, propagação e distribuição; no entanto, a manutenção de coleções de plantas no campo é dispendiosa e também vulnerável à pragas e doenças, além de desastres ambientais, que podem colocar em risco as coleções (HÖFER; HANKE, 2017; PATHIRANA et al., 2016; NIINO; ARIZAGA, 2015; POPOVA et al., 2015; SOUZA et al., 2015; MARKOVIĆ et al., 2013a; CARIMI, PATHIRANA; CARRA, 2011). Duplicatas de segurança *in vitro*, em condições normais ou em crescimento lento, têm sido utilizados para preservação a médio prazo de germoplasma de videira (MAIA et al., 2015; POSTMAN et al., 2006) e macieira (DOBRÁNSZKI e DA SILVA, 2010; FORSLINE et al., 2010). As plantas cultivadas *in vitro* são mantidas em ambiente estéril, livres de patógenos e técnicas de cultura de tecidos permitem a rápida multiplicação clonal em um espaço reduzido (BETTONI et al., 2015a; DAL BOSCO et al., 2015). Quando comparado com coleções a campo, duplicatas de segurança *in vitro* podem facilitar a troca de materiais entre instituições de forma mais segura. Entretanto apresentam algumas limitações, como a

manutenção frequente das culturas e um custo alto, além do risco de contaminação por falhas técnicas e pode ocorrer variação somaclonal durante os frequentes sub-cultivos (MATHEW et al., 2018; WANG et al., 2014; HASSAN; HAGGAG, 2013).

Neste sentido, outras estratégias de conservação *ex situ* para os recursos genéticos de plantas devem ser consideradas que, além da manutenção do material vegetal por longos períodos de tempo, preservem a estabilidade genética durante o armazenamento (HALMAGYI et al., 2010).

2.2 CRIOPRESERVAÇÃO

A criopreservação, que consiste na conservação de material biológico em nitrogênio líquido (NL, -196 ºC) ou vapor de nitrogênio líquido (VNL, -160 ºC para -196 ºC), é atualmente o único método seguro para conservação a longo prazo de material vegetal, principalmente pela manutenção da integridade genética de materiais criopreservados. Nessas condições, tecidos armazenados em NL ou VNL ficam preservados sem sofrer divisões celulares e os processos metabólicos são praticamente paralisados. Adicionalmente, problemas como contaminação e perda de material genético são superados, além de necessitar pequeno espaço para conservação de um grande número de acessos com mínima manutenção (WANG et al., 2018a; SOUZA et al., 2018; BI et al., 2017; REED et al., 2017; MARKOVIĆ et al., 2014; ENGELMANN, 2011; BENSON, 2008).

Técnicas de criopreservação vêm sendo utilizadas em diversas espécies de importância econômica, incluindo frutíferas, dentre as quais estão a videira (PATHIRANA et al., 2016; MARKOVIĆ et al., 2013a) e a macieira (LI et al., 2015; FENG et al., 2013; CONDELLO et al., 2011). Como fonte de material biológico para a criopreservação podem ser utilizadas células, tecidos, órgãos e organismos viáveis (REED, 2017); porém, tecidos organizados, como meristemas ou gemas dormentes, são preferíveis principalmente quando o objetivo é manter as características da planta matriz (BI et al., 2017; WANG et al., 2014).

Os primeiros relatos da aplicação de técnicas de criopreservação em brotos de macieira foram na década de 1980, no cv. Jonathan (KUO e LINEBERGER, 1985). Desde então vários protocolos *in vitro* foram desenvolvidos, como encapsulamento-desidratação (NIINO; SAKAI, 1992a; WU et al., 1999; PAUL et al., 2000; FENG et al., 2013; LI et al., 2014; FENG et al., 2014), vitrificação (NIINO et al., 1992b; WU et al.,

1999; KUSHNARENKO; ROMADANOVA; REED, 2009); encapsulamento-vitrificação (PAUL et al., 2000) e mais recentemente vitrificação em gotas (HALMAGYI et al., 2010; HALMAGYI; DELIU; ISAC, 2010; CONDELLO et al., 2011; FENG et al., 2014; LI et al., 2015).

Atualmente na Unidade de Pesquisa e Preservação de Germoplasma de Plantas do Departamento de Agricultura dos Estados Unidos (National Center for Genetic Resources and Preservation – NCGRP-USDA) em Fort Collins, Colorado, existe um criobanco de germoplasma de *Malus*, onde 2.335 clones são mantidos por meio da técnica de criopreservação de gemas dormentes (VOLK et al., 2015). Nesse caso, o material vegetal utilizado é coletado diretamente no campo, em período de dormência, sendo as gemas desidratadas para um conteúdo de umidade que varia de 25-30 % que em seguida são resfriadas em congelador programável, em taxas de resfriamento de -1 ºC h^{-1} ou -5 ºC dia^{-1} até atingir -30 ºC, então são armazenadas em NL ou VNL. No momento da regeneração as gemas são reidratadas e diretamente enxertadas em porta-enxerto (WANG et al., 2018a; VOLK; JENDEREK; CHAO, 2017a, 2008; TOWILL et al., 2004).

No entanto, a técnica de criopreservação de gemas dormentes é adequada somente para espécies que apresentam dormência. Gemas dormentes criopreservadas apresentam maior velocidade de recuperação e menor custo quando comparado com técnicas de criopreservação que utilizam como fonte de explantes material *in vitro* (JENDEREK; REED, 2017; LAMBARDI et al., 2011). Dessa forma, é evidente que a escolha da técnica de criopreservação deve estar estreitamente relacionada com o objetivo do trabalho de criopreservação.

A macieira é uma das culturas mais estudadas em relação à criopreservação; nas últimas décadas as pesquisas estão focadas em procedimentos de criopreservação que tenham um alcance amplo para várias espécies (WANG et al., 2018a; BENELLI; DE CARLO; ENGELMANN, 2013). Ao longo desses anos, protocolos eficientes de criopreservação de macieira foram elaborados com sucesso, utilizando pólen (XU et al., 2014; VOLK, 2011), sementes (MICHALAK; PLITTA-MICHALAK; CHMIELARZ, 2015), gemas dormentes (VOLK; JENDEREK; CHAO, 2017a) e explantes *in vitro* (LI et al., 2015; FENG et al., 2014); atualmente grandes criobancos de *Malus* estão estabelecidos (WANG et al., 2018a).

Para a videira, os primeiros relatos da aplicação da criopreservação em explantes (meristemas axilares) foram na década de 1990, utilizando a técnica de

encapsulamento-desidratação, e nos anos seguintes outros trabalhos foram desenvolvidos com a mesma técnica (PLESSIS; LEDDET; DEREUDDRE, 1991,1993; WANG et al., 2000; ZHAO et al., 2001; WANG et al., 2003; BAYATI; SHAMS-BAKHSH; MOIENI, 2011; MARKOVIĆ et al., 2013a). Outros protocolos ou técnicas também foram investigados: vitrificação (MATSUMOTO; SAKAI, 2003; WANG et al., 2003; GANINO et al., 2012; BETTONI et al. 2015a), encapsulamento- vitrificação (WANG et al., 2004; GRIBAUDO et al., 2012) e mais recentemente vitrificação em gotas (MARKOVIĆ et al., 2013a; PATHIRANA et al. 2015; 2016; BETTONI et al., 2018a).

Para a videira, resultados limitados foram obtidos por meio da criopreservação de gemas dormentes. Há apenas um registro que foi publicado quase 30 anos atrás, que demonstra a baixa viabilidade de espécies de *Vitis* após a criopreservação (ESENSEE; STUSHNOFF, 1990. Os resultados com técnicas de criopreservação em *Vitis* têm sido mais promissores quando são utilizados explantes *in vitro*. Apesar dos vários protocolos de criopreservação de videira disponíveis, criobancos de *Vitis* spp. não têm sido implementados. Algumas publicações demonstram baixos níveis de regeneração após a exposição em NL (GANINO et al., 2012; BENELLI; LAMBARDI; Fabbri, 2003) e, em grande parte destas, os pesquisadores se concentraram no desenvolvimento de procedimentos usando um número limitado de espécies (BI et al., 2017; BETTONI et al., 2016). Associado a tudo isso, está o principal fator que ainda limita a implementação de criobancos de videira: a especificidade de genótipos para um determinado protocolo (BI et al., 2017; PATHIRANA et al., 2016; BENELLI; DE CARLO; ENGELMANN, 2013; BENSON, 2008). Assim, pesquisas futuras devem focar no desenvolvimento de protocolos que sejam aplicáveis para várias espécies visando facilitar a criação de criobancos de videira.

2.2.1 Fatores-chave básicos que afetam a eficiência de procedimentos de criopreservação

Nas últimas décadas, pesquisadores de todo o mundo buscam estratégias para otimizar os métodos de criopreservação. Dentre os fatores que são entendidos como determinantes para o sucesso de um protocolo de criopreservação está a qualidade do material que será submetido ao procedimento criogênico; portanto, o estado

fisiológico adequado é um pré-requisito de plantas que serão doadoras de explantes (LI et al., 2017; MARKOVIĆ et al., 2014; ELGELMANN, 2011; SAKAI; ENGELMANN, 2007; REED, 2001).

Durante a criopreservação, os meristemas são submetidos a uma série de etapas como excisão, lesão osmótica, dessecação e tratamento a ultrabaixa temperatura; esses procedimentos impõem estresse ao material vegetal, devido à produção de espécies reativas de oxigênio que causam danos oxidativos e podem tornar o maquinário celular inviável (MATHEW et al., 2018; GILL; TUTEJA, 2010; UCHENDU et al., 2010a). Uma abordagem recente que vem sendo estudada é adição de antioxidantes em meios de cultura onde são cultivadas as plantas doadoras de explantes (PATHIRANA et al., 2016; REN et al., 2014) e em meios de cultura durante as etapas da criopreservação (BETTONI et al., 2018a). O efeito positivo da adição de agentes antioxidantes ou compostos anti-estresse tem sido observado na melhoria do crescimento e desenvolvimento das plantas de videira (VOLK; SHEPHERD; BONNART, 2018; BETTONI et al., 2018a; PATHIRANA et al., 2016; SHEPHERD et al., 2013), kiwi (MATHEW et al., 2018), amora (UCHENDU et al., 2010ab) e framboesa (WANG; VALKONEN, 2009a), após a exposição ao NL; dessa forma, esses componentes possivelmente estarão presentes em pesquisas futuras com criopreservação.

O sucesso de protocolos de criopreservação está ligado principalmente ao status hídrico que o material biológico apresenta antes do tratamento em NL (BETTONI et al., 2016). Alguns materiais, como sementes ortodoxas, gemas dormentes e pólen, passam por um processo de desidratação natural no ambiente e, dessa forma, são passíveis de criopreservação sem nenhum pré-tratamento; porém, a maioria dos materiais biológicos que são submentidos à criopreservação possuem alto conteúdo de água, o que os torna extremamente sensíveis ao congelamento (ENGELMANN, 2011). Dessa forma, duas principais estratégias de criopreservação para tecidos de plantas são comumente utilizadas: adição de soluções vitrificantes, como solução de vitrificação de planta 2 (PVS2) (SAKAI; KOBAYASHI; OIYAMA, 1990), que fazem com que a água livre presente nos tecidos sofra uma transição da fase líquida para um estado vítreo, evitando a formação de cristais de gelo quando o material é exposto ao NL (FAHY et al., 1984); e remoção de parte da água das células por meio da desidratação de explantes encapsulados ao ar em câmara de fluxo laminar ou em sílica gel (LI et al., 2014; MARKOVIĆ et al., 2013a; FENG et al., 2013;

WANG et al., 2003; WANG et al., 2000). Ambas as abordagens têm o mesmo objetivo de reduzir ou impedir a formação de cristais de gelo nos tecidos (ROMADANOVA; KUSHNARENKO; KARASHOLAKOVA, 2017). Dessa forma, o maior desafio é encontrar um equilíbrio na desidratação, de modo que não ocorram danos nos tecidos promovidos por uma retirada excessiva da água ou efeitos deletérios causados pela formação de cristais de gelo no interior da célula durante o congelamento, que causam ruptura do sistema de membranas celulares e assim colapso e morte de células (MATSUMOTO, 2017a; VOLK, 2010; WANG et al., 2009) ou toxicidade causado pela exposição excessiva à soluções vitrificantes (GANINO et al., 2012; SAKAI et al., 2000).

Além do status fisiológico das plantas doadoras de explantes, adição de antioxidantes, status hídrico do material biológico, o funcionamento de protocolos de criopreservação depende também da definição de meio adequaldo de regeneração (BI et al., 2017; BETTONI et al., 2016). Dessa forma, é imprescindível que, anterior à aplicação de técnicas de criopreservação a rota de multiplicação do material esteja estabelecida, de modo que não ocorram problemas de regeneração resultantes da utilização de um protocolo de regeneração inadequado. O uso de reguladores de crescimento em meios de regeneração, mesmo quando utilizado em pequenas concentrações, tem-se mostrado fundamental; a adição de citocininas em combinação ou não com auxinas teve efeito positivo no desenvolvimento de explantes de várias espécies de plantas, dentre elas a videira (MARCOVIĆ et al., 2014; WANG et al., 2003a) e macieira (FENG et al., 2014; LI et al., 2014). No entanto, quando esses reguladores de crescimento são utilizados em concentrações inadequadas podem estimular o desenvolvimento de calos (WANG et al., 2000; LAMBARDI; FABBRI; CACCAVALE, 2000). A capacidade de regeneração das culturas por meio de crescimento direto de estrutura diferenciada, sem o desenvolvimento de calo, é fundamental para evitar problemas de variação somaclonal, principalmente quando se utilizam estruturas diferenciadas como fonte de explante (MATSUMOTO, 2017; CHAROENSUB; HIRAI; SAKAI, 2004; HARDING, 2004). Portanto, é evidente que ensaios de criopreservação bem sucedidos são dependentes de protocolos de cultura de tecidos bem estabelecidos; assim, qualquer espécie que possui um protocolo de cultura de tecidos funcional pode ser passível de criopreservação (ENGELMANN, 2011; 2014).

2.2.2 Principais técnicas de criopreservação utilizando tecidos meristemáticos como fonte de explantes

Métodos de criopreservação confiáveis que resultem em níveis altos de regeneração, ou seja, ≥40% na pós-criopreservação, e técnicos qualificados são fundamentais para o desenvolvimento de coleções de genes bem sucedidas (VOLK et al., 2016). Corroborando a isto, o acesso à essas metodologias de criopreservação entre laboratórios irá proporcionar o uso generalizado dessas tecnologias (REED et al., 2004).

Métodos bem sucedidos de criopreservação são dependentes de explantes de qualidade, provenientes na maioria das vezes de plantas matrizes cultivadas *in vitro* e que tolerem os tratamentos que removem água livre das células por desidratação ou por tratamento com soluções de vitrificação (VOLK; SHEPHERD; BONNART, 2018). Atualmente, para a videira e a macieira, esses objetivos são alcançados utilizando os métodos baseados em encapsulamento-desidratação (WANG et al., 2018a; BETTONI et al., 2018b; BI et al., 2017; BETTONI et al., 2016; LI et al., 2014; 2015; FENG et al., 2014; 2013); e vitrificação (WANG et al., 2018a; BI et al., 2017; VOLK et al., 2018; BETTONI et al., 2018a; PATHIRANA et al., 2016; BETTONI et al., 2016; LI et al., 2015; FENG et al, 2014; MATSUMOTO; SAKAI, 2003).

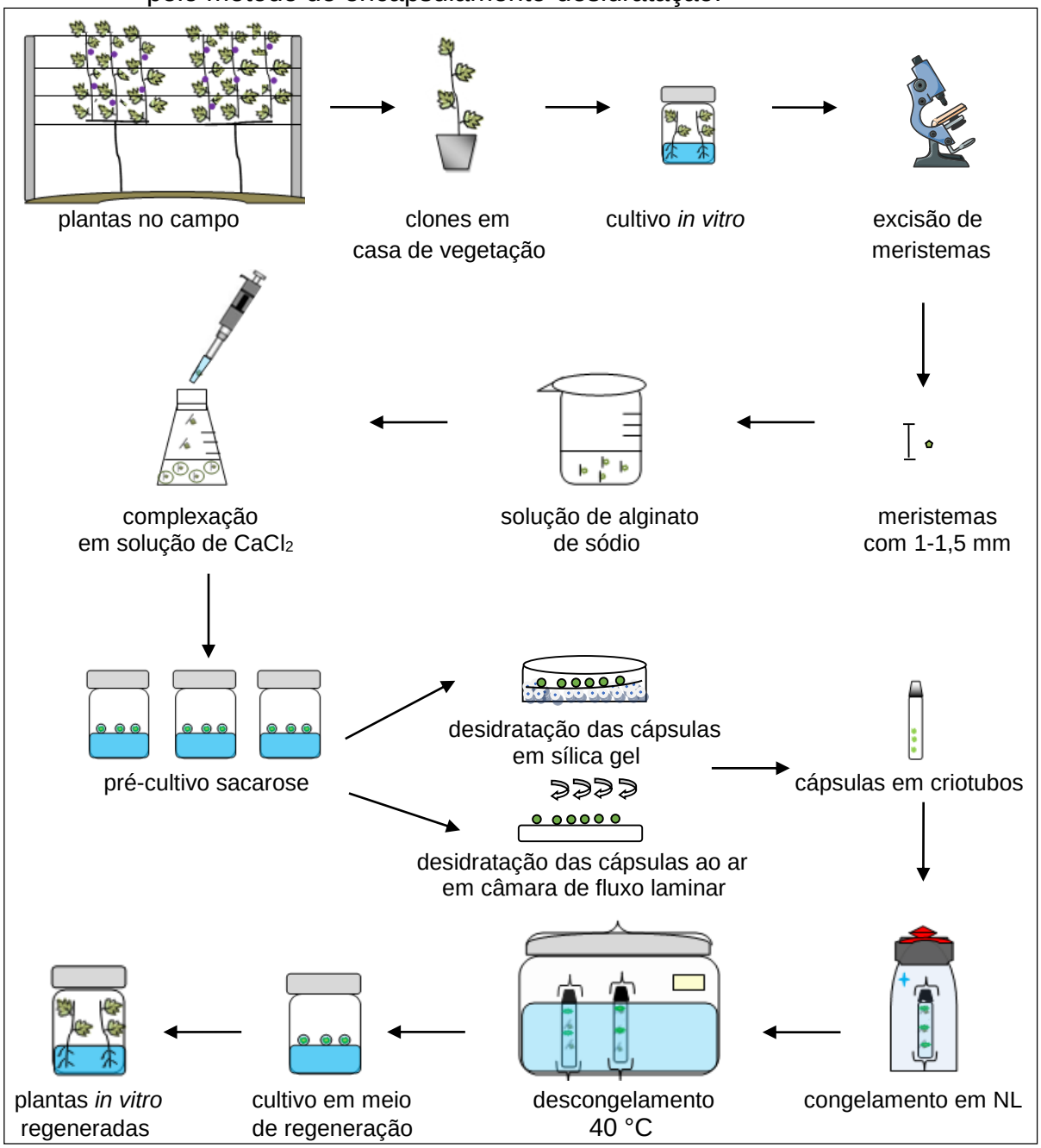
2.2.2.1 Método de encapsulamento-desidratação

A criopreservação pelo método de encapsulamento-desidratação é baseada em técnica empregada na produção de sementes sintéticas (BENELLI; DE CARLO; ENGELMANN, 2013). Protocolos de encapsulamento-desidratação envolvem as seguintes etapas: excisão dos meristemas das culturas *in vitro*; encapsulamento; pré-cultivo em concentrações fixas ou crescentes de sacarose; desidratação; congelamento a -196 °C; descongelamento em banho-maria a 40 °C e cultivo em meio de regeneração (Figura 1) (BETTONI et al., 2016).

Após a excisão dos meristemas das culturas *in vitro*, o método consiste na formação de um endosperma artificial em torno dos ápices meristemáticos, composto pelo agente geleificante alginato de sódio (3 %), que quando em contato com solução

de cloreto de cálcio polimeriza, produzindo pequenas pérolas (GONZÁLEZ-BENITO; CLAVERO-RAMÍREZ; LÓPES-ARANDA, 2004; FENG et al., 2013).

Figura 1 – Representação esquemática do protocolo de criopreservação de plantas pelo método de encapsulamento-desidratação.



Fonte: Elaborado pelo autor, 2018.

Na videira, em sequência, as cápsulas são pré-cultivadas em meios de cultura com concentrações crescentes de sacarose, que variam de 0,25, 0,50, 0,75 e 1,0 M por quatro dias, uma concentração por dia, ou concentrações fixas de sacarose (WANG et al., 2003; MARKOVIĆ et al., 2013a). Para a macieira, os meristemas são

pré-cultivados em 0,5 M de sacarose por 7 dias (LI et al., 2014). O pré-cultivo é fundamental para os explantes encapsulados resistirem à desidratação e ao congelamento. A técnica de encapsulamento permite com que as altas concentrações de sacarose não causem lesões nos tecidos (ENGELMANN et al., 2008), que seriam altamente prejudiciais ou mesmo letais para explantes não encapsulados (MATSUMOTO; SAKAI, 2003).

Após o pré-cultivo, as cápsulas são parcialmente desidratadas em corrente de ar, em uma câmara de fluxo laminar (FENG et al., 2013), ou em recipientes com sílica gel (MARKOVIĆ et al., 2013a). Após a desidratação, as cápsulas são transferidas para criotubos estéreis para tratamento no NL; quando necessário são descongeladas em banho-maria por 3 min a 40 °C e inoculadas em meio de cultura de regeneração (WANG et al., 2003).

2.2.2.2 Método de vitrificação em gotas

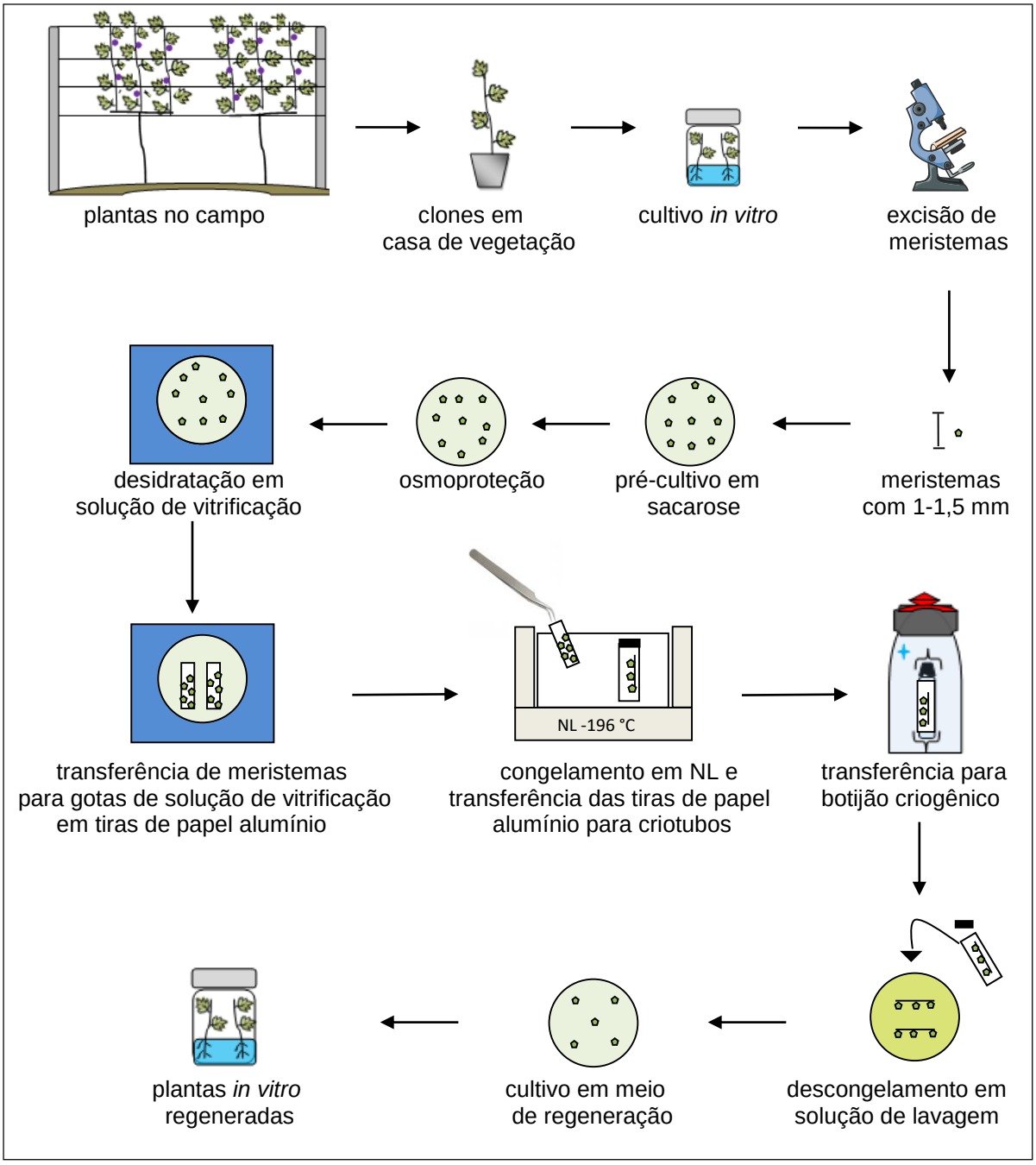
O método de vitrificação em gotas foi aplicado pela primeira vez por Schafermenuhr et al. (1994) para a criopreservação de meristemas de batata. Ele combina a aplicação de soluções de vitrificação altamente concentradas com congelamento e descongelamento a taxas ultrarrápidas, que são fundamentais para resultados bem sucedidos em protocolos de criopreservação (SOUZA et al., 2015; PANIS et al., 2011; KIM et al., 2006; PANIS; PIETTE; SWENNEN, 2005).

Este método envolve o tratamento das amostras em soluções crioprotetoras, que inclui a exposição à solução de glicerol e sacarose (solução de carregamento), seguido por desidratação em solução de vitrificação a 0 ou 22/25 ºC (VOLK; SHEPHERD; BONNART, 2014). Diferentes soluções de vitrificação foram desenvolvidas por vários grupos de pesquisa em todo o mundo; entre elas, as soluções de vitrificação a base de glicerol, PVS2 (SAKAI; KOBAYASHI; OIYAMA, 1990) e PVS3 (solução de vitrificação de plantas 3, NISHIZAWA et al., 1993) são as mais frequentemente utilizadas. A solução de PVS2 consiste de 30% glicerol, 15% etileno glicol (EG), 15% dimetilsulfóxido (DMSO) e 0,4 M de sacarose; PVS3 é composto de 50% de sacarose e 50% de glicerol (NISHIZAWA et al., 1993; SAKAI; KOBAYASHI; OIYAMA, 1990). Após tratamento em solução de vitrificação, os explantes são inseridos individualmente em gotas de solução de vitrificação, que são

colocadas sobre uma tira de folha de alumínio esterilizada e, em seguida, imersas diretamente em NL (MATSUMOTO, 2017). Durante o descongelamento, a remoção do crioprotetor é conseguida usando-se uma solução de lavagem e, em seguida, os explantes são transferidos para recuperação em meios de cultura (Figura 2) (BENELLI; DE CARLO; ENGELMANN, 2013). A principal vantagem desta técnica, em relação ao método clássico de vitrificação, é a possibilidade de alcançar ultrarrápidas taxas de resfriamento/aquecimento devido ao pequeno volume de solução de vitrificação em que os explantes são colocados, diferentemente do método de vitrificação, no qual os explantes são acondicionadas em criotubos com um volume ligeiramente maior de solução de vitrificação, tornando as etapas de congelamento e descongelamento mais demoradas (MATSUMOTO, 2017; PANIS; PIETTE; SWENNEN, 2005). Dessa forma, o fator-chave para a implementação do método de vitrificação em gotas é a otimização do procedimento de desidratação química, a fim de evitar lesões por toxicidade ou estresse osmótico excessivo durante o tratamento dos explantes com a solução de vitrificação. Isso se dá por meio da definição de um tempo ótimo de exposição dos propágulos à solução de vitrificação, bem como da temperatura dessa solução, que por sua vez está diretamente relacionada com a velocidade de permeabilidade da solução no tecido vegetal (VOLK et al., 2014a, b; SAKAI; ENGELMANN, 2007).

O método de vitrificação em gotas tem se mostrado eficiente para diferentes espécies de plantas e, em algumas publicações, são apresentados resultados relevantes para diversos acessos. Utilizando o método de vitrificação em gotas para *Musa* spp., Panis et al. (2005) relataram uma taxa média de regeneração de 53% em 56 acessos; para *Citrus* spp., Volk et al. (2017b) obtiveram taxa média de regeneração de 56% em 142 acessos; para *Ananas* spp., Souza et al. (2015) observaram uma taxa média de sobrevivência de 57% em 16 acessos; para *Solanum* spp., Panta et al. (2015) relataram uma taxa média de regeneração de 51% em 755 acessos. Atualmente, o método de vitrificação em gotas é considerado a estratégia mais promissora para superar os problemas relacionados a espécie ou genótipo especificidade, que muitas vezes é considerado o fator limitante para o estabelecimento de bancos criogênicos (BI et al., 2017; WANG et al., 2014; PANIS; PIETTE; SWENNEN, 2005; PANIS et al., 2011).

Figura 2 – Representação esquemática do protocolo de criopreservação de plantas pelo método de vitrificação em gotas.



Fonte: Elaborado pelo autor,2018.

2.2.2.3 Método de crio-placas de alumínio (D e V crio-placa)

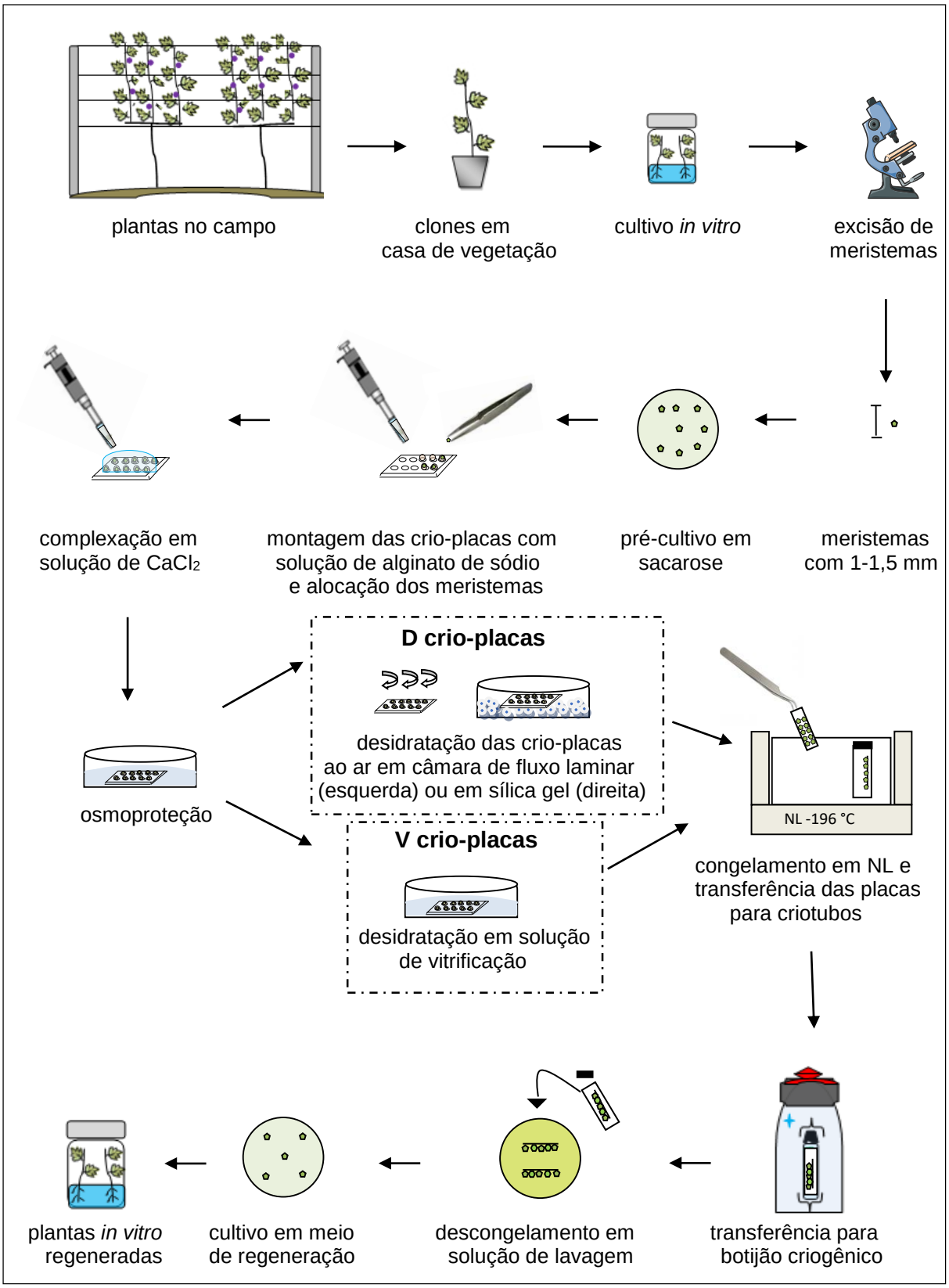
Considerada a mais recente otimização do método de vitrificação em gotas, as crio-placas de alumínio foram desenvolvidas com base nos métodos de vitrificação em PVS2 e desidratação ao ar, conhecidas como V crio-placa (YAMAMOTO et al., 2011) e D crio-placa (NIINO et al., 2013), respectivamente.

Nos métodos de criopreservação em crio-placas os meristemas pré-cultivados em sacarose (0,3 M) são aderidos aos poços presentes nas crio-placas de alumínio, encapsulando-os em gotículas de alginato de cálcio. Em sequência, as crio-placas contendo os meristemas são transferidas para solução de glicerol e sacarose (solução de carregamento), seguido por desidratação em solução de vitrificação (V crio-placa) ou em cabine de fluxo laminar (D crio-placa). Então, os meristemas desidratados são imersos diretamente em nitrogênio líquido (Figura 3) (MATSUMOTO; NIINO, 2017).

As principais vantagens dos métodos de crio-placa são a facilidade de manipulação dos explantes depois de aderidos às crio-placas, já que as amostras podem ser transferidas facilmente entre soluções com o mínimo de danos e rapidamente. O método proporciona taxas rápidas de congelamento e descongelamento, o que resulta em elevados níveis de recuperação para materiais que foram criopreservados (MATSUMOTO et al., 2015; NIINO et al., 2013; YAMAMOTO et al., 2012a). Até o presente, os métodos de crio-placas tem sido aplicados com sucesso para uma gama diversificada de recursos genéticos, incluindo *Dianthus caryophyllus* L. (SEKIZAWA et al., 2011), *Tanacetum cinerariifolium* (YAMAMOTO et al., 2011), *Mentha* spp. (YAMAMOTO et al., 2012a), *Morus* spp. (YAMAMOTO et al., 2012b), *Fragaria ananassa* (YAMAMOTO et al., 2012c), *Juncus decipiens* (NIINO et al., 2013), *Perilla frutescens* (MATSUMOTO et al., 2014), *Phoenix dactylifera* (SALMA et al., 2014), *Cleome rosea* (CORDEIRO et al., 2015), *Saccharum officinarum* (RAFIQUE et al., 2015; 2016), *Solanum tuberosum* (ARIZAGA et al., 2017a; Yamamoto et al., 2015), *Prunus* spp. (VUJOVIĆ et al., 2015), *Clinopodium odorum* (ELGELMANN-SYLVESTRE; ELGELMANN, 2015), *Diospyros kaki* (MATSUMOTO et al., 2015), *Vaccinium virgatum* (DHUNGANA et al., 2017), *Ullucus tuberosus* (ARIZAGA et al., 2017b) e *Passiflora pohlii* (SIMÃO et al., 2017).

Embora a eficácia dos métodos de V e D crio-placa tenham sido publicadas em várias espécies de plantas, até o presente, não há relato de criopreservação de *Vitis* e *Malus* usando os métodos com crio-placas de alumínio.

Figura 3 – Representação esquemática do protocolo de criopreservação de plantas pelo método de crio-placas de alumínio.



Fonte: Elaborado pelo autor, 2018.

2.3 CRIOTERAPIA

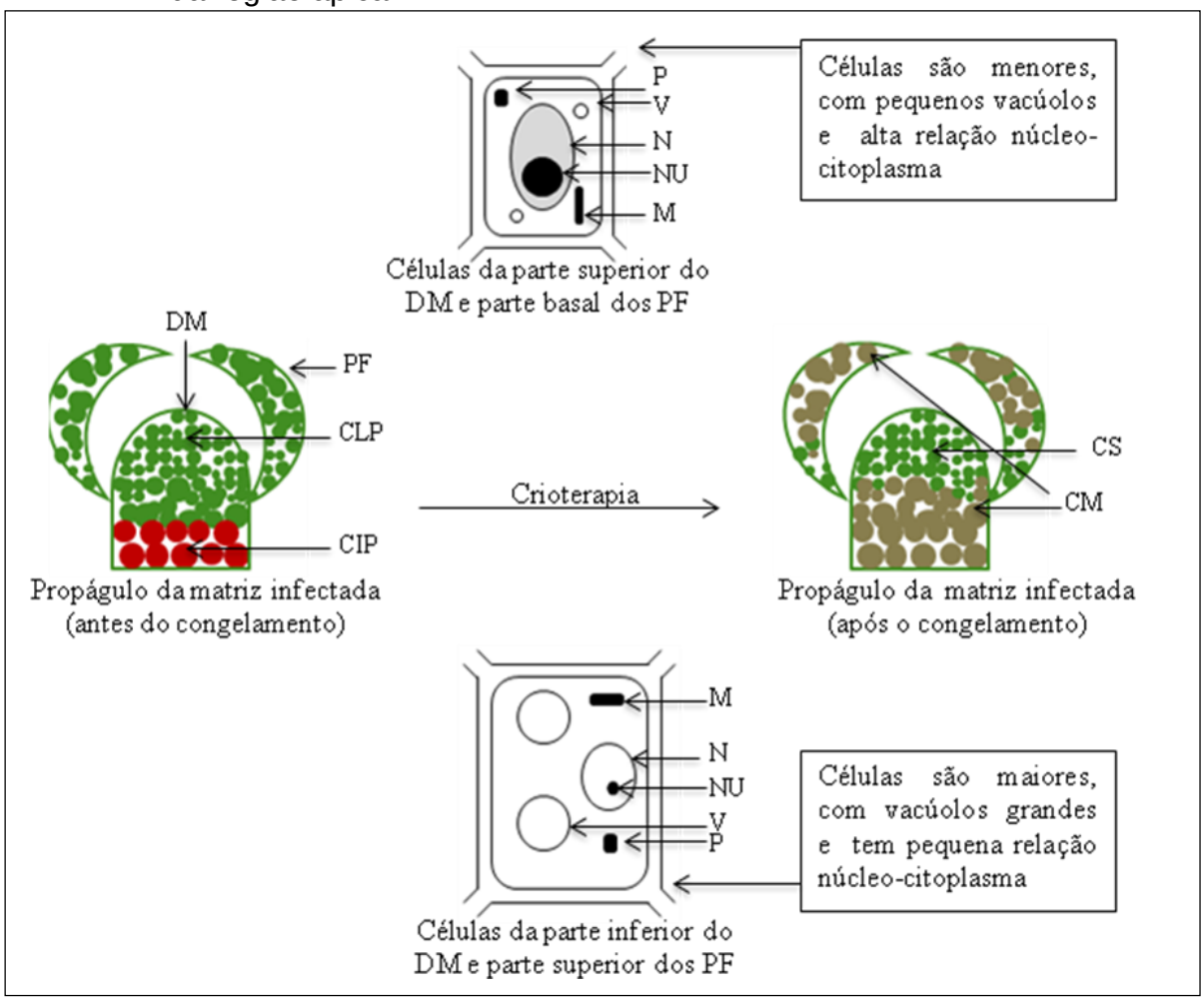
A criopreservação vai além da conservação de material biológico, tecidos *in vitro* inicialmente infectados por vírus, quando criopreservados, regeneraram plantas isentas de vírus (BRISON et al., 1997; WANG et al., 2003; VIEIRA, et al., 2015; PATHIRANA et al., 2015; ROMADANOVA et al., 2016; BETTONI et al., 2018b). Desse modo, surgiu a denominação crioterapia, que fornece em curto espaço de tempo alta frequência de plantas regenerantes livres de vírus (WANG; VALKONEN, 2009b; BETTONI et al., 2016).

Métodos de crioterapia são dependentes da disponibilidade de protocolos de criopreservação na espécie desejada; nessa situação, o material biológico é exposto ao tratamento em nitrogênio líquido (NL) a -196 ° C por um curto período, normalmente 1–24 h (PATHIRANA et al., 2015; WANG et al., 2014). Os explantes utilizados são tecidos organizados, que consistem em segmentos apicais ou laterais contendo a região meristemática (PATHIRANA et al., 2016) e a dimensão do material excisado não é um fator limitante para erradicação do patógeno como ocorre em métodos tradicionais de erradicação do vírus, como na cultura de meristemas (WANG et al., 2003). Após a crioterapia as células sobreviventes se localizam no domo meristemático, região que não possui vascularização. As partículas virais, em teoria, não conseguem infectar o domo meristemático, pois se movimentam na planta através do sistema vascular; por outro lado, as outras células e tecidos mais distantes do meristema, nos quais os vírus podem estar presentes, são mortas pelo efeito letal da temperatura ultrabaixa (WANG et al. 2009; LI; HARTUNG; LEVY, 2006). Portanto, a técnica é beneficiada pelas diferenças físico-anatômicas entre porção de células meristemáticas e os demais tecidos; células meristemáticas são pequenas, altamente adensadas, possuem vacúolos menores e maior relação núcleo-citoplasma do que células que estão fora da região meristemática (Figura 4) (WANG; VALKONEN, 2009a).

O primeiro relato da eficiência da crioterapia para eliminação de vírus foi em porta-enxerto de *Prunus* (BRISON et al., 1997) e desde então vem crescendo a aplicação da crioterapia em diversas espécies de importância econômica, como *Musa* spp. (HELLIOT et al., 2002), *Vitis vinifera* (WANG et al., 2003; BAYATI; SHAMS-BAKHSH; MOIENI, 2011; PATHIRANA et al., 2015), *Fragaria ananassa* (CAI et al., 2008), *Solanum tuberosum* (WANG et al., 2006; BAI et al., 2010; 2012; YI et al. 2014),

Rubus idaeus (WANG & VALKONEN 2009b; WANG et al., 2008), *Ipomea batatas* (WANG; VALKONEN, 2008ab), *Dioscorea opposite* (HEE; KYOON; KEUN, 2013), *Allium sativum* (VIEIRA et al., 2015), *Malus* spp. (ROMADANOVA et al., 2016; LI et al., 2016; BETTONI et al., 2018b; WANG et al., 2018b) (Tabela 1).

Figura 4 – Esquema representativo das diferenças físico-anatômicas de células da região próxima ao domo meristemático e de outras células mais distantes da região apical.



Fonte: Wang e Valkonen (2009a).
DM, domo meristemático; PF, primórdio foliar; CLP, células livres de patógeno; CIP, células infectadas por patógeno; CS, células sobreviventes; CM, células mortas; M, mitocôndria; V, vacúolo; NU, núcleo; N, nucléolo; P, proplastídio.

Tabela 1 – Eficiência de métodos de crioterapia para erradicação de vírus em plantas.

Vírus		Espécie	Método de Crioterapia	Referência
Plum pox virus	PPV	*Prunus* sp.	VI	Brison et al. (1997)
Banana streak virus	BSV	*Musa* sp.	VI	Helliot et al. (2002)
Cucumber mosaic virus	CMV	*Musa* sp.	VI	Helliot et al. (2002)
Grapevine virus A	GVA	*Vitis vinifera*	EN-DE; VI	Wang et al. (2003b)
Potato virus Y	PVY	*Solanum tuberosum*	EN-DE; VI; DR-VI	Wang et al. (2006a)
Potato leafroll virus	PLRV	*Solanum tuberosum*	EN-DE; VI; DR-VI	Wang et al. (2006a)
Strawberry mild yellow-edge virus	SMYEV	*Fragaria ananassa*	VI	Cai et al. (2008)
Sweet potato feathery mottle virus	SPFMV	*Ipomoea batatas*	EN-VI	Wang and Valkonen (2008a)
Sweet potato chlorotic stunt virus	SPCSV	*Ipomoea batatas*	EN-VI	Wang and Valkonen (2008a)
*Raspberry bushy dwarf virus**	RBDV	*Rubus idaeus*	T-EN-VI	Wang et al. (2008)
*Raspberry bushy dwarf virus**	RBDV	*Rubus idaeus*	T-EN-VI	Wang and Valkonen (2009b)
Grapevine virus A	GVA	*Vitis vinifera*	EN-DE	Bayati et al. (2011)
Potato virus X	PVX	*Solanum tuberosum*	DR-VI	Bai et al. (2010; 2012)
Potato spindle tuber viroid	PSTVd	*Solanum tuberosum*	DR-VI	Bai et al. (2012)
Yam mosaic virus	YMV	*Dioscorea opposita*	EN-DE	Hee et al. (2013)
Potato leafroll virus	PLRV	*Solanum tuberosum*	DR-VI; EN-VI	Yi et al. (2014)
Potato virus Y	PVY	*Solanum tuberosum*	DR-VI; EN-VI	Yi et al. (2014)
Onion yellow dwarf virus	OYDV	*Allium sativum*	VI	Vieira et al (2015)

(Continua na próxima página)

Continuação Tabela 1 ...

Vírus		Espécie	Método de Crioterapia	Referência
Leek yellow strip virus	LYSV	*Allium sativum*	VI	Vieira et al (2015)
Garlic common latent virus	GarCLV	*Allium sativum*	VI	Vieira et al (2015)
Grapevine associated virus 1	GLRaV-1	*Vitis vinífera*	DR-VI	Pathirana et al. (2013; 2015)
Grapevine associated virus 2	GLRaV-2	*Vitis vinifera*	DR-VI	Pathirana et al. (2013; 2015)
Grapevine associated virus 3	GLRaV-3	*Vitis vinifera*	DR-VI	Pathirana et al. (2013; 2015)
Grapevine fanleaf virus	GFLV	*Vitis vinifera*	DR-VI	Marković et al. (2015)
Apple chlorotic leaf spot virus	ACLSV	*Malus* sp.	VI	Romadanova et al. (2016)
Apple chlorotic leaf spot virus	ACLSV	*Malus* sp.	EN-DE	Bettoni et al. (2018)
Apple stem pitting virus	ASPV	*Malus* sp.	VI	Romadanova et al. (2016)
Apple stem pitting virus	ASPV	*Malus* sp.	EN-DE	Li et al. (2016)
Apple stem pitting virus	ASPV	*Malus* sp.	EN-DE	Bettoni et al. (2018)
Apple stem grooving virus	ASGV	*Malus* sp.	VI	Romadanova et al. (2016)
Apple stem grooving virus	ASGV	*Malus* sp.	EN-DE	Bettoni et al. (2018)
*Apple stem grooving virus******	ASGV	*Malus* sp.	T-DR-VI	Wang et al. (2018b) *****
Apple mosaic virus	ApMV	*Malus* sp.	VI	Romadanova et al. (2016)

Fonte: Elaborado pelo autor, 2018.
VI: Vitrificação; EN-DE: Encapsulamento-Desidratação; EN-VI: Encapsulametno-Vitrificação; DR-VI: Vitrificação em Gotas; T: Termoterapia.
***** Termoterapia seguida por crioterapia

A crioterapia provou ser uma estratégia eficaz para produzir plantas de macieiras (ROMADANOVA et al., 2016; BETTONI et al., 2018b) e de videiras (PATHIRANA et al., 2015) livres de vírus. Estudos mostraram que as frequências de plantas livres de vírus variaram de acordo com o genótipo e tipo de vírus. Esses resultados podem estar associados à morfologia viral e ou a capacidade de movimentação de cada partícula viral via proteínas de movimento (SAREILA et al., 2004). Em especial, no caso da macieira, há trabalhos que relatam que a crioterapia foi eficiente na erradicação de uma determinada espécie viral mas falhou completamente para a outra. Li et al. (2016) mostraram que porta-enxertos de maciera 'M9' e 'M26' criopreservados por encapsulamento-desidratação tiveram uma frequência de 85% de erradicação de *Apple stem pitting virus* (ASPV) porém a técnica não produziu nenhuma planta isenta de *Apple stem grooving virus* (ASGV). Da mesma forma Wang et al. (2018b) mostraram que plantas de 'Gala' (*Malus* X *domestica*) criopreservadas por vitrificação em gotas estavam ainda infectadas com ASGV após a exposição em NL. Estudos de imunolocalização do vírus ASGV, em explantes de ‘Gala’ criopreservadas, relevaram que as partículas virais estavam presentes em parte da região meristemática e dos primórdios foliares, deixando apenas algumas camadas superiores de células do domo meristemático livres do vírus (LI et al., 2016).

Apesar de técnicas de crioterapia serem baseadas em protocolos de criopreservação, os parâmetros ideais para a criopreservação não são os melhores para a crioterapia; quando o objetivo é produzir plantas livres de vírus, deve ser reduzido ao máximo o número de células do meristema (crio-lesão) após a exposição ao NL e, portanto, melhorar a frequência de erradicação do vírus. Embora seja necessária alguma sobrevivência/regeneração, os altos níveis de regeneração não são fundamentais para o sucesso das aplicações de crioterapia. Se apenas uma ou algumas plantas livres de vírus serem recuperadas, o método foi aplicado com sucesso e plantas saudáveis adicionais podem ser propagadas a partir dessas plantas limpas.

Os estudos de Li et al. (2016) e Wang et al. (2018b), que relataram a ineficiência de técnicas de criopreservação para erradicação de ASGV, baseiam-se em metodologias que foram ideais para a criopreservação de *Malus*. Assim, elevados níveis de regeneração após exposição em nitrogênio líquido foram encontrados; isso, por sua vez, poderia estar associado à ineficiência dos métodos utilizados na erradicação do ASGV. Portanto etapas durante o procedimento criogênico devem ser

melhoradas visando a redução de células meristemáticas que se recuperam após a exposição ao NL, isso poderia melhorar a eficiência de erradicação do vírus.

Uma estratégia que têm se mostrado eficiente na erradicação de vírus que apresentam capacidade de invadir a região meristemática, considerados de difícil controle, é a associação de termoterapia e crioterapia. O tratamento térmico proporcionado pela termoterapia reduz o movimento de partículas virais para a região meristemática por inibição da replicação viral (WANG et al., 2006b), dessa forma a área livre de vírus é maior do que sem tratamento térmico, melhorando assim a chance de erradicação do vírus por crio-lesão em células diferenciadas infectadas (WANG et al., 2008). Esta combinação de tratamento mostrou-se eficaz em maçã 'Gala' infectada por ASGV (WANG et al., 2018b), em framboesa infectada por *Raspberry bushy dwarf virus* (RBDV) (WANG et al., 2008) e em alho infectado com complexo viral *Onion yellow dwarf virus* (OYDV), *Leek yellow strip virus* (LYSV) and *Garlic common latent virus* (GCLV) (VIEIRA et al., 2015).

Observando os resultados de décadas de pesquisa, a crioterapia pode ser considerada como um método prático e promissor que pode auxiliar os métodos tradicionais de erradicação de vírus em material vegetal infectado (WANG; WALKONEN, 2009b; BETTONI et al., 2016). Isso pode facilitar a produção de plantas livres de vírus em escala comercial, gerando material vegetal de alta qualidade; além disso, a crioterapia pode ser considerada um método de rápido desenvolvimento e de baixo custo quando comparado como os métodos tradicionais de erradicação de vírus, oferece a vantagem de não requerer equipamento caro e após o estabelecimento do protocolo para a espécie desejada é simples de reproduzir.

2.4 MÉTODOS TRADICIONAIS PARA PRODUÇÃO DE PLANTAS LIVRES DE VÍRUS

A aquisição de materiais vegetais com origem genética comprovada e com a garantia de bom estado fitossanitário é importante para o sucesso no estabelecimento e manutenção de pomares. Na prática, muitas vezes é difícil encontrar materiais com esses padrões. A produção de mudas de qualidade fitossanitária pode ser feita utilizando-se técnicas de cultivo *in vitro* associadas a terapias de erradicação de viroses.

Os métodos tradicionais utilizados para obtenção de material vegetal sadio, ou seja, isento de vírus e/ou bactérias e fungos patogênicos, incluem cultura de meristemas e termoterapia seguida de cultura de meristemas (DÍAS-BARRITA et al., 2007; MALIOGKA et al., 2009; 2015). Há relatos científicos do sucesso das técnicas em diversas espécies; no entanto, em termos funcionais algumas peculiaridades dificultam o amplo uso dessas ferramentas biotecnológicas (WANG et al., 2014). A cultura de meristemas vem sendo utilizada há décadas, porém a capacidade de eliminação dos vírus está associado à dimensão do tecido excisado e, para a maioria das espécies, o tamanho recomendado é extremamente pequeno, de 0,1 a 0,3 mm (WANG; VALKONEN, 2008a). Além dos problemas mecânicos com a remoção do meristema, a baixa taxa de regeneração e, principalmente, a não garantia de remoção das partículas virais são os gargalos dessa técnica (FACCIOLI; MARANI, 1998; DÍAS-BARRITA et al., 2007; WANG et al., 2016). A aplicação da termoterapia associado à cultura de meristemas é um processo oneroso, exige equipamento específico, o tempo de tratamento é dependente do tipo de vírus e nem todos os vírus são eliminados por meio da técnica; além disso, muitas plantas hospedeiras são termossensíveis (BHOJWANI; DANTU, 2013; HU et al., 2015).

Outros métodos são apontados como alternativos para a eliminação de vírus, entre os quais estão embriogênese somática (GAMBINO; DI MATTEO; GRIBAUDO, 2009) e quimioterapia (HU et al., 2015). A embriogênese somática, além de um método demorado, é difícil de ser executado e, no final, pode causar a variação somaclonal em plantas regeneradas. Protocolos de quimioterapia foram aplicadas com sucesso para remover o vírus do enrolamento da folha em videira (*Grapevine leafroll-associated virus* - GLRaV-1 e 3) (PANATTONI et al., 2011) e em macieira para os vírus da mancha clorótica foliar (*Apple chlorotic leaf spot virus* - ACLSV), do acanalamento do tronco (*Apple stem grooving virus* - ASGV) e da canelura do tronco (*Apple stem pitting virus* - ASPV) (HU et al., 2015). O modo de ação dos compostos antivirais utilizados na quimioterapia, geralmente, consiste na inibição da replicação viral por inibição da síntese de ácidos nucléicos. Embora existam relatos promissores na utilização da quimioterapia em tecidos vegetais, também há relatos demonstrando alta fito-toxicidade induzida por compostos antivirais. Além disso, respostas variáveis são observadas entre as diferentes cultivares e não há nenhuma pesquisa que demonstre que compostos antivirais não provoquem alteração mutagênica. Em geral,

todas as técnicas acima mencionadas têm as suas desvantagens e cada uma delas necessita ser otimizada (SKIADA et al., 2013).

2.5 MOLÉSTIAS VIRAIS EM MACIEIRA E VIDEIRA

No Brasil, a ocorrência de vírus em culturas propagadas vegetativamente é generalizada, impulsionada pelos métodos de propagação, quando não há seleção sanitária das plantas matrizes. Um fator preocupante é que a disseminação e o acúmulo de títulos de vírus nas plantas são beneficiados pelo emprego de material propagativo infectado, o que contribui para dispersão e contaminação de novas áreas (WANG; VALKONEN, 2009b; BASSO et al., 2015). Macieiras e videiras são infectadas por um grande número de vírus. Até o presente, pelo menos 12 e 65 espécies virais distintas pertencentes à diferentes famílias foram relatadas infectando macieira (NISAR, 2013) e videiras (MARTELLI, 2014; CHIUMENTI et al., 2016; BASSO; FAJARDO; SALDARELLI, 2017), respectivamente.

Macieiras infectadas com vírus apresentam problemas de incompatibilidade de enxertia, redução do vigor, menor produtividade e qualidade dos frutos, além de maior suscetibilidade a outros agentes fitopatogênicos (NICKEL et al., 2001; CIEŚŃINSKA; RUTKOWSKI, 2008; GUERRA et al., 2012; KUMAR et al., 2014). Em macieira, as estirpes virais podem causar uma variação de sintomas, que vão desde plantas sintomáticas, onde as folhas possuem distorções e manchas irregulares, os frutos podem apresentar redução do tamanho e diminuição da qualidade; e plantas assintomáticas, que não expressam os sintomas característicos, estas constituem uma fonte de inóculo com graves consequências para a pomicultura (BARBA; ILARDI; PASQUINI, 2015). Dentre os vírus que expressam os sintomas a campo, destaca-se o vírus do mosaico da macieira (ApMV, *Apple mosaic virus*, família *Bromoviridae*, gênero *Ilarvirus*); já entre os latentes, encontram-se o vírus do acanalamento do tronco (ASGV, família *Betaflexiviridae*, gênero *Capillovirus*), o vírus das caneluras do tronco (ASPV, família *Betaflexiviridae*, gênero *Foveavirus*) e o vírus da mancha clorótica foliar (ACLSV, *Apple chlorotic leaf spot virus*, família *Betaflexiviridae*, gênero *Trichovirus*) (NICKEL et al., 2001). ASGV, ASPV e ACLSV são os principais patógenos virais em macieiras responsáveis por substanciais perdas na indústria mundial da maçã (FAJARDO; NICKEL, 2014). Nenhum vetor é atualmente conhecido por disseminar

ASGV, ASPV e ACLSV. Portanto, a disseminação se dá apenas através de material propagado infectado (MARTELLI et al., 2007, NICKEL e FAJARDO, 2014; BARBA, ILARDI e PASQUINI, 2015, HU et al., 2017). Plantas infectadas por essas espécies virais são geralmente assintomáticas, comumente ocorrendo como infecções mistas, e podem se tornar aparentes quando plantas hospedeiras são enxertadas em porta-enxertos sensíveis (BARBA; ILARDI; PASQUINI, 2015).

Apesar do grande número de viroses que podem infectar plantas de videira, somente um número restrito pode ser considerado de importância econômica. Dentre as doenças viróticas da videira, as relacionadas com o enrolamento da folha (GLRaV, *Grapevine leafroll-associated virus*, família *Closteroviridae*, gênero *Closterovirus*), canelura do caule (GRSPaV, *Grapevine rupestris stem pitting-associated virus*, família *Betaflexiviridae*, gênero *Foveavirus*) e a degenerescência da videira (GFLV, *Grapevine fanleaf virus*, família *Comovirinae*, gênero *Nepovirus*) são as de maior expressão, seja pela maior incidência ou pelos prejuízos que causam. Plantas de videira infectadas apresentam crescimento reduzido, diminuição da produtividade e qualidade; prejuízos podem chegar até 70 % de redução da produção e redução do teor de açúcar em até 4° Brix, além da diminuição da vida útil do pomar, seguido de declínio e morte das plantas (FAJARDO et al., 2004; 2007; BASSO et al., 2010; GIOVANNINI, 2014). Do mesmo modo que ocorre na macieira, as estirpes virais podem causar uma ampla gama de sintomas, que variam entre as espécies, como sintomas de amarelecimento das folhas, faixas amarelas brilhantes perto das nervuras e bifurcação nas nervuras. Algumas cultivares de origem americana e híbridos, especialmente porta-enxertos, não demonstram os sintomas característicos decorrentes de infecções virais, que permanecem em estado latente (SHATNAWI et al., 2011). Espécies de insetos, bem como nematóides são conhecidos como vetores de vírus em videira, de forma semi-persistente. Cochonilhas e pulgões são vetores eficientes de *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine virus E* (GVE), GLRaV-1, GLRaV-3 e GLRaV-4 (BELLI et al., 1994; CABALEIRO; SEGURA, 1997; TSAI et al., 2010); nematóides das espécies *Xiphinema index* e *Xiphinema italiae* são vetores de GFLV e, cigarrinhas de *Grapevine cabernet franc-associated virus* (GCFaV) e *Grapevine redleaf-associated virus* (GRLaV) (BASSO; FAJARDO; SALDARELLI, 2017).

Independente da espécie vegetal, uma vez que o plantio seja realizado com material propagativo infectado com espécies virais é impossível erradicar os vírus.

Portanto, materiais para formação de jardins clonais e plantas matrizes, que são fornecedoras de propágulos, devem estar isentos de agentes patogênicos de etiologia viral (WANG et al., 2014). O fornecimento de materiais de plantio livres de vírus é fundamental para a produção sustentável e uma medida efetiva para o controle de vírus em espécies propagadas vegetativamente.

3 DROPLET-VITRIFICATION OF TWELVE *VITIS* TAXA USING APICAL SHOOT TIPS DERIVED FROM *IN VITRO* GROWN PLANTS[1]

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3.1 ABSTRACT

We report a method to cryopreserve twelve *Vitis* species using shoot tips excised from *in vitro*-grown plants. Uniform 1 mm apical shoot tips were obtained from nodal sections cultured from *in vitro* stock plants. Shoot tips were precultured for 3 days on medium containing 0.3 M sucrose, salicylic acid, glutathione (reduced form), and ascorbic acid. They were then treated with loading solution for 20 min at 22ºC followed by half-strength PVS2 for 30 min, and full-strength PVS2 treatments ranging from 60 to 105 min at 0 ºC. After exposure to LN on foil strips, shoot tips were warmed/diluted in unloading solution for 20 min and placed on recovery medium. Our results demonstrate a wide applicability of this technique with regrowth levels at least of 43 % in 13 genotypes representing 12 *Vitis* species. Histological observations revealed that cryopreserved shoot tips appear to have a high degree of cellular preservation in both the meristematic regions and at the base of leaf primordia. Some treatments were independently replicated by two technicians to determine the transferability of the methods. We demonstrate that the droplet-vitrification procedure can be successfully replicated by technical staff and suggest that this *Vitis* cryopreservation method may soon be ready for implementation in genebanks.

Keywords*:* Genebank. Grapevine. Histological observation. Plant vitrification solution.

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3.2 INTRODUCTION

Grapes (*Vitis*) are one of the most economically important fruit crops cultivated and consumed worldwide (OIV, 2017). *Vitis* has a rich genetic diversity; there are more than 70 species within the genus, with most of the globally important cultivars assigned to *Vitis vinifera* (LI et al., 2017). Wild *Vitis* species, as well as *Muscadinia rotundifolia,* have been used for hybrid grape breeding as new cultivars and rootstocks, many of which provide resistance to pests and diseases (PERTOT et al., 2017; SMITH et al., 2016; MIGICOVSKY et al., 2016; EIBACH et al., 2007).

The availability of and easy access to *Vitis* genetic resources are essential for future breeding program advances (RONALD, 2011). *Vitis* genebank collections are traditionally conserved in field repositories. Field maintenance is costly and time-consuming, requires extensive acreage, and plants are vulnerable to abiotic stresses as well as biotic threats (PATHIRANA et al., 2016; MARKOVIĆ et al., 2013a; BENELLI; DE CARLO; ENGELMANN, 2013; REED et al., 20014). *In vitro* culture back-up is one alternative to short-term preservation of *Vitis* germplasm (MAIA et al., 2015; POSTMAN et al., 2006), but it has some limitations because tissue culture is labor-intensive, and cultures could become contaminated and undergo somaclonal variation (MATHEW et al., 2018; SOUZA et al., 2015).

Cryopreservation is the storage of biological materials in liquid nitrogen (LN, -196 °C) or liquid nitrogen vapor (LNV, -165 to -190 °C). Cryopreservation methods have been used to securely conserve portions of plant genebank collections for the long-term. Under cryopreserved conditions, viable propagules are preserved in a state where cellular divisions and metabolic processes are minimized (WANG et al., 2018a; BENELLI; DE CARLO; ENGELMANN, 2013). Compared to the cost of creating duplicated field collections, it is cost effective to maintain collections for extended lengths of time in LN (REED, 2017; VOLK et al., 2014a; BENSON, 2008).

Although there are many established cryobanks that conserve vegetative propagules such as shoot tips or dormant buds of clonally propagated genetic resources (WANG et al., 2014; TOWILL et al., 2004), to the best of our knowledge, *Vitis* cryo-storage has not been fully implemented within genebanks. Limited results were obtained when *Vitis* dormant buds were cryopreserved (ESENSEE; STUSHNOFF, 1990). Results have been more promising with respect to *Vitis* shoot tip cryopreservation. Several papers have reported the successful cryopreservation of

Vitis shoot tips (BI et al., 2017; HASSAN; HAGGAG, 2013; PATHIRANA et al., 2016); however, genotype-specific responses have made it difficult to implement (PATHIRANA et al., 2016; BENELLI; DE CARLO; ENGELMANN, 2013; BENSON, 2008; MARKOVIĆ et al., 2013b; GANINO et al., 2012; BENELLI; LAMBARDI; FABBRI, 2003). Most *Vitis* cryopreservation research has focused on the development of procedures using a limited number of species (BI et al., 2017; BETTONI et al., 2016).

Access to reliable cryopreservation methods that result in high levels of viability (≥40% after LN exposure) and highly skilled staff are key to the development of successful base collections (VOLK et al., 2016; REED et al., 2004). Herein, we focus on developing a droplet-vitrification procedure to cryopreserve *Vitis* shoot tips from *in vitro* stock plants that is widely applicable to many species.

3.3 MATERIALS AND METHODS

3.3.1 Plant material and pre-treatments

All plant materials were originally received from the USDA-ARS National Clonal Germplasm Repository for Tree Fruit, Nut Crops and Grapes in Davis, CA, USA and introduced into *in vitro* conditions. *Vitis* accessions include *Vitis vinifera* cvs. 'Chardonnay' and 'Riesling', *V. actinifolia* (DVIT 2594.1), *V. aestivalis* (DVIT 1408), *Vitis* sp. (DVIT 1445), *V. flexuosa* (DVIT 1385), *V. palmata (DVIT 2228.1), V. riparia* (PI 588214), *V. rupestris* (DVIT 8166), *V. sylvestris* (DVIT 2426.1), *V. ficifolia* DVIT 2008.1*, V. treleasi* (DVIT 1410) and *V.* × *novae angelae* (DVIT 1457).

Stock cultures were maintained and subcultured every 8 to 12 weeks in glass culture vessels (89 x 170 mm) on MS medium (MURASHIGE and SKOOG, 1962), containing 30 g L^{-1} sucrose, 0.175 mg L^{-1} IAA (3-indoleacetic acid) and 2.5 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving). Cultures were grown at 25 °C, under a photoperiod of 16 h light day^{-1} with a light intensity of 40 µM m^{-2} s^{-1}.

Nodal sections were obtained from 2- to 3-month old *in vitro* stock plants and placed into 100 × 25 mm plastic Petri dishes with 50 mL pretreatment medium (MS salts/vitamins supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} N^6-benzyladenine (BA^3), 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form) and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving)) with a density of 40 nodal sections per plate, and cultured for 2 weeks in the same conditions as the *in vitro* stock cultures.

Nodal sections were cold acclimated at 5 ^{o}C with a 16 h photoperiod for an additional 2 weeks (when applied).

3.3.2 Preculture and cryopreservation

Uniform apical shoot tips (1 mm length and width) were excised from nodal sections that either had or had not been cold acclimated. Shoot tips were cultured on preculture medium (half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form) and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving)) for 3 days at 25 ^{o}C in darkness. They were then placed in loading solution (half-strength MS + 2M glycerol + 0.4 M sucrose at pH 5.7 (pH 6.94 prior to autoclaving)) for 20 min at 22^{o}C followed by half-strength PVS2 (filter sterilized solution consisting half-strength MS + 15% (w/v) glycerol + 7.5% (w/v) ethylene glycol (EG) +7.5% (w/v) dimethyl sulfoxide (DMSO) + 0.4 M sucrose at pH 5.8, (MATSUMOTO; SAKAI, 2003) for 30 min at 22^{o}C and full-strength PVS2 (filter sterilized solution, half-strength MS + 30% (w/v) glycerol + 15% (w/v) EG +15% (w/v) DMSO + 0.4 M sucrose at pH 5.8, SAKAI; KOBAYASHI; OIYAMA, 1990) at 0 ^{o}C for 60, 75, 90, or 105 min. Two minutes before the end of each treatment, PVS2-treated shoot tips were placed onto a thin layer of PVS2 on sterile aluminum foil strips (6 x 25 mm) and then plunged into liquid nitrogen (LN).

3.3.3 Regrowth after cryoexposure

After one hour of LN exposure, the aluminum foil strips with shoot tips were warmed quickly by inverting the strips into unloading solution (half-strength MS + 1.2 M sucrose at pH 5.7 (pH 7.5 prior to autoclaving)) at 22^{o}C and diluted for 20 min.

For recovery, shoot tips were placed onto recovery medium 1 (1/2X MS macroelements, 1X MS microelements, and *Vitis* vitamins (100 mg L^{-1} myo inositol, 10 mg L^{-1} thiamine HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} Ca pantothenate, 0.01 mg L^{-1} biotin, 2 mg L^{-1} glycine, without NH_4, supplemented with 0.6 M sucrose and 8 g L^{-1} agar at pH 5.7 (pH 7.0 prior to autoclaving)) overnight in the dark and then transferred to recovery medium 2 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins), without NH_4, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA[3] and 8 g L^{-1} agar at ph 5.7 (pH 6.5 prior to autoclaving)) and cultured for

2 weeks at 25 ^{o}C in darkness. They were then transferred onto recovery medium 3 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA[3] and 8 g L^{-1} agar at pH 5.7 (pH 6.5 prior to autoclaving)) and grown in the light at 25 ^{o}C (40 µM m^{-2} s^{-1}, 16 h photoperiod).

3.3.4 Application of the droplet-vitrification technique to additional *Vitis* species

The applicability of the droplet vitrification protocol was evaluated for *in vitro* source plants of five additional *Vitis* species: *V. vinifera* cv. 'Riesling', *V. actinifolia* (DVIT 2594.1), *V. ficifolia* (DVIT 2008.1), *V. treleasi* (DVIT 1410) and *V.* × *novae angelae* (DVIT 1457). This experiment was performed as described above, except that shoot tips were exposed to PVS2 for 90 min at 0 ^{o}C.

3.3.5 Regrowth and data analysis

Shoot tip survival (green cell mass or leaf tissue) and regrowth (organized shoots with at least one leaf) were measured eight weeks after plating on regrowth medium. Each experiment was performed with two replicates of 20 shoot tips for each treatment. Means and standard errors were calculated across experimental replicates, and analized using ANOVA and means separation tests were performed using the Tukey's mean separation test. $P \leq 0.05$ was considered significantly different.

3.3.6 Histological studies of cryopreserved shoot tips

Histological studies were performed to observe cellular structural modifications before and after LN exposure using *Vitis flexuosa* shoot tips excised from *in vitro* source plants. Shoot tips were taken at different points of the cryopreservation protocol including (1) control, shoot tips excised from pre-treatment nodal sections, (2) excised shoot tips precultured for 3 days, (3) shoot tips treated with cryoprotectants, not exposed to LN, and diluted with 1.2 M sucrose, (4) shoot tips treated with cryoprotectants, not exposed to LN, and diluted with 1.2 M sucrose and incubated on recovery medium for 6 days; (5) shoot tips treated with cryoprotectants, exposed to LN, and diluted with 1.2 M sucrose, (6) shoot tips treated with cryoprotectants, exposed to LN, and diluted with 1.2 M sucrose and incubated on recovery medium for 6 days.

Fifteen shoot tips per treatment were placed into fixative (1.25% glutaraldehyde, 2% paraformaldehyde, and 50 mM Pipes buffer [piperazine N, *N*-bis (2-ethanesulfonic acid)] overnight at 22°C. Shoot tips were rinsed three times (10 min each) in 50 mM Pipes buffer and post-fixed with 2% osmium tetroxide in 50 mM Pipes buffer overnight at 22°C. Shoot tips were then rinsed three times (10 min each) in 50 mM Pipes buffer and dehydrated with 30, 50, 70, 90, 100, 100% ethanol for 15 min each. Then, samples were infiltrated with Spurr resin (Electron Microscopy Sciences, Hatfield, PA), in an ethanol: Spurr resin ratio of 3:1 for 4 h, followed by 2:1 for 3 h, 1:1 for 4h, 1:2 for 4 h, 1:3 for 4 h and kept in 100% resin for 72 h (the resin was replaced every 24 hours). Samples were then transferred to fresh resin, placed into embedding moulds polymerised at 65 °C for 24 h. Thin sections (1 µm) were made with glass knives on an RMC MT-X microtome (Ventana Medical Systems Inc., Tucson, AZ) and mounted on glass slides and stained with methylene blue. Sections were visualized with an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan) and images were captured using a Leica MC170 HD digital camera (Wetzlar, Germany).

3.4 RESULTS

3.4.1 Survival and regrowth of cryopreserved *Vitis* shoot tips from *in vitro* stock plants

The first series of experiments (Tables 2-5) were performed by a single technical expert (J. Bettoni) to identify optimum PVS2 exposure durations and the effect of cold acclimation on shoot tip survival and regrowth after cryoexposure. J. Bettoni then applied the optimized method, with 90 min full-strength PVS2 exposure, to five additional *Vitis* accessions (Table 6). Cryopreservation experiments were then performed by a second technical expert (R. Bonnart) using the same five additional accessions (Tables 7-10) to determine the extent that the method could be successfully performed by others.

Table 2 – Survival level (%) of shoot tips excised from nodal sections sourced from eight *Vitis* species grown *in vitro* with 60, 75, 90 and 105 min of PVS2 exposure, with and without LN exposure, and no cold acclimation.

Species	Identifier	-LN PVS2 exposure duration (min) 60	75	90	105
V. vinifera	Chardonnay	100 ± 0 a	100 ± 0 a	98 ± 3 ab	93 ± 3 abc
V. aestivalis	DVIT 1408	95 ± 5 ab	98 ± 3 ab	95 ± 0 ab	83 ± 3 bcd
Vitis sp.	DVIT 1445	95 ± 0 ab	95 ± 0 ab	98 ± 2 ab	93 ± 3 abc
V. flexuosa	DVIT 1385	78 ± 3 cd	90 ± 0 abc	93 ± 3 abc	88 ± 3 abcd
V. palmata	DVIT 2228.1	100 ± 0 a	98 ± 3 ab	93 ± 3 abc	95 ± 5 ab
V. riparia	PI 588214	90 ± 5 abc	90 ± 0 abc	85 ± 0 abcd	73 ± 3 d
V. rupestris	DVIT 8166	90 ± 5 abc	83 ± 3 bcd	83 ± 3 bcd	90 ± 0 abc
V. sylvestris	DVIT 2426.1	100 ± 0 a	88 ± 3 abcd	93 ± 8 abc	95 ± 5 ab
Average		93 ± 3	93 ± 2	92 ± 2	88 ± 3

Species	Identifier	+LN PVS2 exposure duration (min) 60	75	90	105
V. vinifera	Chardonnay	93 ± 3 ab	95 ± 5 a	83 ± 3 abcde	78 ± 3 abcdef
V. aestivalis	DVIT 1408	75 ± 0 abcdefg	85 ± 5 abcd	90 ± 0 ab	78 ± 3 abcdef
Vitis sp.	DVIT 1445	63 ± 3 defg	88 ± 3 abc	90 ± 3 ab	85 ± 5 abcd
V. flexuosa	DVIT 1385	65 ± 0 cdefg	78 ± 3 abcdef	75 ± 0 abcdefg	70 ± 0 bcdefg
V. palmata	DVIT 2228.1	53 ± 3 g	80 ± 0 abcdef	75 ± 5 abcdefg	60 ± 5 efg
V. riparia	PI 588214	73 ± 3 abcdefg	78 ± 3 abcdef	80 ± 5 abcdef	58 ± 8 fg
V. rupestris	DVIT 8166	73 ± 3 abcdefg	70 ± 10 bcdefg	65 ± 5 cdefg	65 ± 5 cdefg
V. sylvestris	DVIT 2426.1	83 ± 3 abcde	83 ± 8 abcde	85 ± 0 abcd	75 ± 0 abcdefg
Average		72 ± 4	82 ± 3	80 ± 3	71 ± 3

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at $P<0.05$ using Tukey's mean separation test.

Table 3 – Regrowth level (%) of shoot tips excised from nodal sections sourced from eight *Vitis* species grown *in vitro* with 60, 75, 90 and 105 min of PVS2 exposure, with and without LN exposure, and no cold acclimation.

Species	Identifier	-LN			
		PVS2 exposure duration (min)			
		60	75	90	105
V. vinifera	Chardonnay	90 ± 0 a	85 ± 5 ab	73 ± 3 abc	73 ± 3 abcd
V. aestivalis	DVIT 1408	73 ± 3 abcd	70 ± 5 abcd	75 ± 5 abc	50 ± 5 bcde
Vitis sp.	DVIT 1445	80 ± 5 abc	85 ± 0 ab	92 ± 2 a	80 ± 5 abc
V. flexuosa	DVIT 1385	63 ± 8 abcde	65 ± 5 abcde	65 ± 5 abcde	68 ± 3 abcde
V. palmata	DVIT 2228.1	80 ± 5 abc	83 ± 13 abc	75 ± 5 abc	68 ± 18 abcde
V. riparia	PI 588214	48 ± 3 cde	38 ± 3 de	38 ± 3 de	33 ± 8 e
V. rupestris	DVIT 8166	60 ± 5 abcde	53 ± 3 bcde	65 ± 5 abcde	58 ± 3 abcde
V. sylvestris	DVIT 2426.1	78 ± 8 abc	68 ± 13 abcde	70 ± 0 abcde	80 ± 5 abc
Average		71 ± 5	68 ± 6	69 ± 5	63 ± 6

Species	Identifier	+LN			
		PVS2 exposure duration (min)			
		60	75	90	105
V. vinifera	Chardonnay	53 ± 3 abcde	68 ± 3 ab	58 ± 3 abcd	50 ± 0 abcdef
V. aestivalis	DVIT 1408	38 ± 3 defgh	58 ± 3 abcd	58 ± 3 abcd	33 ± 8 efgh
Vitis sp.	DVIT 1445	48 ± 3 abcdefg	50 ± 5 abcdef	72 ± 2 a	53 ± 8 abcde
V. flexuosa	DVIT 1385	33 ± 3 efgh	43 ± 3 cdefgh	48 ± 3 abcdefg	43 ± 3 cdefgh
V. palmata	DVIT 2228.1	30 ± 5 efgh	53 ± 3 abcde	53 ± 3 abcd	45 ± 5 bcdefgh
V. riparia	PI 588214	23 ± 3 h	28 ± 3 fgh	35 ± 5 defgh	25 ± 5 gh
V. rupestris	DVIT 8166	38 ± 8 defgh	43 ± 8 cdefgh	43 ± 8 cdefgh	33 ± 3 efgh
V. sylvestris	DVIT 2426.1	33 ± 3 efgh	50 ± 5 abcdef	63 ± 3 abc	48 ± 3 abcdefg
Average		37 ± 3	49 ± 4	53 ± 4	41 ± 3

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN/PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at $P<0.05$ using Tukey's mean separation test.

Table 4 – Survival level (%) of shoot tips excised from nodal sections sourced from eight *Vitis* species grown *in vitro* with 60, 75, 90 and 105 min of PVS2 exposure, with and without LN exposure, and 2 weeks of cold acclimation.

Species	Identifier	-LN								
		PVS2 exposure duration (min)								
		60			75			90			105		
V. vinifera	Chardonnay	98 ± 3	a		95 ± 5	a		95 ± 5	a		98 ± 3	a	
V. aestivalis	DVIT 1408	93 ± 3	a		95 ± 5	a		95 ± 5	a		75 ± 5	a	
Vitis sp.	DVIT 1445	95 ± 5	a		90 ± 10	a		88 ± 13	a		85 ± 0	a	
V. flexuosa	DVIT 1385	95 ± 5	a		100 ± 0	a		98 ± 3	a		95 ± 0	a	
V. palmata	DVIT 2228.1	98 ± 3	a		88 ± 3	a		88 ± 3	a		85 ± 5	a	
V. riparia	PI 588214	85 ± 5	a		95 ± 5	a		80 ± 0	a		78 ± 3	a	
V. rupestris	DVIT 8166	95 ± 5	a		90 ± 0	a		95 ± 5	a		83 ± 3	a	
V. sylvestris	DVIT 2426.1	93 ± 8	a		98 ± 3	a		98 ± 3	a		93 ± 8	a	
Average		94 ± 1			94 ± 1			92 ± 2			86 ± 3		

Species	Identifier	+LN								
		PVS2 exposure duration (min)								
		60			75			90			105		
V. vinifera	Chardonnay	83 ± 3	ab		98 ± 3	a		85 ± 5	ab		85 ± 15	ab	
V. aestivalis	DVIT 1408	90 ± 5	ab		93 ± 3	ab		85 ± 10	ab		75 ± 5	ab	
Vitis sp.	DVIT 1445	95 ± 5	ab		83 ± 3	ab		90 ± 5	ab		70 ± 5	ab	
V. flexuosa	DVIT 1385	75 ± 5	ab		88 ± 8	ab		75 ± 0	ab		80 ± 10	ab	
V. palmata	DVIT 2228.1	80 ± 10	ab		78 ± 3	ab		78 ± 8	ab		58 ± 8	b	
V. riparia	PI 588214	83 ± 8	ab		75 ± 0	ab		65 ± 0	ab		68 ± 8	ab	
V. rupestris	DVIT 8166	85 ± 5	ab		90 ± 10	ab		85 ± 0	ab		75 ± 0	ab	
V. sylvestris	DVIT 2426.1	93 ± 8	ab		95 ± 0	ab		88 ± 13	ab		90 ± 10	ab	
Average		85 ± 2			87 ± 3			81 ± 3			75 ± 4		

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN/PVS2 exposure combinations are significantly different at $P<0.05$ using Tukey's mean separation test.

Table 5 – Regrowth level (%) of shoot tips excised from nodal sections sourced from eight *Vitis* species grown *in vitro* with 60, 75, 90 and 105 min of PVS2 exposure, with and without LN exposure, and 2 weeks of cold acclimation.

Species	Identifier	-LN			
		PVS2 exposure duration (min)			
		60	75	90	105
V. vinifera	Chardonnay	85 ± 0 ab	85 ± 0 ab	83 ± 3 abc	83 ± 8 abc
V. aestivalis	DVIT 1408	60 ± 0 abcdefgh	72 ± 3 abcdef	63 ± 3 abcdefgh	48 ± 3 fgh
Vitis sp.	DVIT 1445	78 ± 3 abcde	68 ± 3 abcdefg	55 ± 15 cdefgh	58 ± 8 bcdefgh
V. flexuosa	DVIT 1385	88 ± 3 a	85 ± 5 ab	75 ± 5 abcdef	80 ± 0 abcd
V. palmata	DVIT 2228.1	75 ± 5 abcdef	68 ± 8 abcdefg	68 ± 13 abcdefg	60 ± 0 abcdefgh
V. riparia	PI 588214	48 ± 3 fgh	53 ± 3 defgh	43 ± 3 gh	38 ± 3 h
V. rupestris	DVIT 8166	70 ± 5 abcdefg	60 ± 0 abcdefgh	70 ± 5 abcdefg	53 ± 3 defgh
V. sylvestris	DVIT 2426.1	70 ± 5 abcdefg	68 ± 3 abcdefg	73 ± 8 abcdef	50 ± 0 efgh
Average		72 ± 5	70 ± 4	66 ± 4	58 ± 6
Species	Identifier	+LN			
		PVS2 exposure duration (min)			
		60	75	90	105
V. vinifera	Chardonnay	58 ± 3 abcde	70 ± 0 a	58 ± 8 abcde	45 ± 0 abcdefg
V. aestivalis	DVIT 1408	48 ± 3 abcdefg	58 ± 3 abcde	40 ± 5 cdefg	28 ± 8 g
Vitis sp.	DVIT 1445	55 ± 5 abcdef	58 ± 3 abcde	53 ± 3 abcdefg	38 ± 3 defg
V. flexuosa	DVIT 1385	55 ± 5 abcdef	65 ± 5 abc	53 ± 3 abcd	55 ± 10 abcdef
V. palmata	DVIT 2228.1	55 ± 5 abcdef	60 ± 0 abcd	58 ± 3 abcdefg	30 ± 5 fg
V. riparia	PI 588214	33 ± 3 efg	43 ± 3 bcdefg	33 ± 3 efg	28 ± 3 g
V. rupestris	DVIT 8166	45 ± 0 abcdefg	55 ± 5 abcdef	58 ± 8 abcde	40 ± 5 cdefg
V. sylvestris	DVIT 2426.1	55 ± 5 abcdef	68 ± 3 ab	58 ± 8 abcde	48 ± 3 abcdefg
Average		50 ± 3	59 ± 3	51 ± 3	39 ± 4

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at $P<0.05$ using Tukey's mean separation test.

Across the species evaluated by the first technical expert (*V. vinifera* 'Chardonnay', *V. aestivalis, Vitis* sp. (DVIT 1445)*, V. flexuosa, V. palmata, V. riparia, V. rupstris, and V. sylvestris*), shoot tips that were not exposed to LN (-LN) and not-cold acclimated had higher levels of survival (88 to 93%) and regrowth (63 to 71%) compared to the corresponding LN treatment (+LN) (71 to 82% survival and 37 to 53% regrowth (Tables 2,3). Similarly, shoot tips that were not exposed to LN and were cold-acclimated had higher levels of survival (86 to 94%) and regrowth (58 to 72%) compared to the corresponding LN treatment (+LN) (75 to 87% survival and 39 to 59% regrowth (Tables 4, 5).

For the non-cold acclimated, non-LN treated shoot tips, there were some species-specific responses. *V. flexuosa* had the lowest survival levels after 60 min PVS2 (78%), *V. rupestris* had the lowest survival levels after 75 or 90 min PVS2 (83%), and the lowest survival levels were 83% for *V. aestivalis* and 73% for *V. riparia* after 105 min PVS2 (Table 2). Regrowth levels for the non-cold acclimated and non-LN treated shoot tips averaged 71% for 60 min PVS2, 68% for 75 min PVS2, 69% for 90 min PVS2, and 63% for 105 min PVS2. The non-cold acclimated shoot tips with the lowest regrowth levels (-LN) were for *V. riparia* with 60 min PVS2 (48%), *V. riparia* (38%) and *V. rupestris* (53%) with 75 min PVS2, *V. riparia* with 90 min PVS2 (38%), and *V. aestivalis* (50%) and *V. riparia* (33%) with 105 min PVS2 (Table 3, Figure 5). Overall, the *V. riparia* accession responded less positively to the PVS2 treatments than the other species.

With LN, and without cold acclimation, the average survival level across the eight species was 72% for 60 min PVS2, 82% for 75 min PVS2, 80% for 90 min PVS2, and 71% for 105 min PVS2 (Table 2). With LN, and without cold acclimation, regrowth levels were 37% for 60 min PVS2, 49% for 75 min PVS2, 53% for 90 min PVS2, and 41% for 105 min PVS2 (Table 3). For 90 min PVS2 exposures, accessions ranged from 35% regrowth (*V. riparia*) to 72% regrowth (*Vitis* sp. (DVIT 1445)) (Table 3).

There were no differences in the survival and regrowth levels of –LN shoot tips that were cold acclimated at 5^{o}C for 2 weeks and those that were not cold-acclimated for the corresponding PVS2 exposure durations. Survival levels were all 75% or higher with cold acclimation and no LN exposure (Table 4). With cold acclimation and -LN, regrowth levels were 72% for 60 min PVS2, 70% for 75 min PVS2, 66% for 90 min PVS2, and 58% for 105 min PVS2 (Table 5). The lowest regrowth levels (-LN) were obtained for *V. riparia* with 48% after 60 min PVS2 and 53% after 75 min PVS2, *Vitis*

sp. (DVIT 1445) with 55% and *V. riparia* with 43% after 90 min PVS2, and *V. aestivalis* (48%), *Vitis* sp. (DVIT 1445) (58%), *V. riparia* (38%), *V. rupestris* (53%), and *V. sylvestris* (50%) after 105 min PVS2 (Table 5). Although there weren't statistically significant differences between the survival levels of cold-acclimated and non-cold-acclimated shoot tips after LN exposure, there was a trend to have more uniform responses in the cold-acclimated treatments.

Overall, the highest regrowth levels with LN were obtained after 90 min PVS2 and no cold acclimation: *V. vinifera* 'Chardonnay' (58%), *V. aestivalis* (58%), *Vitis* sp. (DVIT 1445) (72%), *V. flexuosa* (48%), *V. palmata* (53%), *V. riparia* (35%), *V. rupestris* (43%), and *V. sylvestris* (63%) (Table 3, Figure 5). In general, shoot tips excised from cold acclimated plants needed shorter PVS2 exposure time than shoot tips that were not cold acclimated; the highest regrowth levels were obtained after 75 min PVS2 with cold acclimation: *V. vinifera* 'Chardonnay' (70%), *V. aestivalis* (58%), *Vitis* sp. (DVIT 1445) (58%), *V. flexuosa* (65%), *V. palmata* (60%), *V. riparia* (43%), *V. rupestris* (55%), and *V. sylvestris* (68%) (Table 5). With the 90 min +LN treatment without cold acclimation, seven of the eight species met our goal of 40% regrowth (Table 3). With 75 min +LN treatment with cold acclimation, all eight species met our goal of 40% regrowth (Table 5).

3.4.2 Application of the droplet-vitrification procedure to additional *Vitis* species using 90 min full-strength PVS2 and no cold acclimation

The droplet-vitrification procedure, with no cold acclimation and 90 min full-strength PVS2, was tested using five additional genotypes (*V. actinifolia*, *V. ficifolia, V. treleasi, V. vinifera* 'Riesling', and *V.* × *novae angelae*) to determine its applicability to additional species. In the control treatment (-LN), shoot tips exposed to full-strength PVS2 for 90 min had an average survival of 95% and an average regrowth of 82%. The corresponding cryoexposed shoot tip survival levels ranged from 73% for *V.* × *novae angelae* to 83% for *V. vinifera* 'Riesling' and regrowth levels ranged from 43% for *V. treleasi* to 68% for *V. vinifera* 'Riesling'. Across the five *Vitis* genotypes, the average level of survival was 78% and the average level of regrowth was 57% (Table 6).

Table 6 – Survival and Regrowth levels (%) of shoot tips excised from nodal sections sourced from five *Vitis* species grown *in vitro* with 90 min PVS2 exposure, with and without LN exposure, without cold acclimation.

Species	Identifier	-LN		+LN	
		Survival %	Regrowth %	Survival %	Regrowth %
V. actinifolia	DVIT 2594.1	90 ± 0 a	73 ± 3 a	78 ± 8 a	63 ± 3 ab
V. ficifolia	DVIT 2008.1	95 ± 0 a	83 ± 3 a	75 ± 10 a	53 ± 3 bc
V. treleasii	DVIT 1410	98 ± 3 a	83 ± 8 a	80 ± 5 a	43 ± 3 c
V. vinifera	Riesling	98 ± 3 a	88 ± 3 a	83 ± 3 a	68 ± 3 ab
V. × novae angelae	DVIT 1457	93 ± 3 a	83 ± 3 a	73 ± 8 a	58 ± 3 ab
Average		95 ± 1	82 ± 1	78 ± 1	57 ± 0

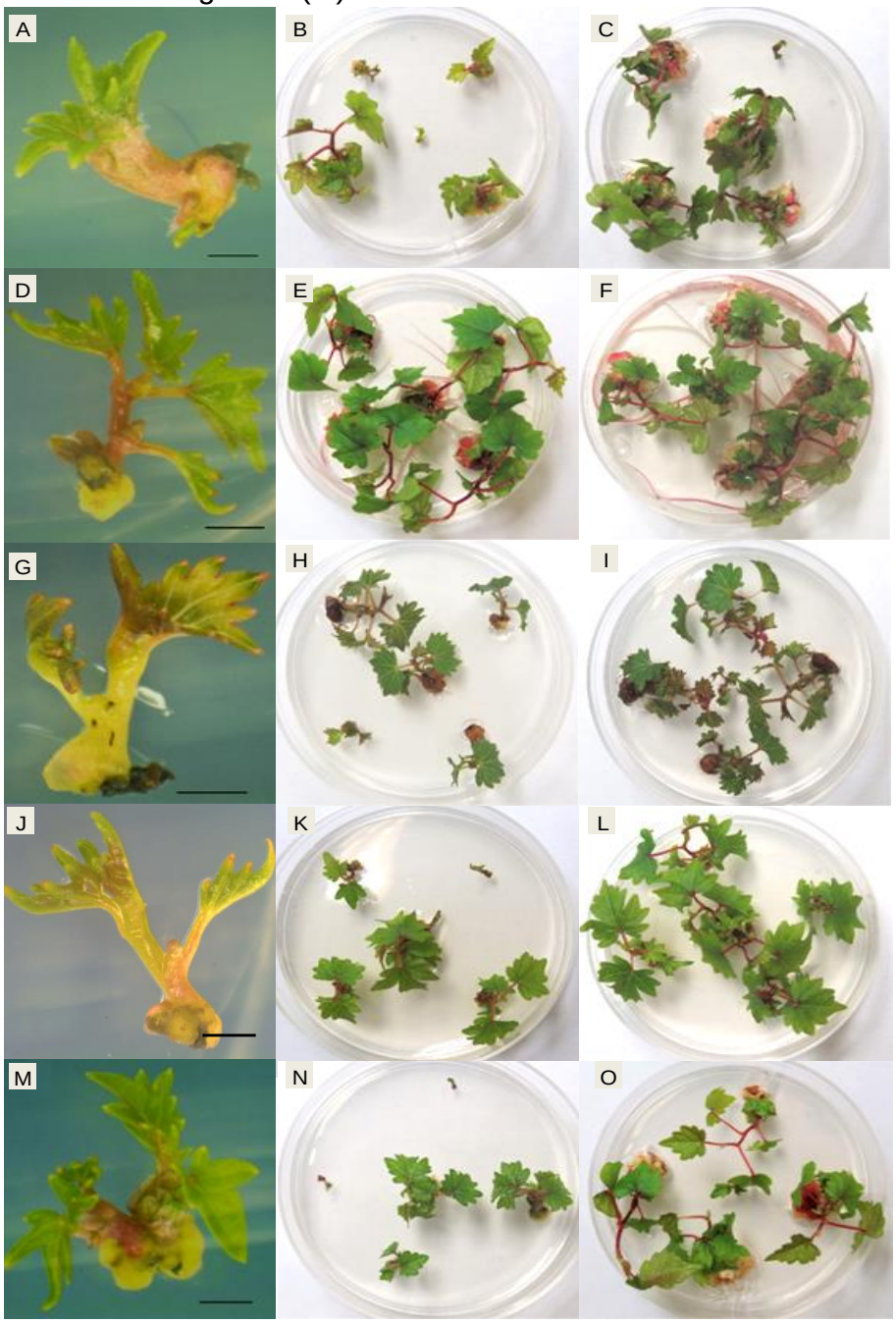
Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within each column are significantly different at P<0.05 using Tukey's mean separation test.

3.4.3 Replication of the droplet-vitrification method by a second technical expert

A second technical expert (R. Bonnart) assessed the survival and regrowth levels in shoot tips of *V. actinifolia, V. ficifolia, V. treleasii, V. vinifera* 'Riesling', *V.* × *novae angelae* that were excised from nodal sections either with or without cold acclimation, exposed to full-strength PVS2 for 60, 75, or 90 min, either with (+LN) or without (-LN) liquid nitrogen exposure (Tables 7-10).

Average survival levels of the non-cold-acclimated and non-LN treated shoot tips of *V. actinifolia, V. treleasii, V. vinifera* 'Riesling', and *V.* × *novae angelae* were 90% for 60 min PVS2, 88% for 75 min PVS2, and 91% for 90 min PVS2 exposure durations (Table 7). Average regrowth levels of the non cold-acclimated and non-LN treated shoot tips for the same four accessions were 74% for 60 min PVS2, 65% for 75 min PVS2, and 64% for 90 min PVS2 exposure durations (Table 8). With LN exposure and no cold acclimation, average shoot tip survival levels were 82% for 60 min PVS2, 78% for 75 min PVS2, and 77% for 90 min PVS2 exposure durations (Table 7) and average shoot tip regrowth levels were 54% for 60 min PVS2, 49% for 75 min PVS2, and 48% for 90 min PVS2 exposure durations (Table 8). In non-cold-acclimated shoot tips, there were no statistically significant differences among accessions with respect to survival and regrowth levels and PVS2 treatments (Tables 7, 8).

Figure 5 – Grapevine plants recovered after 90 min PVS2 treatment and liquid nitrogen (+LN) or without liquid nitrogen (-LN) exposure. *V. aestivalis* shoot tips exposed to LN and recovered for 30 days (**A**) and 2 months (**B**) and without LN exposure after 2 months of regrowth (**C**). *Vitis* sp. (DVIT 1445) shoot tips exposed to LN and recovered for 30 days (**D**) and 2 months (**E**) and without LN exposure after 2 months of regrowth (**F**). Chardonnay shoot tips exposed to LN and recovered for 30 days (**G**) and 2 months (**H**) and without LN exposure after 2 months of regrowth (**I**). *V. flexuosa* shoot tips exposed to LN and recovered for 30 days (**J**) and 2 months (**K**) and without LN exposure after 2 months of regrowth (**L**). *V. palmata* shoot tips exposed to LN and recovered for 42 days (**M**) and 2 months (**N**) and without LN exposure after 2 months of regrowth (**O**).



Source: Prepared by the author, 2018.
Scale Bars: **A=** 1 mm; **D, G, J, M=** 2 mm.

Table 7 – Survival level (%) of shoot tips excised from nodal sections sourced from four *Vitis* species grown *in vitro* with 60, 75 and 90 min of PVS2 exposure, with and without LN exposure, and no cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		60	75	90	60	75	90
V. actinifolia	DVIT 2594.1	93 ± 3 a	88 ± 8 a	93 ± 8 a	90 ± 0 a	85 ± 5 a	80 ± 0 a
V. treleasii	DVIT 1410	87 ± 7 a	87 ± 7 a	90 ± 0 a	70 ± 10 a	73 ± 8 a	68 ± 13 a
V. vinifera	'Riesling'	96 ± 4 a	87 ± 2 a	91 ± 6 a	82 ± 2 a	86 ± 2 a	85 ± 5 a
V. × novae angelae	DVIT 1457	85 ± 11 a	91 ± 1 a	90 ± 5 a	85 ± 0 a	69 ± 1 a	77 ± 13 a
Average		90 ± 2	88 ± 2	91 ± 2	82 ± 2	78 ± 1	77 ± 3

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at P<0.05 using Tukey's mean separation test.

Table 8 – Regrowth level (%) of shoot tips excised from nodal sections sourced from four *Vitis* species grown *in vitro* with 60, 75 and 90 min of PVS2 exposure, with and without LN exposure, and no cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		60	75	90	60	75	90
V. actinifolia	DVIT 2594.1	75 ± 10 a	70 ± 10 a	65 ± 10 a	73 ± 13 a	62 ± 18 a	58 ± 2 a
V. treleasii	DVIT 1410	57 ± 4 a	47 ± 7 a	44 ± 4 a	34 ± 1 a	32 ± 2 a	40 ± 0 a
V. vinífera	'Riesling'	83 ± 3 a	64 ± 9 a	69 ± 19 a	50 ± 6 a	54 ± 6 a	35 ± 5 a
V. × novae angelae	DVIT 1457	83 ± 7 a	82 ± 7 a	78 ± 2 a	58 ± 3 a	50 ± 6 a	59 ± 7 a
Average		74 ± 2	65 ± 1	64 ± 4	54 ± 3	49 ± 4	48 ± 1

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at P<0.05 using Tukey's mean separation test.

Table 9 – Survival level (%) of shoot tips excised from nodal sections sourced from four *Vitis* species grown *in vitro* with 60, 75 and 90 min of PVS2 exposure, with and without LN exposure, and 2 weeks of cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		60	75	90	60	75	90
V. actinifolia	DVIT 2594.1	94 ± 7 a	94 ± 0 a	95 ± 5 a	80 ± 0 a	90 ± 5 a	98 ± 3 a
V. ficifolia	DVIT 2008.1	83 ± 18 a	75 ± 0 a	79 ± 9 a	63 ± 3 a	60 ± 15 a	69 ± 1 a
V. treleasii	DVIT 1410	100 ± 0 a	95 ± 5 a	100 ± 0 a	59 ± 14 a	72 ± 12 a	78 ± 3 a
V. vinifera	'Riesling'	100 ± 0 a	95 ± 5 a	93 ± 8 a	78 ± 3 a	85 ± 5 a	88 ± 13 a
Average		94 ± 4	90 ± 1	92 ± 2	70 ± 3	77 ± 3	83 ± 3

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN/PVS2 exposure combinations are significantly different at P<0.05 using Tukey's mean separation test.

Table 10 – Regrowth level (%) of shoot tips excised from nodal sections sourced from four *Vitis* species grown *in vitro* with 60, 75 and 90 min of PVS2 exposure, with and without LN exposure, and 2 weeks of cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		60	75	90	60	75	90
V. actinifolia	DVIT 2594.1	84 ± 4 a	80 ± 13 a	76 ± 11 a	54 ± 7 ab	85 ± 0 a	70 ± 5 ab
V. ficifolia	DVIT 2008.1	54 ± 19 a	58 ± 8 a	73 ± 8 a	45 ± 5 b	38 ± 13 b	47 ± 3 b
V. treleasii	DVIT 1410	90 ± 0 a	73 ± 8 a	72 ± 2 a	42 ± 7 b	43 ± 13 b	43 ± 3 b
V. vinifera	'Riesling'	76 ± 9 a	77 ± 4 a	65 ± 15 a	63 ± 3 ab	73 ± 3 ab	68 ± 3 ab
Average		76 ± 4	72 ± 2	71 ± 3	51 ± 1	60 ± 3	57 ± 1

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN /PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at P<0.05 using Tukey's mean separation test.

In shoot tips that were cold acclimated and non-LN treatment the average survival levels were 94, 90, and 92% corresponding to 60, 75, and 90 min PVS2 exposure, respectively (Table 9). With LN exposure, cold-acclimated shoot tips had average survival levels of 70% for 60 min PVS2, 77% for 75 min PVS2, and 83% for 90 min PVS2 exposure durations (Table 9). There were significant differences among accessions with respect to regrowth levels of cold-acclimated shoot tips after LN exposure (Table 9). With cold-acclimation and LN exposure, *V. actinifolia* (85% regrowth) and *V. vinifera* cv 'Riesling' (73% regrowth) had the highest regrowth levels and *V. ficifolia* had the lowest regrowth levels (38%) after 75 min PVS2 exposure (Table 10).

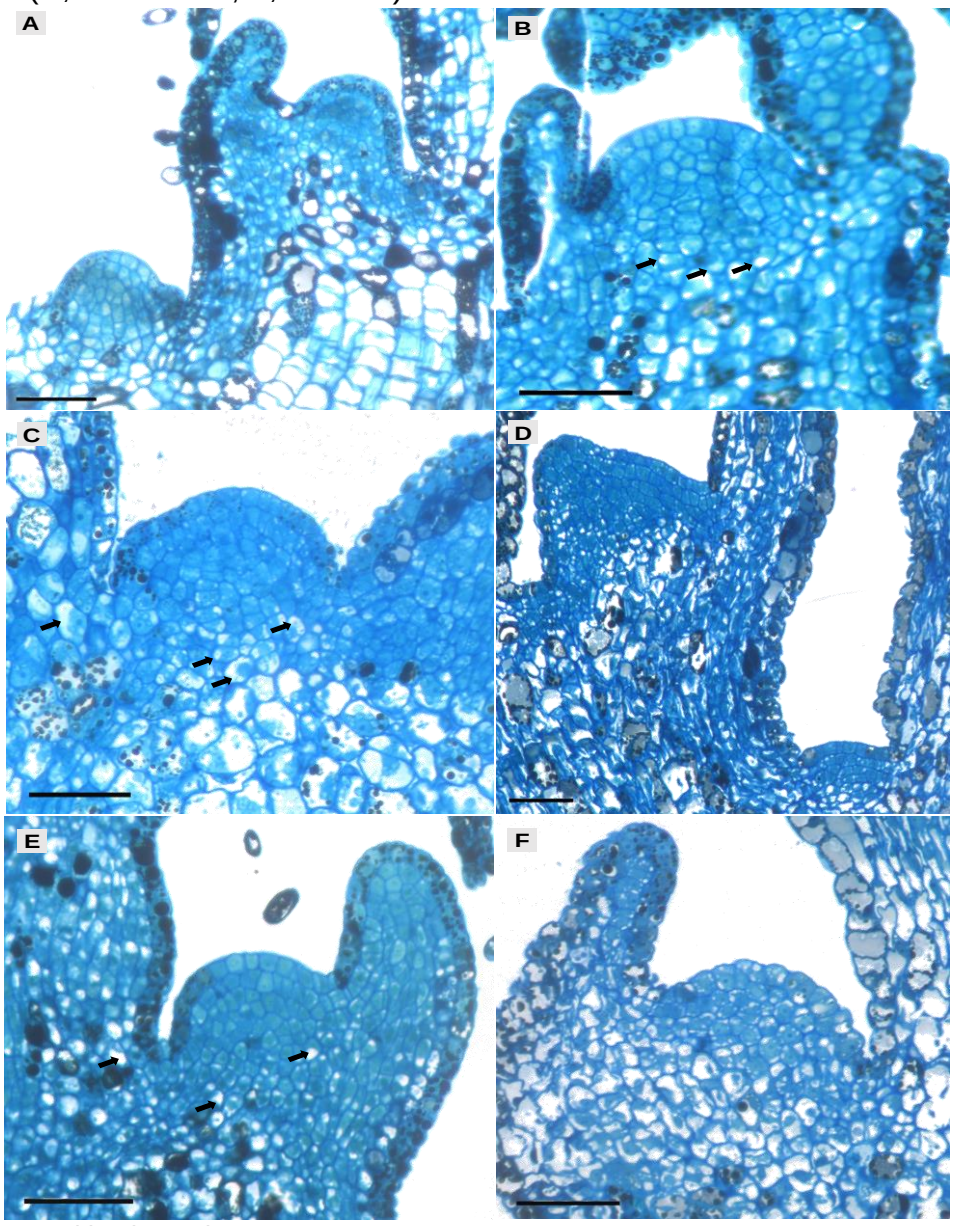
Comparisons between the two technical experts for the same *Vitis* accessions (Table 6 vs Tables 7,8) revealed no statistical differences between the survival and regrowth levels of *V. actinifolia*, *V. treleasii*, *V. vinifera* 'Riesling', and *V.* × *novae angelae* shoot tips cryopreserved (+LN) that were exposed to full-strength PVS2 for 90 minutes, without cold-acclimation. Technical expert 1 had an average survival level of 78% and average regrowth level of 58% for the 4 accessions, and Technical expert 2 had an average survival of 77% and average regrowth level of 48% for the same accessions.

3.4.4 Histological studies of cryopreserved shoot tips

Histological studies were performed using 1 mm *Vitis flexuosa* shoot tips. Representative shoot tip cross sections for each cryoprotective and recovery treatment are shown in Figure 6. In some cases, both a primary apical meristem (AM) as well as one or more lateral meristems (LM) were observed in the shoot tip cross sections (Figure 6A). Shoot tip meristem cells were unplasmolyzed after 3 days of preculture (Figure 6B), 90 min PVS2 (Figure 6C), 90 min PVS2 + 6 days of recovery (Figure 6D), 90 min PVS2 + LN (Figure 6E), and 90 min PVS2 + LN + 6 days of recovery (Figure 6F). In contrast, some of the non-meristematic cells exhibited plasmolysis in the various treatments (arrows, Figure 6B-F). Histological observations revealed a high level of cellular preservation of the apical dome (AD) region and at the base of the leaf primordia after LN exposure whereas cells in the lower part of AD and in the older leaf primordia (LPs) exhibited some damage (Figure 6E). After LN exposure and 6 days of

regrowth, we observed shoot tips with signs of cellular division, as well as some surrounding damaged cells (Figure 6F).

Figure 6 – Histological observations in *Vitis flexuosa* shoot tips after successive steps of cryopreservation procedure. Cross section of *Vitis* shoot tips sampled at (**A**) control, shoot tips excised from pre-treatment nodal sections; (**B**) shoot tips precultured on medium for 3 days; shoot tips treated with cryoprotectants (30 min at 22ºC in half-strength PVS2 followed by full-strength PVS2 at 0 ºC for 90 min), not exposed or exposed to LN, and then diluted with 1.2 M sucrose (**C**, without LN; **E**, with LN); shoot tips treated with cryoprotectants and were either not exposed or exposed to LN, diluted with 1.2 M sucrose and that were incubated in recovery medium for 6 days (**D**, without LN; **F**, with LN).



Source: Prepared by the author, 2018.
AM, apical meristem; LM, lateral meristems; LP, leaf primordia. Arrows, regions where plasmolysis has occurred. Scale Bars: **A-F=** 50 µm.

3.5 DISCUSSION

Cryopreservation is a reliable and cost-effective method for the long-term storage of vegetatively propagated plant germplasm: once collections are established in LN, cryopreserved collections require minimal space, labor, and maintenance (WANG et al., 2018a; BENELLI; DE CARLO; ENGELMANN, 2013). In this paper, we present a droplet vitrification technique effective for the cryopreservation of the *Vitis* species using apical shoot tips from *in vitro* plants. We demonstrate that this method results in regrowth levels of at least of 43% for thirteen genotypes, two of which are *V. vinifera* cultivars, representing 12 *Vitis* species.

Vitis shoot tip cryopreservation protocols have previously been reported using encapsulation-dehydration (MARKOVIĆ et al., 2013a; BAYATI; SHAMS-BAKHSH; MOIENI, 2011; WANG et al., 2003a; ZHAI et al., 2003; ZHAO et al., 2001; PLESSIS; LEDDET; DEREUDDRE, 1991, 1993), encapsulation-vitrification (GRIBAUDO et al., 2012; BENELLI; LAMBARDI; FABBRI, 2003), vitrification (BETTONI et al., 2015b; HASSAN; HAGGAG, 2013; GANINO et al., 2012; SHATNAWI et al., 2011; MATSUMOTO; SAKAI, 2003; WANG et al., 2003a, b), and droplet vitrification (PATHIRANA et al., 2015, 2016; MARKOVIĆ et al., 2013a, b; MARKOVIĆ et al., 2014) methods. The droplet vitrification technique is simple and effective and has been applied to a wide range of plant genera (MATHEW et al., 2018; VOLK et al., 2017b; SOUZA et al., 2015; LI et al., 2015; WHITAKER et al., 2010; PANIS; PIETTE; SWENNEN, 2005). In *Vitis*, previous reports focused on developing and applying methods primarily to *V. vinifera* cultivars, and it has been difficult to extend these procedures to the diverse range of *Vitis* species conserved within plant genebanks (BI et al., 2017; BETTONI et al., 2016). Our goal was to identify a protocol that effectively cryopreserved diverse *Vitis* species to help eliminate the genotype-specific responses that limit the application of *Vitis* cryopreservation in genebanks.

Vitis cryopreservation protocols have been established using apical and axillary shoot tips excised directly from 3 week-old up to 5 month-old *in vitro* stock plants (BENELLI; LAMBARDI; FABBRI, 2003; MATSUMOTO; SAKAI, 2003; WANG et al., 2000) or axillary shoot tips excised directly from 1 year-old greenhouse plants (HASSAN; HAGGAG, 2013). In a recent study, Pathirana et al. (2016) found that shoot tips located at different positions along the shoot have differing regrowth responses to PVS2 exposure. The quality and physiological state of the stock cultures was thought

to be a key factor in successful *Vitis* cryopreservation (BI et al., 2017; MARKOVIĆ et al., 2014; ENGELMANN, 2011). In our protocol, we used *in vitro* plants as source materials. Nodal sections from *in vitro* plants were placed on shooting medium for 2 weeks to generate shoots from which uniform 1 mm apical shoot tips could be excised. Previously, Marković et al. (2014) observed shoot tips sampled from microcuttings had higher regrowth compared to shoot tips sampled directly from *in vitro* stock plants.

Successful cryopreservation procedures are dependent upon having optimized pre-treatment conditions (VOLK; SHEPHERD; BONNART, 2018; VOLK; CASPERSEN, 2017c). Pre-treatments can differ by species and may include cold treatment exposure (MATHEW et al., 2018); adding osmotic agents, such as sorbitol and sugars (LYNCH et al., 2011); antioxidants, such as glutathione reductase and ascorbic acid (MATHEW et al., 2018; REED, 2014; SHEPHERD; BONNART; VOLK, 2013; VOLK; SHEPHERD; BONNART, 2018) and elicitors of defense-related proteins in plants, such as salicylic acid (PATHIRANA et al., 2016; VOLK; SHEPHERD; BONNART, 2018). We previously determined that osmotic agents and salicylic acid improved the cryopreservability of *Vitis* species (VOLK; SHEPHERD; BONNART, 2018). The positive effect of the addition of antioxidants and salicylic acid in pre-treatment and preculture media on plantlet growth and development after LN exposure has been reported for kiwifruit (MATHEW et al., 2018), grapevine (PATHIRANA et al., 2016; SHEPHERD; BONNART; VOLK, 2013; VOLK; SHEPHERD; BONNART, 2018), *Arabidopsis* (CHEN et al., 2015), blackberry (UCHENDU et al., 2010a, b) and raspberry (WANG; VALKONEN, 2009a).

There are reports that the addition of BA^3 to the shooting medium has a positive effect on regrowth after LN exposure (MARKOVIĆ et al., 2014). In addition to BA^3, we included salicylic acid, glutathione (reduced form) and ascorbic acid in the pretreatment medium in an attempt to reduce the formation of reactive oxygen species (ROS) during cryoprotectant and LN exposure (CHEN et al., 2015; WHITAKER et al., 2010; ROACH et al., 2008).

The influence of a cold acclimation pretreatment (5 ^oC for 2 weeks) prior to shoot tip desiccation was also assessed. Our results showed the use of cold pre-treatment did not significantly improve the survival and regrowth of *Vitis* shoot tips after cryopreservation. For some species, cold acclimation pre-treatments improved the regrowth and quality of regenerated plants after cryopreservation (CHANG; REED, 2000, 2001; KACZMARCZYK et al., 2008; MATHEW et al., 2018; PANTA et al., 2015;

KUSHNARENKO; ROMADANOVA; REED, 2009), and may be helpful for the cryoprocessing of some untested *Vitis* species and/or cultivars.

Herein, we investigated whether the droplet-vitrification procedure described could be performed by another technician in the laboratory. We obtained similar regrowth levels for the cryopreserved shoot tips across all the evaluated accessions when the experiments were performed by two technicians with skills in tissue culture and cryopreservation procedures. The reproducibility of results across technicians helps demonstrate that the described procedures can be replicated, thus increasing the likelihood of success for genebanks that may adopt this method for Vitis cryopreservation (REED et al., 2004; REED, 2001).

Histological observations indicated that the cells in the apical dome and the base of the leaf primordia appeared to retain their structural integrity during the cryopreservation and recovery processes. We observed that the meristematic cells had minimal damage after LN exposure. This makes our *Vitis* cryopreservation procedure a candidate for use in cryotherapy research, where pathogens are eliminated from infected clonal plant materials as a result of liquid nitrogen exposure (BETTONI et al., 2018b; BETTONI et al., 2016; WANG et al., 2014). The implementation of the cryopreservation protocol to a wide range of genetic diversity, as we have presented, may facilitate the use of cryotherapy methods to eradicate viruses (BETTONI et al., 2018b; BETTONI et al., 2016; PATHIRANA et al., 2015; BAYATI; SHAMS-BAKHSH; MOIENI, 2011; WANG; VALKONEN, 2009a).

We describe a droplet-vitrification cryopreservation technique with high regrowth levels for the cryopreservation of *Vitis* species that makes use of shoot tips derived from *in vitro* stock plants. Procedural validation and technology transfer are necessary for the routine implementation of methods in multiple genebanks (REED et al., 2004). The high regrowth levels that we obtained in cryopreserved shoot tips when experiments were independently performed by two technicians with skills in tissue culture and cryopreservation procedures suggest that this method may soon be ready for implementation for genebank *Vitis* collections.

4 MODIFICATIONS TO A *VITIS* SHOOT TIP CRYOPRESERVATION PROCEDURE: EFFECT OF SHOOT TIP SIZE AND USE OF CRYOPLATES[2]

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4.1 ABSTRACT

Cryopreservation has been considered a preferred method for the long-term storage of plant germplasm, especially to efficiently conserve and maintain the genetic integrity of genebank materials. Droplet vitrification procedures have been developed to cryopreserve *Vitis* shoot tips from *in vitro*-grown plants. This research focused on optimizing shoot tip sizes for droplet vitrification and the feasibility of using cryo-plates for *Vitis* cryopreservation. Uniform apical shoot tips were obtained from nodal sections cultured from *in vitro* grown stock plants of *V. aestivalis* and *Vitis* sp. (DVIT 1445). Shoot tips were precultured for 3 days on medium containing 0.3 M sucrose, salicylic acid, glutathione (reduced form), and ascorbic acid. They were cryopreserved using either droplet vitrification on aluminum foil strips or by placement in calcium alginate gel in the wells of aluminium cryo-plates. Shoot tips were then treated with loading solution followed by PVS2 treatment prior to liquid nitrogen (LN) exposure. Shoot tips were warmed in unloading solution and placed on recovery medium. The highest regrowth levels of cryopreserved shoot tips were obtained using 1 mm shoot tips and a PVS2 exposure for 90 min at 0°C with the droplet vitrification method on aluminum foil strips or by using 30 min of PVS2 at 22°C using V cryo-plates. Shoot tip size is an important factor in the cryopreservability of *Vitis* shoot tips; 1 mm shoot tips were the most successful for the droplet vitrification cryopreservation method that was tested. In addition, the V cryo-plate cryopreservation technique described herein can be easily executed and results in high regrowth levels (≥70%) with quality plants obtained from cryo-exposed shoot tips, making it a practical and promising *Vitis* cryopreservation methodology.

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Keywords*:* Cryo-plate. Vitrification. Genebanking. Grapevine.

4.1 INTRODUCTION

Traditionally, genebank collections of vegetatively propagated crops, including grapevine, have been maintained primarily in field collections and in *in vitro* culture collections (POSTMAN et al., 2006). *Vitis* field collections are readily available for use in breeding programs and propagation; however, they are expensive to maintain and are vulnerable to abiotic stresses and biotic threats, such as pathogens and pests (PATHIRANA et al., 2016; MARKOVIĆ et al., 2013a). *In vitro* culture back-up has been be used for short-term preservation of *Vitis* germplasm (MAIA et al., 2015), but it has some limitations because it is labor-intensive, has a high cost of maintenance, has a risk of microbial contamination and technical failures, and may experience somaclonal variation (MATHEW et al., 2018). Alternative *ex situ* conservation strategies for plant genetic resources should be considered that allow for plant materials to be secured for long periods of time and preserve the genetic integrity during storage (HALMAGYI et al., 2010).

Cryopreservation, the storage of biological materials in liquid nitrogen (LN, -196 °C) or liquid nitrogen vapor (LNV, -165 to -190 °C), has been considered to be a preferred method for the long-term storage of plant germplasm, especially for maintaining genetic integrity. In liquid nitrogen, plant tissue is preserved in a state where cellular divisions and metabolic processes cease and the risk of contamination is minimized (WANG et al., 2018a). It is also cost effective for long-term maintenance (BENSON, 2008).

Several protocols have been developed for *Vitis* shoot tip cryopreservation including encapsulation-dehydration (PLESSIS; LEDDET; DEREUDDRE, 1991; WANG et al., 2003b; BAYATI; SHAMS-BAKHSH; MOIENI, 2011; MARKOVIĆ et al., 2013a), vitrification (MATSUMOTO; SAKAI, 2003; WANG et al., 2003b), encapsulation-vitrification (GRIBAUDO et al., 2012), and more recently, droplet-vitrification (PATHIRANA et al. 2015, 2016; MARKOVIĆ et al., 2013a, b). To the best of our knowledge, implementation of *Vitis* cryo-storage as a routine preservation method is still limited.

We previously reported the successful cryopreservation of 12 *Vitis* species using a droplet vitrification technique that utilized apical shoot tips excised from nodal

sections that were grown on medium supplemented with antioxidants (BETTONI et al., 2018c). This previous work focused on using 1 mm shoot tips. In the current research, we compare 1, 1.5, and 2 mm shoot tip sizes to determine if larger, more easily excised shoot tips can be cryopreserved using the same optimized method.

Recently, novel cryopreservation protocols using aluminum cryo-plates were developed, based on PVS2 vitrification (V cryo-plate) (YAMAMOTO et al., 2011) and air dehydration (D cryo-plate) (NIINO et al., 2013) methods. The aluminium cryo-plate methods have been successfully reported with a diverse range of genetic resources including carnation (SEKIZAWA et al., 2011), chrysanthemum (YAMAMOTO et al., 2011), mint (YAMAMOTO et al., 2012a), mulberry (YAMAMOTO et al., 2012b), strawberry (YAMAMOTO et al., 2012c), mat rush (NIINO et al., 2013), perilla (MATSUMOTO et al., 2014), date palm (SALMA et al., 2014), sugarcane (RAFIQUE et al., 2015; 2016), potato (ARIZAGA et al., 2017a; Yamamoto et al., 2015), plum (VUJOVIĆ et al., 2015), *Clinopodium odorum* (ELGELMANN-SYLVESTRE; ELGELMANN, 2015), persimmon (MATSUMOTO et al., 2015), blueberry (DHUNGANA et al., 2017), ulluco (ARIZAGA et al., 2017b) and wild passionfruit (SIMÃO et al., 2017). We modified our previously reported *Vitis* droplet cryopreservation protocols to identify a V cryo-plate cryopreservation method that could be applied to *Vitis* shoot tips, in which there is less shoot tip handling during the cryopreservation process (YAMAMOTO et al., 2011).

In the cryo-plate cryopreservation method, shoot tips are adhered to wells in aluminium cryo-plates by encapsulating them in droplets of calcium alginate. The main advantages of the cryo-plate method are that samples can be easily transferred between solutions with minimal damage and that rapid cooling and warming rates can be obtained as shoot tips are exposed to and warmed from liquid nitrogen (MATSUMOTO et al., 2015; NIINO et al., 2013; YAMAMOTO et al., 2012a).

4.2 MATERIALS AND METHODS

4.2.1 Plant Material and pre-treatments

Vitis accessions *V. aestivalis* (DVIT 1408) and *Vitis* sp. (DVIT 1445) were originally received from the USDA-ARS National Clonal Germplasm Repository for Tree Fruit, Nut Crops and Grapes in Davis, CA, USA. *In vitro* stock cultures were maintained and subcultured every 8-12 weeks in glass culture vessels (89 x 170 mm) on MS medium (MURASHIGE; SKOOG, 1962), containing 30 g L^{-1} sucrose, 0.175 mg L^{-1} IAA^3 (3-Indoleacetic acid) and 2.5 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving). Cultures were placed in a growth room at 25 °C, under a photoperiod of 16 h light day^{-1} with a light intensity of 40 µM m^{-2} s^{-1}.

4.2.2 Pre-treatment and preculture

Nodal sections were obtained from 2- to 3-month old *in vitro* stock plants and placed on 50 mL pretreatment medium (MS containing 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA^3 (N^6-benzyladenine), 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form) and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving)) in plastic Petri dishes (100 x 25 mm) at a density of 40 nodal sections per plate, and cultured for 2 weeks in the same conditions as the *in vitro* stock cultures (Figure 7B-C). Nodal sections were cold acclimated at 5ºC with a 16 h photoperiod for an additional 2 weeks (when applied).

Apical shoot tips (1-2 mm) (Figure 7D) were excised from nodal sections and pre-cultured on preculture medium (half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form) and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving)) for 3 days at 25 ºC in darkness.

4.2.3 Droplet vitrification cryopreservation and warming

This experiment was carried out with the aim of optimizing the shoot tip size. Apical shoot tips with lengths of 1, 1.5 or 2 mm were excised from nodal sections either with or without cold acclimation. Shoot tips were pre-cultured as described above and

then placed in loading solution (half-strength MS + 2M glycerol + 0.4 M sucrose at pH 5.7 (pH 6.9 prior to autoclaving)) for 20 min at 22 ^{o}C followed by half-strength PVS2 (filter sterilized solution consisting half-strength MS + 15% (w/v) glycerol + 7.5% (w/v) ethylene glycol (EG) +7.5% (w/v) dimethyl sulfoxide (DMSO) + 0.4 M sucrose at pH 5.8, MATSUMOTO; SAKAI et al., 2003) for 30 min at 22^{o}C and full-strength PVS2 (filter sterilized solution, half-strength MS + 30% (w/v) glycerol + 15% (w/v) EG +15% (w/v) DMSO + 0.4 M sucrose at pH 5.8, SAKAI; KOBAYASHI; OIYAMA, 1990)) at 0 ^{o}C for 90 min. Two minutes before the end of the each treatment, PVS2-treated shoot tips were placed onto a thin layer of PVS2 on sterile aluminum foil strips (6 x 25 mm) and then plunged into liquid nitrogen (LN). After one hour of LN exposure, the aluminum foil strips with shoot tips were warmed quickly by inverting the strips into unloading solution (half-strength MS + 1.2 M sucrose at pH 5.7 (pH 7.5 prior to autoclaving)) at 22^{o}C for 20 min.

For recovery, shoot tips were placed onto recovery medium 1 (1/2X MS macroelements, 1X MS microelements, and *Vitis* vitamins (100 mg L^{-1} myo inositol, 10 mg L^{-1} thiamine HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} Ca pantothenate, 0.01 mg L^{-1} biotin, 2 mg L^{-1} glycine, without NH_4, supplemented with 0.6 M sucrose and 8 g L^{-1} agar at pH 5.7 (pH 7.0 prior to autoclaving)) overnight in the dark and shoot tips were or were not removed from alginate beads and transferred to recovery medium 2 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins), without NH_4, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA^3 and 8 g L^{-1} agar at pH 5.7 (pH 6.5 prior to autoclaving)) and cultured for 2 weeks at 25 ^{o}C in darkness. They were then transferred onto recovery medium 3 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA^3 and 8 g L^{-1} agar at pH 5.7 (pH 6.5 prior to autoclaving)) and grown in the same conditions as the *in vitro* stock cultures.

4.2.4 V cryo-plate cryopreservation and warming

The aluminium cryo-plates used for the V cryo-plate protocol were nº 2 (37 x 7 x 0.5 mm), containing 12 circular wells with a diameter of 1.5 mm and a depth of 0.75 mm (NIINO; YAMAMOTO, 2017). The V cryo-plate procedures were derived from a protocol described by Yamamoto et al. (2012a). The V cryo-plates used were kindly provided by Prof. Takao Niino (University of Tsukuba, Tsukuba, Japan).

Droplets (2 µL) of 2 % sodium-alginate solution (medium viscosity- 3,500 cps; Sigma® A-2033) in a calcium-free MS basal medium supplemented with 0.4 M sucrose at pH 5.7 (pH 6.4 prior to autoclaving) were placed into each well into the cryo-plates. Precultured apical shoot tips (1 mm) were placed individually into each well, and 1.5 µL sodium-alginate solution was added to cover the shoot tips completely (Figure 7E). The calcium chloride solution (0.1 M calcium chloride in MS basal medium supplemented with 0.4 M sucrose at pH 5.7 (pH 6.4 prior to autoclaving)) was added dropwise to the cryo-plate until all the wells were covered and kept at 22 ºC for 20 min for polymerization (Figure 7F). The solution was then removed gently from the cryo-plate with a micropipette.

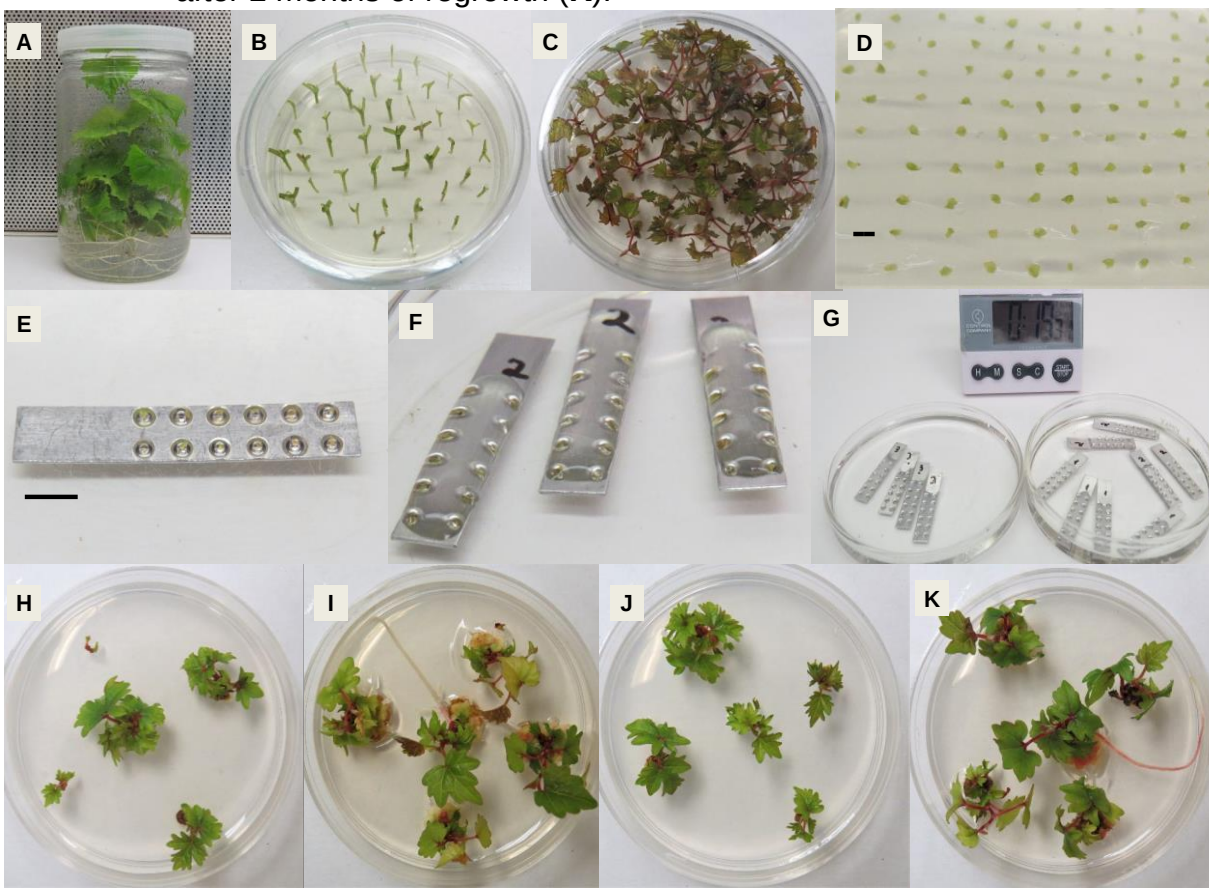
Cryo-plates with shoot tips were placed into 25 mL loading solution in Petri dishes for 30 min at 22 ºC. Cryo-plates were removed from the loading solution, tapped on filter paper in a Petri dish to remove the excess of the loading solution and placed into 25 mL full-strength PVS2 solution in a Petri dish at 22 ºC for 30, 40 or 50 minutes (Figure 7G). Cryo-plates were then tapped on filter paper in a Petri dish to remove the excess of the PVS2 and plunged into LN for 1h and warmed into 25 mL unloading solution in Petri dishes at 22 ºC for 20 minutes. Alginate beads were then detached from the cryo-plates and shoot tips were extracted from alginate beads and transferred onto the recovery medium as used for the droplet-vitrification method.

In an additional experiment, shoot tips were treated on the cryo-plates as described, except that shoot tips were placed in PVS2 at 22 ºC for 40 min and plunged into LN for 1h and warmed in unloading solution, then shoot tips were not extracted from the alginate beads and transferred onto the recovery medium.

4.2.5 Regrowth and data analysis

Shoot tip survival (green cell mass or leaf tissue) and regrowth (organized shoots with at least one leaf) were evaluated 8 weeks after plating on regrowth medium. Each experiment was performed with two replicates of 20 shoot tips for each treatment. Statistical analysis was performed using Tukey’s test to compare the means and determine significant differences ($P \leq 0.05$).

Figure 7 – The V cryo-plate procedure for *in vitro* grapevine. Nodal sections were excised from *in vitro* stock plants (**A**) and placed on pretreatment medium (**B**) and grown for 2 weeks (**C**). Shoot tips were incubated on the preculture medium (**D**), placed on sodium alginate solution in the wells of the cryo-plate (**E**), then polymerized with calcium chloride solution (**F**) and osmoprotected on loading solution (**G**-left) followed by full-strength PVS2 (**G**-right) at 22 ºC. They were then plunged into LN. After 1 h, shoot tips adhering to cryo-plates were warmed into unloading solution at 22ºC and then transferred to recovery medium. *V. aestivalis* and *Vitis* sp. (DVIT 1445) shoot tips were grown on medium to assess regrowth. *V. aestivalis* shoot tips exposed to LN and recovered for 2 months (**H**) and without LN exposure after 2 months of regrowth (**I**). *Vitis* sp. (DVIT 1445) shoot tips exposed to LN and recovered for 2 months (**J**) and without LN exposure after 2 months of regrowth (**K**).



Source: Prepared by the author, 2018.
Scale Bars: **D=** 2 mm; **E=** 7 mm.

4.3 RESULTS

4.3.1 Effect of shoot-tip size on survival and regrowth levels

Vitis shoot tips that were not exposed to LN (-LN) had higher levels of survival and regrowth compared to the corresponding LN treatment (+LN) (Table 11, 12) when the droplet vitrification method was applied. There were no overall significant differences among shoot tip sizes across the -LN treatments with respect to survival or regrowth. The 5 ^{o}C 2 week cold acclimation treatment did not significantly affect shoot tip survival and regrowth levels in –LN shoot tips, independent of the shoot tip size (Table 11, 12).

Shoot tip size significantly affected survival and regrowth levels after liquid nitrogen exposure, with increased shoot tip size significantly decreasing the regrowth levels (Table 11, 12). There were no differences between +LN shoot tips that were cold acclimated and those that were not cold-acclimated. With LN exposure, shoot tips that were 1 mm and 1.5 mm had higher survival levels (86 and 76%, respectively) than shoot tips that were 2 mm (53%) (Table 11). After LN exposure, the highest regrowth levels occurred with 1 mm shoot tips; regrowth levels for *V. aestivalis* and *Vitis* sp. (DVIT 1445) ranged from 53 to 70% for +LN shoot tips that were not cold-acclimated and from 48 to 58% for +LN shoot tips that were cold-acclimated (Table 12). Regrowth levels decreased when shoot tips were longer than 1 mm; average regrowth levels were 38 and 23% when shoot tips 1.5 and 2 mm in size were cryopreserved, respectively (Table 12).

Table 11 – Survival level (%) of shoot tips excised with 1, 1.5 and 2 mm from nodal sections sourced from *V. aestivalis* and *Vitis* sp. (DVIT 1445) grown *in vitro* with 90 min of PVS2 exposure, with and without LN exposure, using the droplet vitrification technique.

Species	Identifier	Cold Acclimation	-LN			+LN		
			1 mm	1.5 mm	2 mm	1 mm	1.5 mm	2 mm
V. aestivalis	DVIT 1408	No	95 ± 0 a	98 ± 3 a	100 ± 0 a	88 ± 3 ab	70 ± 0 bcd	55 ± 5 d
Vitis sp.	DVIT 1445	No	95 ± 5 a	100 ± 0 a	100 ± 0 a	93 ± 3 a	78 ± 3 abc	70 ± 5 bcd
V. aestivalis	DVIT 1408	Yes	100 ± 0 a	100 ± 0 a	98 ± 3 a	78 ± 3 abc	80 ± 0 abc	50 ± 5 d
Vitis sp.	DVIT 1445	Yes	98 ± 3 a	98 ± 3 a	100 ± 0 a	85 ± 0 abc	65 ± 5 cd	53 ± 8 d
Average			97 ± 1	99 ± 1	99 ± 1	86 ± 1	73 ± 1	53 ± 1

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at P<0.05 using the Tukey's mean separation test.

Table 12 – Regrowth level (%) of shoot tips excised with 1, 1.5 and 2 mm from nodal sections sourced from *V. aestivalis* and *Vitis* sp. (DVIT 1445) grown *in vitro* with 90 min of PVS2 exposure, with and without LN exposure, using the droplet vitrification technique.

Species	Identifier	Cold Acclimation	-LN			+LN		
			1 mm	1.5 mm	2 mm	1 mm	1.5 mm	2 mm
V. aestivalis	DVIT 1408	No	78 ± 3 ab	95 ± 0 ab	100 ± 0 a	53 ± 3 abc	30 ± 0 de	13 ± 3 e
Vitis sp.	DVIT 1445	No	75 ± 5 b	88 ± 8 ab	93 ± 8 ab	70 ± 0 a	43 ± 3 bcd	35 ± 10 cd
V. aestivalis	DVIT 1408	Yes	78 ± 3 ab	95 ± 5 ab	95 ± 0 ab	48 ± 3 bcd	38 ± 3 bcd	13 ± 3 e
Vitis sp.	DVIT 1445	Yes	80 ± 5 ab	95 ± 0 ab	98 ± 3 ab	58 ± 3 ab	43 ± 3 bcd	33 ± 4 cde
Average			78 ± 1	93 ± 2	96 ± 2	57 ± 1	38 ± 1	23 ± 2

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at P<0.05 using the Tukey's mean separation test.

4.3.2 Cryopreservation using V cryo-plate method

In vitrification protocols, the length of PVS2 exposure must be optimized to obtain a level of regrowth after LN exposure (NIINO; YAMAMOTO, 2017; WANG et al., 2003b). Herein, we compared the response of shoot tips to 30, 40, and 50 min PVS2 treatments (at 22ºC) with and without cryoexposure using aluminum cryo-plates. There were no overall significant differences among PVS2 exposure durations across the -LN or +LN treatments with respect to survival levels of the cultivars (Table 13). Shoot tip survival levels were between 90-100% and 85-95% across the three PVS2 exposure times for shoot tips with and without LN, respectively (Table 13).

Regrowth levels of shoot tips that were not cryoexposed were 78, 75 and 55% for *V. aestivalis* were 78, 78 and 50% for *Vitis* sp. (DVIT 1445) after PVS2 exposure for 30, 40 and 50 minutes, respectively. Although not statistically significant, there were lower viabilities with longer PVS2 exposures. For example, survival of both *V. aestivalis* and *Vitis* sp. (DVIT 1445) was 78% after 30 min PVS2 exposures, and 55% for *V. aestivalis* and 50% for *Vitis* sp. (DVIT 1445) after 50 min PVS2 exposures. Overall, the highest regrowth levels of cryopreserved shoot tips were obtained after 30 min of PVS2 exposure at 22ºC using the cryo-plates. Regrowth was 70% for *V. aestivalis* and 73% for *Vitis* sp. (DVIT 1445), with no significant genotypic differences (Table 14).

There were no significant differences among the regrowth levels of shoot tips that were removed from the alginate beads or that remained in the alginate beads (Table 15). There were also no statistically significant differences between *Vitis* species with respect to survival and regrowth levels (Table 13). Without LN exposure, the survival level of *V. aestivalis* was 98% and the survival level of *Vitis* sp. (DVIT 1445) was 93%. For cryopreserved shoot tips, survival level were 93 for *V. aestivalis* and 95% for *Vitis* sp. (DVIT 1445) (Table 15). Shoot tip regrowth levels -LN were 73% for both accessions, and regrowth levels +LN were 68% for *V. aestivalis* and 70% for *Vitis* sp. (DVIT 1445) (Table 15).

Table 13 – Survival level (%) of shoot tips excised from nodal sections sourced from *V. aestivalis* and *Vitis* sp. (DVIT 1445) grown *in vitro* with 30, 40 and 50 minutes of PVS2 exposure, with and without LN exposure using the V cryo-plate technique. Shoot tips were extracted from alginate buds.

Species	Identifier	-LN			+LN		
		30 min PVS2	40 min PVS2	50 min PVS2	30 min PVS2	40 min PVS2	50 min PVS2
V. aestivalis	DVIT 1408	90 ± 5 a	95 ± 0 a	95 ± 5 a	90 ± 5 a	98 ± 3 a	85 ± 5 a
Vitis sp.	DVIT 1445	100 ± 0 a	98 ± 3 a	93 ± 3 a	95 ± 5 a	93 ± 3 a	90 ± 0 a

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at $P<0.05$ using the Tukey's mean separation test.

Table 14 – Regrowth level (%) of shoot tips excised from nodal sections sourced from *V. aestivalis* and *Vitis* sp. (DVIT 1445) grown *in vitro* with 30, 40 and 50 minutes of PVS2 exposure, with and without LN exposure using the V cryo-plate technique. Shoot tips were extracted from alginate buds.

Species	Identifier	-LN			+LN		
		30 min PVS2	40 min PVS2	50 min PVS2	30 min PVS2	40 min PVS2	50 min PVS2
V. aestivalis	DVIT 1408	78 ± 8 a	75 ± 0 a	55 ± 5 a	70 ± 10 a	73 ± 3 a	48 ± 3 ab
Vitis sp.	DVIT 1445	78 ± 3 a	78 ± 3 a	50 ± 10 a	73 ± 8 a	65 ± 0 ab	38 ± 3 b

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at $P<0.05$ using the Tukey's mean separation test.

Table 15 – Survival and regrowth levels (%) of shoot tips excised from nodal sections sourced from *V. aestivalis* and *Vitis* sp. (DVIT 1445) grown *in vitro* with 40 minutes of PVS2 exposure, with and without LN exposure using the V cryo-plate technique. Shoot tips were not extracted from alginate buds.

Species	Identifier	Survival (%)		Regrowth (%)	
		-LN	+LN	-LN	+LN
V. aestivalis	DVIT 1408	98 ± 3 a	93 ± 3 a	73 ± 3 a	68 ± 3 a
Vitis sp.	DVIT 1445	93 ± 3 a	95 ± 5 a	73 ± 3 a	70 ± 5 a

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at $P<0.05$ using the Tukey's mean separation test.

4.4 DISCUSSION

The quality and physiological state of the stock cultures (BI et al., 2017; MARKOVIĆ et al., 2014; YAMAMOTO et al., 2015) as well as the shoot tip pre-treatment conditions (MATHEW et al., 2018) are likely key factors for successful *Vitis* cryopreservation. We grew *in vitro* derived nodal segments on shooting medium for 2 weeks to produce uniform 1-2 mm apical shoot tips. Salicylic acid, glutathione (reduced form) and ascorbic acid were included in the pre-treatment medium in an attempt to reduce the formation of reactive oxygen species (ROS) during cryoprotectant and LN exposure. Previously, it was observed shoot tips sampled from microcuttings had higher regrowth compared to shoot tips sampled directly on *in vitro* stock plants (MARKOVIĆ et al., 2014). The addition of antioxidants and salicylic acid in pre-treatment and preculture medium also improved shoot tip regeneration levels after LN exposure for grapevine (PATHIRANA et al., 2016), kiwifruit (MATHEW et al., 2018), *Arabidopsis* (CHEN et al., 2015), blackberry (UCHENDU et al., 2010b) and raspberry (WANG; VALKONEN, 2009a).

Plant growth regulators in the culture medium also play an important role in the regrowth levels of cryopreserved shoot tips. The addition of N^6-Benzyladenine (BA^3) to the shooting medium had a positive effect on *Vitis* regrowth after LN exposure (MARKOVIĆ et al., 2014; WANG et al., 2003a). Preliminary research in our laboratory revealed that low BA^3 allowed the development of quality plantlets after cryo-exposure and avoided hyperhydricity or callus formation that is induced when BA^3 concentrations were higher (VOLK et al., 2018); therefore, we minimized BA^3 in the pre-treatment and recovery medium. Shoot tip size also plays a factor in cryopreservability. We found that

1 mm shoot tips were the most successful for the droplet vitrification cryopreservation conditions that were tested.

We identified a V cryo-plate cryopreservation method for *Vitis* cryopreservation that used shoot tips derived from *in vitro* source plants. The high levels of regrowth after shoot tip cryopreservation suggest that this procedure appears to be an efficient and practical method for the cryopreservation of *Vitis* shoot tips, and its use could facilitate the technology transfer among labs. Alginate-bead encased shoot tips on cryo-plates may require less shoot tip handling and minimize damage before LN exposure. In addition, we found that regrowth levels were not affected and it was less time-consuming to not remove the alginate gel from the shoot tips after cryopexposure. For some species that were cryopreserved by D or V cryo-plate, removing the alginate gel from shoot tips significantly increased post-LN regrowth (RAFIQUE et al., 2015, 2016) and it also was necessary for *Vitis* (PLESSIS; LEDDET; DEREUDDRE, 1991) and *Malus* (BETTONI et al., 2018b) shoot tips cryopreserved by the more traditional encapsulation-dehydration technique which makes use of larger alginate beads.

Availability of a simple and efficient cryopreservation protocol developed for long-term *Vitis* preservation may also facilitate the use of cryotherapy methods in the production of virus-free plants (BETTONI et al., 2016; BI et al., 2017). To date, for grapevine studies have shown effectiveness of cryotherapy in the elimination of *Grapevine virus A* (GVA) (WANG et al., 2003b; BAYATI; SHAMS-BAKHSH; MOIENI, 2011), *Grapevine fanleaf virus* (GFLV) (MARKOVIĆ et al., 2015), *Grapevine leafroll associated virus-1* (GLRaV-1) (PATHIRANA et al., 2015), GLRaV-2 (PATHIRANA et al., 2015) and GLRaV-3 (MARKOVIĆ et al., 2015; PATHIRANA et al., 2015).

In this paper, we demonstrated that the V cryo-plate technique is a practical and promising *Vitis* cryopreservation methodology that resulted in high levels of regrowth for *V. aestivalis* and *Vitis* sp. (DVIT 1445) shoot tips after cryoexposure. The V cryo-plate technique offers the advantage of simple and quick handling of samples as well as fast cooling and warming rates, once the shoot tips are mounted on the cryo-plates. We obtained quality plantlets and high regrowth levels (≥70%) were obtained after cryoexposure. Future work will determine the applicability of this procedure across additional *Vitis* species.

Our results also showed that shoot tip size optimization is necessary to enhance regrowth levels after cryopreservation by droplet vitrification; 1 mm shoot tips were the

most successful for the droplet vitrification cryopreservation conditions that were tested.

Access to reliable cryopreservation methods that result in high levels of viability (≥40% after LN exposure) and highly skilled staff are key to the development of successful cryopreserved base collections (REED et al., 2004; VOLK et al., 2016). In this study, we successfully cryopreserved shoot tips from two *Vitis* species, *V. aestivalis* and *Vitis* sp. (DVIT 1445). The V cryo-plate protocol employed was adapted from that published by Yamamoto et al. (2012a). Although the efficacy of the V cryo-plate method has been reported for multiple crops, to our knowledge, this is the first report of cryopreservation of *Vitis* shoot tips using the V cryo-plate method.

5 SUCCESSFUL CRYOPRESERVATION AND HISTOLOGICAL OBSERVATIONS OF *VITIS* SHOOT TIPS ISOLATED FROM GROWTH CHAMBER SOURCE PLANTS [3]

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5.1 ABSTRACT

It is labor-intensive to establish *in vitro* cultures as source materials of shoot tips for the cryopreservation of field collections of fruit crops. We sought to determine if growth chamber *Vitis* plants could serve as the source of shoot tips for cryopreservation. Nodal sections from growth chamber plants were surface-disinfected and placed in tissue culture on pre-treatment medium for 2 weeks. Uniform apical shoot tips (1 mm) were first obtained from the nodal sections and then precultured for 3 days on medium containing 0.3 M sucrose, salicylic acid, glutathione (reduced form), ascorbic acid and plant preservative mixture. They were then treated with loading solution for 20 minutes at 22 °C followed by half-strength PVS2 for 30 minutes, and full-strength PVS2 for 30, 40 or 50 minutes at 0°C. After liquid nitrogen (LN) exposure, shoot tips were warmed/diluted in unloading solution for 20 minutes and placed on recovery medium. This cryopreservation protocol achieved high levels of regrowth in *V. vinifera* 'Chardonnay' and 'Riesling' and *V.* hybrid 'Oppenheim'. Histological observations revealed that shoot tips from growth chamber plants had apical as well as multiple lateral meristems that survived LN exposure. The preservation of multiple meristems in each shoot tip may increase the capacity of shoot tip regeneration in cryopreserved *Vitis* that originates from *ex vitro* sources. The high levels of regrowth after shoot tip cryopreservation using *Vitis* shoot tips derived from growth chamber source plants suggest that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture.

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Keywords: Grapevine. *Ex situ* conservation. Histological observation. Plant vitrification solution.

5.2 INTRODUCTION

Grapevine is one of the most economically important temperate fruit crops. In 2016, grapevines covered 7.52 million hectares and produced 75.8 million tons of fruit worldwide (OIV, 2017). Grapes have a rich genetic diversity; there are about 70 species within the *Vitis* genus (LI et al., 2017). It is essential to have methods available to securely conserve genebank collections of *Vitis* genetic resources for future generations.

Cryopreservation, the storage of biological materials in liquid nitrogen (LN, -196 °C) or liquid nitrogen vapor (LNV, -165 to -190 °C), is a preferred method for the long-term storage of plant germplasm, especially to maintain the genetic integrity of genebank materials and to minimize the risk of biotic and abiotic threats. Under cryopreserved conditions, viable cells, tissues, organs and organisms are preserved in a state whereby cellular divisions and metabolic processes are significantly reduced (WANG et al., 2018a; BENELLI; DE CARLO; ENGELMANN, 2013; BENSON, 2008).

Although there are many established cryobanks that preserve vegetative propagules, such as shoot tips or dormant buds of clonally propagated genetic resources (JENDEREK; REED, 2017; WANG et al., 2014; VOLK et all, 2008; TOWILL et al., 2004), to the best of our knowledge, *Vitis* cryo-storage has not been fully implemented within genebanks. Limited results were obtained when *Vitis* dormant buds were cryopreserved (ESENSEE; STUSHNOFF, 1990). Results have been more promising with respect to *Vitis* shoot tip cryopreservation. Over the years, many cryopreservation techniques have been published; among them, the droplet vitrification technique appears to be the most efficient. Several papers have reported the successful cryopreservation of *Vitis* shoot tips (BI et al., 2017; HASSAN; HAGGAG, 2013; PATHIRANA et al., 2016); however, most *Vitis* cryopreservation research used a limited number of species to develop new procedures (BI et al., 2017; BETTONI et al., 2016). Genotype-specific responses have made those methods difficult to implement (PATHIRANA et al., 2016; BENELLI; DE CARLO; ENGELMANN, 2013; BENSON, 2008; MARKOVIĆ et al., 2013a; GANINO et al., 2012; BENELLI; LAMBARDI; FABBRI, 2003). Reliable cryopreservation methods that result in high

levels of viability (≥40% after LN exposure) and highly skilled staff are key to the development of successful cryopreserved base collections of clonal crops (VOLK et al., 2016; REED et al., 2004).

Previously, most of the studies on *Vitis* cryopreservation protocols reported using explants from *in vitro* cultures, making it is necessary to establish and maintain *in vitro* stock plants. There is only one report that made use of shoot tips from greenhouse plants; the study of Hassan and Haggag (2013) simplified the cryo-procedure, in which shoot tips were sampled directly from greenhouse-grown plants, resulting in less time consuming cryopreservation process. The Hassan and Haggag (2013) protocol resulted in regrowth levels of 40 and 47% for *Vitis vinifera* 'Black Matrouh' and 'Bez El-Anza', respectively, using shoot tips derived directly from greenhouse plants. The quality and physiological state of the stock cultures and explants, as well as the preculture conditions, play key roles and are determinant to the success of *Vitis* cryopreservation techniques (BI et al., 2017).

Herein, we used nodal sections to increase the uniformity of shoot tips from *ex vitro Vitis* source plants. Preculture conditions and the length of the vitrification solution exposure were optimized to identify successful cryopreservation methods for two *Vitis* cultivars and one rootstock.

5.3 MATERIALS AND METHODS

5.3.1 Plant Material and pre-treatments

Growth chamber plants of *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection Oppenheim#4 (DVIT 8121 (SO4; *V. berlandieri* × *V. riparia*)) were originally received from the USDA-ARS National Clonal Germplasm Repository for Tree Fruit, Nut Crops and Grapes in Davis, CA, USA.

Plants were grown in pots in Sun Gro Professional Growing mix (Sunshine VP Metro-Mix 250; Canadian sphagnum peat moss, coarse perlite, vermiculite, dolomitic limestone, long-lasting wetting agent, RESiLIENCE; Sun Gro Horticulture Ltd., Seba Beach, AB, Canada) at 25 °C, in a growth chamber under a photoperiod of 16 h light day^{-1} provided by metal halide and high pressure sodium lamps. Nodal sections approximately 2 cm long containing a single bud were harvested from the growth

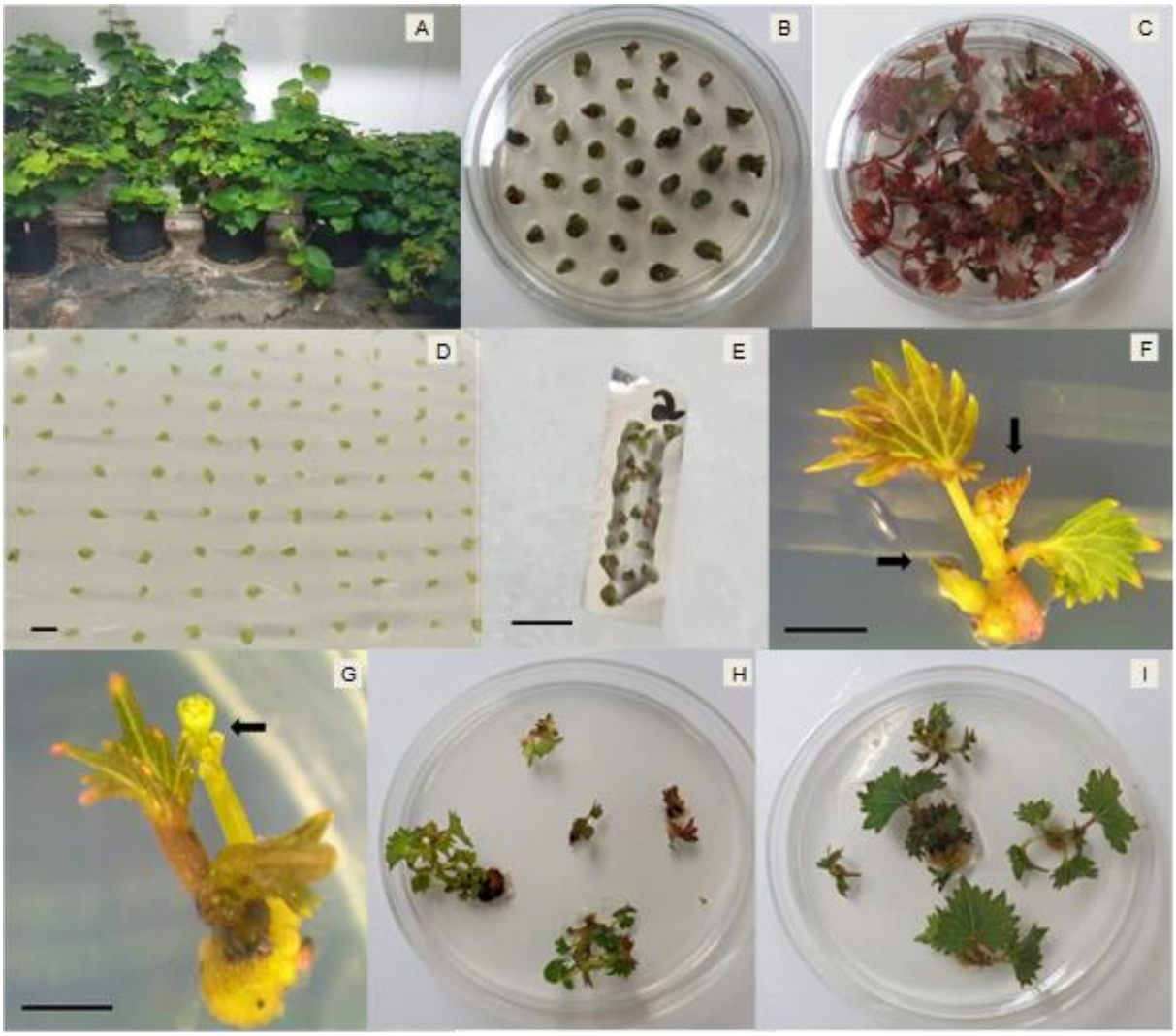
chamber plants (Figure 8A), sterilized with 70% isopropanol for 1 min and rinsed twice for 1 min with distilled water. They were then treated with 5% sodium hypochlorite and 0.1% Tween 20 (v/v) for 5 min and rinsed three times in sterile distilled water.

Nodal sections were placed into 100 × 25 mm plastic Petri dishes with 50 mL pre-treatment medium (MS (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose, 0.2 mg L^{-1} N^6-Benzyladenine (BA^3), 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), 1.5% (v/v) Plant Preservative Mixture (PPM; Plant Cell Technology, WA, 1.5% v/v), and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving)) with 30 nodal sections per plate (Figure 8B). Nodal sections were cultured on pre-treatment medium for 2 weeks in a growth room at 25 °C with a 16 h photoperiod provided by fluorescent lights (40 µM m^{-2} s^{-1}) (Figure 8C). After initial culture at 25°C, cold acclimation treatments were performed by placing the plates of nodal sections in a growth chamber with a constant temperature of 5 °C with a 16 h photoperiod for an additional two weeks.

5.3.2 Preculture and cryopreservation

Uniform apical shoot tips (1 mm) (Figure 8D) were excised from nodal sections that either had or had not been cold acclimated. Shoot tips were cultured on preculture medium (half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), 1.5% (v/v) PPM and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving)) for 3 days at 25 ^oC in darkness. They were then placed in loading solution (half-strength MS + 2M glycerol + 0.4 M sucrose at pH 5.7 (pH 6.9 prior to autoclaving)) for 20 min at 22^oC followed by half-strength PVS2 (filter sterilized solution consisting half-strength MS + 15% (w/v) glycerol + 7.5% (w/v) ethylene glycol (EG) +7.5% (w/v) dimethyl sulfoxide (DMSO) + 0.4 M sucrose at pH 5.8, MATSUMOTO; SAKAI, 2003) for 30 min at 22^oC and full-strength PVS2 (filter sterilized solution, half-strength MS + 30% (w/v) glycerol + 15% (w/v) EG +15% (w/v) DMSO + 0.4 M sucrose at pH 5.8, SAKAI; KOBAYASHI; OIYAMA, 1990) at 0 ^oC for 30, 40, or 50 min. Two minutes before the end of each treatment, PVS2-treated shoot tips were placed onto a thin layer of PVS2 on sterile aluminum foil strips (6 x 25 mm) (Figure 8E) and then plunged into liquid nitrogen (LN).

Figure 8 – Droplet vitrification procedure of *Vitis* shoot tips from growth chamber source plants. Nodal sections were excised from growth chamber plants **(A),** surface-disinfected and placed on pretreatment medium **(B),** and grown for 2 weeks **(C)**. Shoot tips were incubated on the preculture medium for three days **(D)**. Shoot tips were placed on foil strips in a thin layer of PVS2 prior to LN exposure **(E)**. *Vitis vinifera* 'Riesling' shoot tips were recovered for 40 days after cryoexposure exhibiting multiple shoots **(F)** and flower buds **(G)**. Petri plates of *Vitis vinifera* 'Riesling' shoot tips grown for two months with cryoexposure **(H)** and without cryoexposure **(I)**. Arrows indicate multiple shoots in **F** and inflorescence in **G**.



Source: Prepared by the author, 2018.
Scale Bars: D, F and **G=** 2 mm; **E**= 5 mm.

After one hour of LN exposure, the aluminum foil strips with shoot tips were warmed quickly by inverting the strips into unloading solution (half-strength MS + 1.2 M sucrose at pH 5.7 (pH 7.5 prior to autoclaving)) and incubating at 22 ^{o}C for 20 min.

For regrowth, shoot tips were placed onto recovery medium 1 (1/2X MS macroelements, 1X MS microelements, and *Vitis* vitamins (100 mg L^{-1} myo inositol, 10 mg L^{-1} thiamine HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} Ca

pantothenate, 0.01 mg L^{-1} biotin, 2 mg L^{-1} glycine, without NH_4, supplemented with 0.6 M sucrose and 8 g L^{-1} agar at pH 5.7 (pH 7.0 prior to autoclaving)) overnight in the dark and then transferred to recovery medium 2 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins), without NH_4, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA^3 and 8 g L^{-1} agar at pH 5.7 (pH 6.5 prior to autoclaving)) and cultured for 2 weeks at 25 ^oC in darkness. They were then transferred onto recovery medium 3 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA^3 and 8 g L^{-1} agar at pH 5.7(pH 6.5 prior to autoclaving)) and grown in the light at 25 ^oC (40 µM m^{-2} s^{-1}, 16 h photoperiod).

5.3.3 Regrowth and data analysis

Shoot tip survival (green cell mass or leaf tissue) and regrowth (organized shoots with at least one leaf) were measured 8 weeks after plating on regrowth medium. Each experiment was performed with at least two replicates of 20 shoot tips for each treatment. Means and standard errors were calculated across experimental replicates; and ANOVA and Tukey's means separation test ($P \leq 0.05$).

5.3.4 Histological studies of cryopreserved shoot tips

Histological studies were performed to observe cellular structural modifications pre- and post-cryopreservation for *Vitis vinifera* 'Riesling' shoot tips that originated from growth chamber source plants. The following treatments were sampled for shoot tips that were not exposed to LN: (1) control, shoot tips excised from pre-treatment nodal sections; (2) shoot tips precultured on preculture medium for 3 days; (3) shoot tips treated with cryoprotectants (30 min at 22^oC in half-strength PVS2 followed by full-strength PVS2 at 0 ^oC for 30 min), not exposed to LN, and diluted with 1.2 M sucrose; (4) shoot tips treated with cryoprotectants (30 min at 22^oC in half-strength PVS2 followed by full-strength PVS2 at 0 ^oC for 30 min), not exposed to LN, and diluted with 1.2 M sucrose and incubated in recovery medium for 6 days. The following treatments were for shoot tips exposed to LN: (5) shoot tips treated with cryoprotectants (30 min at 22^oC in half-strength PVS2 followed by full-strength PVS2 at 0 ^oC for 30 min) exposed to LN, and diluted with 1.2 M sucrose; (6) shoot tips treated with

cryoprotectants (30 min at 22ºC in half-strength PVS2 followed by full-strength PVS2 at 0 ºC for 30 min), exposed to LN, and diluted with 1.2 M sucrose and incubated in recovery medium for 6 days.

Fifteen shoot tips per treatment were placed into fixative (1.25% glutaraldehyde, 2% paraformaldehyde, and 50 mM Pipes buffer [piperazine N, *N*-bis (2-ethanesulfonic acid)] overnight at 22°C. Shoot tips were rinsed three times (10 min each) in 50 mM Pipes buffer and post-fixed with 2% osmium tetroxide in 50 mM Pipes buffer overnight at 22°C. Shoot tips were then rinsed three times (10 min each) in 50 mM Pipes buffer and dehydrated with 30, 50, 70, 90, 100, 100% ethanol for 15 min each. Samples were infiltrated with Spurr resin (Electron Microscopy Sciences, Hatfield, PA), in an ethanol: resin ratio of 3:1 for 4 h, followed 2:1 for 3 h, 1:1 for 4h, 1:2 for 4 h, 1:3 for 4h and kept in 100% resin for 72 h (fresh resin was added every 24 hours). Samples were then transferred to fresh resin, placed into embedding containers, and the resin was polymerised at 65 °C for 24 h. Thin sections (1 µm) were made with glass knives on an RMC MT-X microtome (Ventana Medical Systems Inc., Tucson, AZ) and mounted on glass slides. Sections were stained with methylene blue, visualized with an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan) and images captured using a Leica MC170 HD digital camera (Wetzlar, Germany).

5.4 RESULTS

5.4.1 Survival and regrowth of cryopreserved *Vitis* shoot tips from growth chamber source plants

Shoot tips excised from nodal sections derived from growth chamber plants were cryopreserved with and without two weeks of cold acclimation. Preliminary results revealed that shoot tips derived from growth chamber plants could not tolerate PVS2 incubation durations that were as long as those for shoot tips derived from *in vitro* source cultures (data not shown); however, shorter incubation times were sufficient for adequate levels of shoot tip regrowth after LN exposure (Tables 16-19). Average survival levels across the three accessions of the non-cold-acclimated shoot tips were 96, 93, and 85%, corresponding to 30, 40, and 50 min of PVS2 exposure and survival levels were 95, 96, and 88%, for 30, 40 and 50 min PVS2 exposure, respectively, for the cold acclimated shoot tips (Tables 16 and 17). With LN exposure, the survival levels

averaged 93, 92, and 82% for the non-cold acclimated shoot tips for 30, 40 and 50 min PVS2 exposures, respectively, and 90, 90, and 81% for the 30, 40 and 50 min PVS2 exposures with cold acclimation. In the +LN treatments, there were no significant differences among the survival levels of the cultivars, PVS2 treatments, or +/- cold acclimation (Tables 16 and 17).

Regrowth levels were lower when shoot tips from growth chamber-sourced shoot tips were exposed to LN compared to those with no LN exposure. There were no statistically significant differences in regrowth levels between shoot tips excised from nodal sections with or without cold acclimation (Tables 18 and 19). For the non-cold acclimated shoot tips, average regrowth levels were 73, 72, and 63% for the 30, 40 and 50 min PVS2 exposures without LN. In contrast, regrowth levels after LN exposure were and 48, 50, and 41% for the 30, 40 and 50 min PVS2. Without cold acclimation and with LN exposure, *V. vinifera* cv. 'Riesling' had the highest regrowth levels (62%) after a 30 min PVS2 and *V. vinifera* cv. 'Chardonnay' had the lowest regrowth levels after a 40 (37%) or 50 (30%) min PVS2 exposure (Table 18). For the cold acclimated shoot tips, average regrowth levels were 74, 69, and 60% for the 30, 40 and 50 min PVS2 exposures without LN and 55, 49, and 46% for the 30, 40, and 50 min PVS2 exposures with LN, respectively. *Vitis vinifera* 'Riesling' had regrowth of 68%, 60%, and 46% for 30 min, 40 min, and 50 min PVS2 exposures, respectively. Although there weren't statistically significant differences between the regrowth levels of cold-acclimated and non-cold-acclimated shoot tips after LN exposure, there was a trend to have more uniform responses in the cold-acclimated treatments (Table 19).

For the shoot tips that were not cold acclimated, *V. vinifera* cvs. 'Chardonnay' and 'Riesling' achieved at least 40% regrowth with 30 min PVS2 exposures and *V. hybrid* 'Oppenheim' achieved at least 40% regrowth with 40 min PVS2 exposure (Table 18). With 30 min PVS2 exposure and +LN treatment with cold acclimation, all three cultivars had regrowth levels of at least 40% (Table 19).

The optimal PVS2 treatment length was determined to be a 30 min exposure to half-strength PVS2 at 22 ^{o}C, followed by a 30 min exposure to full-strength PVS2 at 0 ^{o}C, which resulted in an average regrowth of 48 and 55% for shoot tips excised from growth chamber-sourced nodal sections without and with cold-acclimation, respectively.

Table 16 – Survival level (%) of shoot tips excised from nodal sections sourced from *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection Oppenheim#4 grown in the growth chamber, 30, 40 and 50 min of PVS2 exposure, with and without LN exposure, and no cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		30	40	50	30	40	50
V. vinifera	Chardonnay	97 ± 2 a	92 ± 3 ab	83 ± 2 b	90 ± 0 a	90 ± 3 a	77 ± 4 a
V. vinifera	Riesling	92 ± 4 ab	94 ± 2 ab	86 ± 2 ab	94 ± 8 a	90 ± 6 a	80 ± 4 a
V. hybrid	Oppenheim #4 DVIT 8121	98 ± 3 a	93 ± 3 ab	90 ± 0 ab	95 ± 5 a	95 ± 0 a	90 ± 0 a
Average		96 ± 2	93 ± 1	86 ± 2	93 ± 2	92 ± 2	82 ± 4

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at P<0.05 using the Tukey's mean separation test.

Table 17 – Survival level (%) of shoot tips excised from nodal sections sourced from *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection Oppenheim#4 grown in the growth chamber, 30, 40 and 50 min of PVS2 exposure, with cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		30	40	50	30	40	50
V. vinifera	Chardonnay	93 ± 3 a	98 ± 3 a	88 ± 3 a	85 ± 5 a	83 ± 8 a	75 ± 10 a
V. vinifera	Riesling	100 ± 0 a	96 ± 0 a	86 ± 6 a	94 ± 6 a	96 ± 0 a	84 ± 4 a
V. hybrid	Oppenheim #4 DVIT 8121	93 ± 8 a	95 ± 0 a	90 ± 0 a	90 ± 0 a	90 ± 5 a	85 ± 5 a
Average		95 ± 2	96 ± 1	88 ± 1	90 ± 3	90 ± 4	81 ± 3

Source: Prepared by the author, 2018.

Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at P<0.05 using the Tukey's mean separation test.

Table 18 – Regrowth level (%) of shoot tips excised from nodal sections sourced from *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection Oppenheim#4 grown in the growth chamber, 30, 40 and 50 min of PVS2 exposure, without cold acclimation.

Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		30	40	50	30	40	50
V. vinifera	Chardonnay	72 ± 4 a	68 ± 2 a	63 ± 6 a	43 ± 4 bc	37 ± 3 bc	30 ± 3 c
V. vinifera	Riesling	76 ± 4 a	72 ± 4 a	64 ± 0 a	62 ± 2 a	64 ± 4 a	50 ± 2 ab
V. hybrid	Oppenheim #4 DVIT 8121	70 ± 0 a	75 ± 7 a	63 ± 4 a	38 ± 3 bc	48 ± 3 abc	43 ± 3 bc
Average		73 ± 2	72 ± 2	63 ± 0	48 ± 7	50 ± 8	41 ± 6

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at P<0.05 using the Tukey's mean separation test.

Table 19 – Regrowth level (%) of shoot tips excised from nodal sections sourced from *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection Oppenheim#4 grown in the growth chamber, 30, 40 and 50 min of PVS2 exposure with and without LN exposure, with cold acclimation.

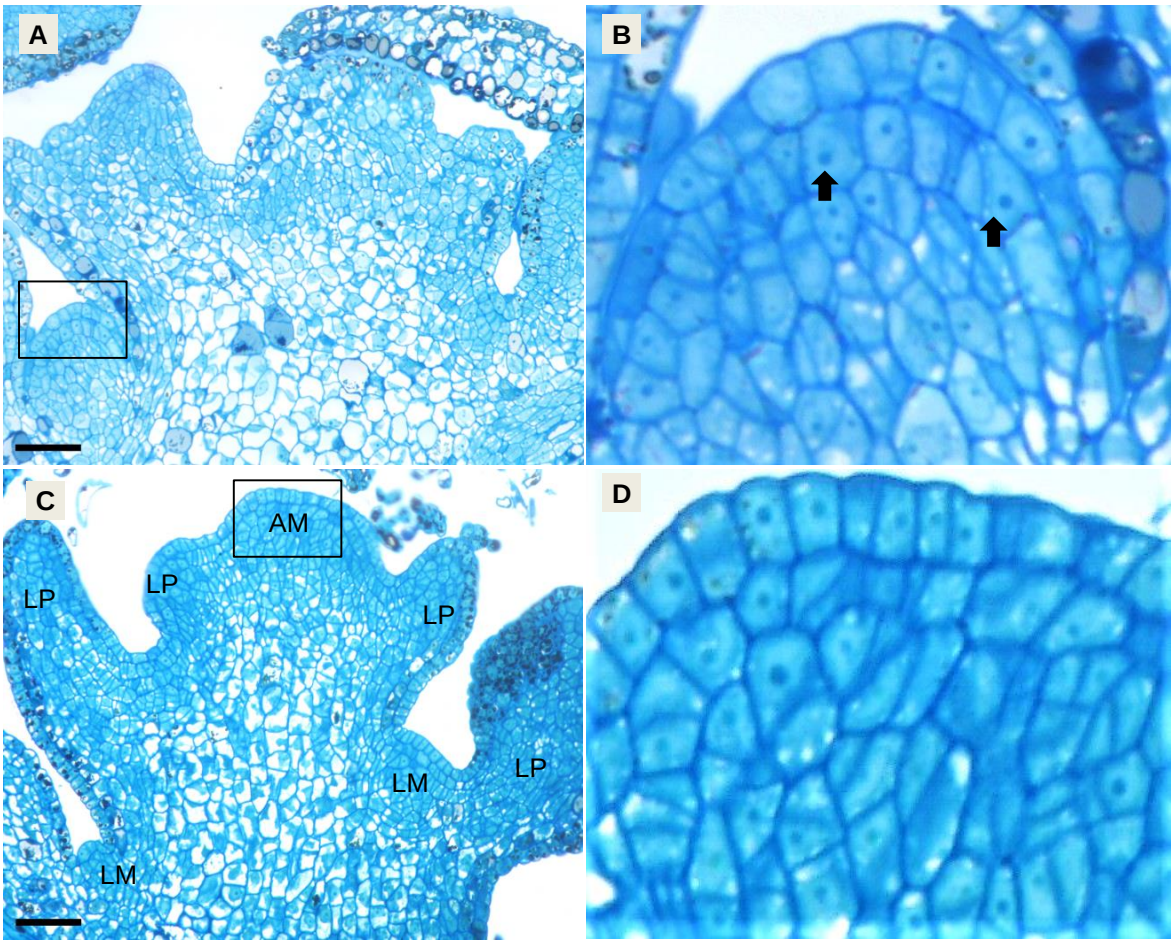
Species	Identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		30	40	50	30	40	50
V. vinifera	Chardonnay	70 ± 0 ab	73 ± 3 ab	55 ± 5 b	48 ± 3 ab	43 ± 8 ab	55 ± 10 ab
V. vinifera	Riesling	84 ± 4 a	70 ± 2 ab	64 ± 4 b	68 ± 0 a	60 ± 4 ab	46 ± 6 ab
V. hybrid	Oppenheim #4 DVIT 8121	68 ± 3 ab	63 ± 3 b	60 ± 5 b	48 ± 3 ab	45 ± 5 ab	38 ± 3 b
Average		74 ± 5	69 ± 3	60 ± 3	55 ± 7	49 ± 5	46 ± 5

Source: Prepared by the author, 2018.
Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at P<0.05 using the Tukey's mean separation test.

5.4.2 Histological studies of cryopreserved shoot tips

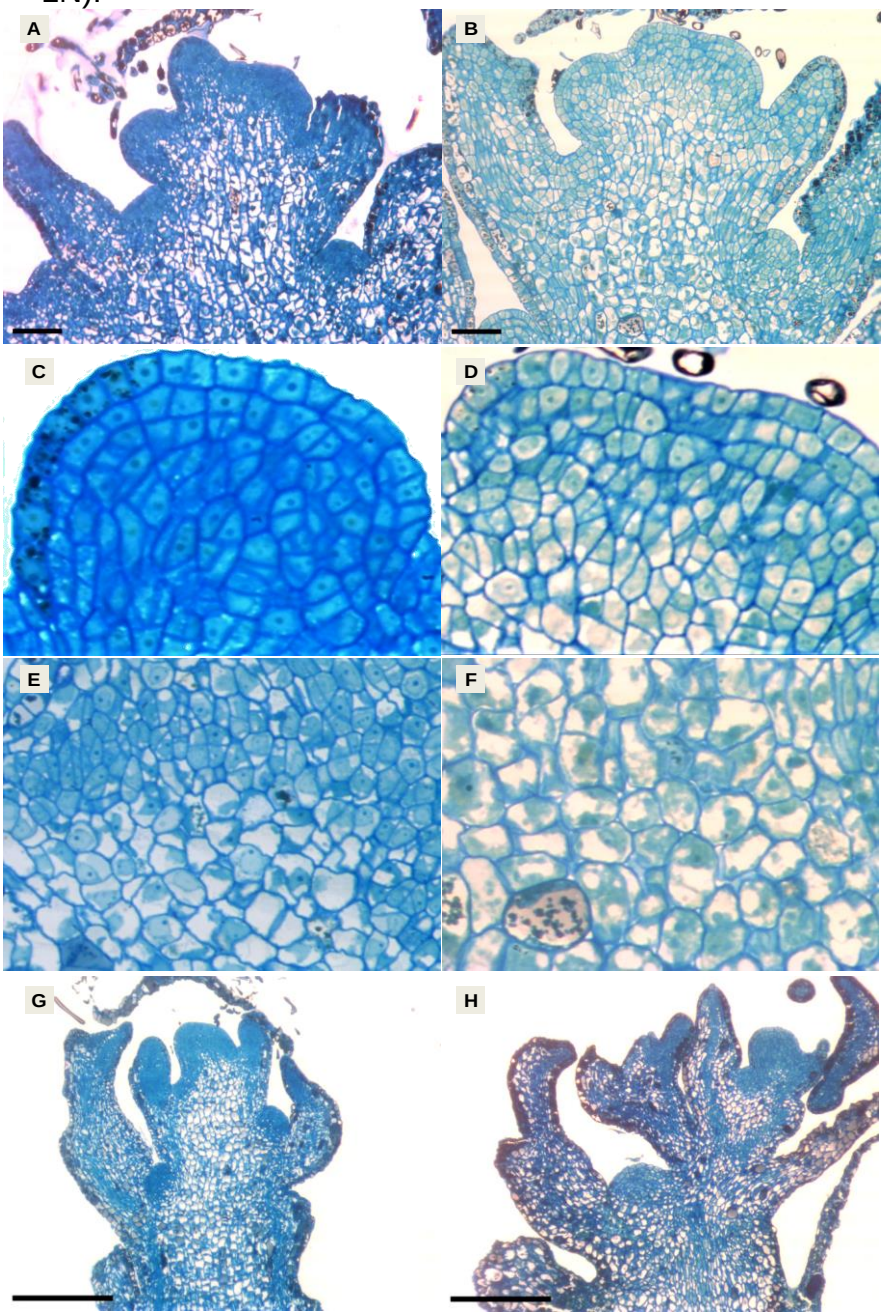
Excised shoot tips of *Vitis vinifera* cultivar 'Riesling' that originated from growth chamber plants were embedded in resin for histological observations. All of the observed shoot tips exhibited apical meristems in addition to multiple lateral meristems that were located in the leaf axes (Figure 9C). Apical meristems exhibited a characteristic dome shape, and were often wider than the lateral meristems. The apical portion of the dome (AD) appeared to have cells that were smaller and more compact than those in the basal region (Figure 9C). Many of the observed apical and lateral meristematic cells showed signs of recent cellular divisions and were highly cytoplasmic, with large nuclei, some showing nucleoli (Figure 9B, 9D).

Figure 9 – Cross section of *Vitis vinifera* 'Riesling' shoot tips sampled at (**A**) control, shoot tips excised from pre-treatment nodal sections; (**B)**, a closer view of a lateral meristem in A; (**C**) shoot tips precultured on medium for 3 days; (**D**) a closer view of an apical meristem in C.



Source: Prepared by the author, 2018.
Black arrows represent some cells showing nucleoli. AM, apical meristem; LM, lateral meristems; LP, leaf primordium. Scale Bars: **A-C=** 50 µm.

Figure 10 – Histological observations in *Vitis vinifera* 'Riesling' shoot tips after successive steps of cryopreservation procedure. Cross section of shoot tips treated with cryoprotectants (30 min at 22ºC in half-strength PVS2 followed by full-strength PVS2 at 0 ºC for 30 min), not exposed or exposed to LN, and then diluted with 1.2 M sucrose (10A, without LN; 10B, with LN). A closer view of apical meristem (10C) and cortex cells (10E) in the non-LN exposed shoot tip from 10A. A closer view of apical meristem (10D) and cortex cells (10F) in the LN exposed shoot tip from 10B. Shoot tips treated with cryoprotectants and were either not exposed or exposed to LN, diluted with 1.2 M sucrose and were incubated in recovery medium for 6 days (10G, without LN; 10H, with LN).



Source: Prepared by the author, 2018.
Asterisks, regions where plasmolysis has occurred. Black arrows represent damaged cells. Scale Bars: **A-B=** 50 µm, **G-H=** 200 µm.

Both apical and lateral meristems retained their cellular integrity after PVS2 exposure, either with or without LN (Figure 10A, 10B, 10C, 10D). Differentiated, vacuolated cortex cells in the PVS2-exposed meristems were plasmolyzed (Figure 10E), while the cortex cells in the PVS2+LN-exposed meristems were lethally damaged, showing evidence of cell membrane disruptions (Figure 10F). After 6 days of recovery, we observed signs of regrowth in the shoot tips that were and were not exposed to liquid nitrogen (Figure 10G, 10H).

5.5 DISCUSSION

We sought to identify a cryopreservation protocol that did not require *Vitis* source plants to be introduced and multiplied in tissue culture prior to shoot tip excision. Nodal sections from growth chamber plants were surface sterilized and then plated on medium to produce uniform shoot tips. These shoot tips were then excised and cryopreserved using a droplet-vitrification procedure that was successfully applied to *V. vinifera* ‘Chardonnay’ and ‘Riesling’ and *V.* hybrid ‘Oppenheim’. Average regrowth levels were 48% after shoot tips were exposed to half-strength PVS2 at 22ºC for 30 min, followed by full-strength PVS2 at 0ºC for 30 min, followed by liquid nitrogen exposure. Our results showed that the use of a cold acclimation treatment did not significantly improve the survival and regrowth of *Vitis* shoot tips after cryopreservation.

The use of explants directly from the greenhouse, or possibly even from field-grown plants, for cryopreservation would alleviate the laborious steps of *in vitro* culture establishment and multiplication, thus reducing the time and increasing the efficiency of cryopreserving cultivars in genebank collections (BETTONI et al., 2018a; VOLK et al., 2018; BI et al., 2017; TOWILL et al., 2004). Previously, Hassan and Haggag (2013) reported the cryopreservation of shoot tips from greenhouse grapevine plants of Egyptian cultivars. These authors used shoot tips derived directly from greenhouse-grown plants, without the incubation of nodal sections on media to produce uniform shoot tips. We found that shoot tips derived directly from growth chamber plants did not exhibit uniform or repeatable responses to cryopreservation treatments (data not shown). Our proposed method of excising shoot tips from pre-treated nodal sections improved the shoot tip quality and reduced the effects of oxidation and microbial contamination.

The quality and physiological state of the explant source material are key factors in successful *Vitis* cryopreservation (VOLK et al., 2018; BI et al., 2017; MARKOVIĆ et al., 2014; ENGELMANN, 2011). In our protocol, nodal sections were harvested from growth chamber plants, then surface sterilized and placed on pre-treatment medium supplemented with BA^3, salicylic acid, glutathione (reduced form), ascorbic acid, and PPM. Within two weeks, uniform apical shoot tips were excised for cryopreservation experiments. At this point, contamination posed the greatest challenge to using nodal sections from growth chamber plants. PPM was added during the pre-treatment and preculture steps to reduce and/or eliminate contamination issues. Preliminary trials revealed that contamination was also reduced when nodal sections were shaken for 2 hours in 5 % (v/v) PPM solution supplemented with full-strength MS salts (no pH adjustment), after nodal section sterilization and prior to plating on to pre-treatment medium (data not shown).

Successful cryopreservation procedures are dependent upon optimal pre-treatment conditions (VOLK et al., 2018; MATHEW et al., 2018). We included salicylic acid, glutathione (reduced form) and ascorbic acid in the pre-treatment medium to reduce the formation of reactive oxygen species (ROS) during cryoprotectant and LN exposure because oxidative damage may affect shoot tip regrowth after LN exposure (UCHENDU et al, 2010a; JOHNSTON; HARDING; BENSON, 2007). The positive effects of adding antioxidants and salicylic acid to pre-treatment and preculture media during the cryopreservation process have been reported with *Vitis* (VOLK et al., 2018; BETTONI et al., 2018a; PATHIRANA et al., 2016; SHEPHERD et al., 2013) and other species (MATHEW et al., 2018; REED, 2014; UCHENDU et al., 2010ab).

We found that a cold acclimation treatment (5 ^{o}C for 2 weeks) did not significantly improve the survival and regrowth of *Vitis* shoot tips after cryopreservation. For some species, cold acclimation treatments improved the regrowth and quality of regenerated plants after cryopreservation (MATHEW et al., 2018; PANTA et al., 2015; KUSHNARENKO; ROMADANOVA; REED, 2009; KACZMARCZYK et al., 2008; CHANG; REED, 2000; 2001). This treatment step could be considered when cryopreserving other *Vitis* cultivars and/or species.

We describe a droplet-vitrification cryopreservation technique for *Vitis* that makes use of shoot tips derived from growth chamber source plants. Nodal sections were cultured on pre-treatment medium for 2 weeks, uniform apical shoot tips were excised and cultured on preculture medium for 3 days, then they were placed into

loading solution for 20 minutes followed by half-strength PVS2 for 30 minutes. The optimal full-strength PVS2 exposure duration was 30 min for shoot tips derived from growth chamber plants. The high level of regrowth after cryoexposure suggests that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture.

It is interesting to note that one of the cultivars included in this work was the rootstock selection 'Oppenheim', with a similar genetic background as 'Kober 5BB' (*V. berlandieri* x *V. riparia*), which was shown by Ganino et al. (2012) to be recalcitrant to cryopreservation. Using our method, this rootstock cultivar had regrowth levels of 48% after cryoexposure.

Histological observations at the light microscope level revealed that *Vitis* shoot tips isolated from growth chamber source plants appeared to have a high degree of cellular preservation in both the apical and lateral meristems. Similar highly preserved apical meristems were observed in cryopreserved mint and citrus shoot tips (VOLK et al., 2017b; VOLK; CASPERSEN, 2007); however, in *Vitis*, we observed multiple preserved lateral meristems within each excised shoot tip. We found that multiple shoots elongated from each growth chamber derived shoot tip, indicating that both the apical and lateral shoot tips survived cryoexposure. This could increase the capacity of shoot tip regeneration in cryopreserved *Vitis* from *ex vitro* sources (Figure 8F). Previous reports of *Vitis* shoot tip cryopreservation using explants derived from *in vitro* source plants exhibited the emergence of a single shoot from the shoot tips, suggesting that only a single meristem exhibited regrowth (PATHIRANA et al., 2016; MARKOVIĆ et al., 2013a).

In addition, we found that some lateral meristems developed floral buds (Figure 8G). Although grape plants produce fruit in the shoot that sprouts in the growth season, the formation of the flower bud occurs over two seasons; where the floral primordia are induced in the first vegetative year and are then differentiated into the inflorescence (MALLET; RABOT; GENY, 2015; VASCONCELOS et al., 2009). Apparently, when nodal sections were excised from growth chamber plants, some buds had already been induced to produce floral primordia.

Cryopreserved genebank collections require less space and fewer resources than traditional field conservation methods, particularly for clonally propagated crops (WANG et al., 2018a; BI et al., 2017; PATHIRANA et al., 2016; BETTONI et al., 2016; BENELLI; DE CARLO; ENGELMANN, 2013; MARKOVIĆ et al., 2013a; REED et al.,

2004). To date, *Vitis* cryopreservation has been considered challenging due to the genotype-specific responses, low levels of regrowth after cryopreservation and the difficulty in acquiring the same results as those that have been published by other laboratories. These characteristics have limited the implementation of grape cryopreservation in genebanks (BI et al., 2017; GANINO et al., 2012). With the application of optimized pre-treatment conditions, improvement of the shoot tip quality and uniformity, and ideal regrowth medium conditions, the current challenges can be overcome.

Availability of a cryopreservation method for *Vitis* may facilitate the use of cryotherapy methods to eradicate viruses (BI et al., 2017; BETTONI et al., 2016). To date, major *Vitis* viral pathogens including *Grapevine leafroll associated virus-1* (GLRaV-1) (PATHIRANA et al., 2015), GLRaV-2 (PATHIRANA et al., 2015), GLRaV-3 (MARKOVIĆ et al., 2015; PATHIRANA et al., 2015), *Grapevine virus A* (GVA) (BAYATI; SHAMS-BAKHSH; MOIENI, 2011; WANG et al., 2003b) and *Grapevine fanleaf virus* (GFLV) (MARKOVIĆ et al., 2015) have been eliminated from grapevines using cryotherapy techniques. The proposed *Vitis* cryopreservation procedure which makes use of growth chamber source plants, possibly in combination with thermotherapy, may further increase the success and applicability of cryotherapy techniques.

6 CRYOTHERAPY BY ENCAPSULATION-DEHYDRATION IS EFFECTIVE FOR *IN VITRO* ERADICATION OF LATENT VIRUSES FROM 'MARUBAKAIDO' APPLE ROOTSTOCK[4]

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6.1 ABSTRACT

Apple stem pitting virus (ASPV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) are several major viral pathogens of apple trees, responsible for substantial damage to the world's apple industry. This study aimed to evaluate the effectiveness of the encapsulation-dehydration cryopreservation technique to eradicate these viral pathogens from *in vitro* shoot tips excised from 'Marubakaido' apple rootstock cultures. Axillary shoot tips were excised from *in vitro* cultures, encapsulated in alginate beads, precultured in MS salts, dehydrated in a laminar flow hood, immersed in liquid nitrogen, then warmed and recovered on medium. After LN exposure, *in vitro* rooting and acclimatization, recovered 'Marubakaido' plants exhibited 52% survival and 35% regrowth without callus formation. After 8 months of regrowth, PCR analyses revealed that all the plants were free of ACLSV and ASPV, but 2 out of 20 recovered plants were still infected with ASGV. This is the first report in Brazil of the application of cryotherapy to eradicate viral complexes in *Malus*. Cryotherapy can facilitate the production of virus-free plants by producing high quality plant material.

Keywords*: Malus*. ASGV. ASPV. ACLSV. Eradication. Cryopreservation.

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6.2 INTRODUCTION

Apples (*Malus* spp.) are among the most important fruit crops cultivated and consumed in the world (VOLK et al., 2015). Apple orchard health and productivity, as well as international import/export, are significantly affected by the presence of viruses (BARBA; ILARDI; PASQUINI, 2015). Plant viruses use chemical components of host cells for their replication and transfer, resulting in the modification of gene expression and protein accumulation, hormonal changes and photosynthetic activity (HULL, 2001; BASSO et al., 2010). Apple trees infected with viruses exhibit reduced vigour, productivity and fruit quality, as well as increased susceptibility to other phytopathogenic agents (NICKEL et al., 2001; CIEŚLIŃSKA; RUTKOWSKI, 2008; GUERRA et al., 2012; KUMAR et al., 2014). *Apple stem grooving virus* (ASGV, genus *Capillovirus*), *Apple stem pitting virus* (ASPV, genus *Foveavirus*) and *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*) are particularly pathogenic to apple trees (LÉMOINE, 1990; FAJARDO; NICKEL, 2014). No vector is currently known to spread ASGV, ASPV and ACLSV. There are spread only through infected propagative material or transmitted by grafting (MARTELLI et al., 2007; NICKEL; FAJARDO, 2014; BARBA; ILARDI; PASQUINI, 2015; HU et al., 2017). Plants infected by these viral species are asymptomatic in tolerant combinations of scion and rootstock, and may become apparent when the host plants are propagated onto alternative rootstocks (BARBA; ILARDI; PASQUINI, 2015). It becomes difficult to control spread of the diseases within infected trees once they are planted in the field. Nurseries seek to supply disease-free materials to their customers (WANG et al., 2014).

In fruit crops, virus-free plant material has traditionally been obtained through meristem culture and thermotherapy, followed by another round of meristem culture (DÍAS-BARRITA et al., 2007; MALIOGKA et al., 2009; 2015). Although these techniques are successful, they are very labor- and time-intensive (WANG et al., 2014). For successful viral eradication through the use of meristem culture, it is usually necessary to excise shoot tips that are 0.1 to 0.3 mm in length (WANG; VALKONEN, 2008a). In addition to the mechanical difficulties of meristem excision, the low regrowth level and, the challenge of viral particle elimination are some bottlenecks of these methods (FACCIOLI; MARANI, 1998; DÍAS-BARRITA et al., 2007; WANG et al., 2016). Thermotherapy associated with meristem culture is also a difficult process that

requires specific equipment and virus-specific treatment conditions. Not all viruses can be successfully eliminated using thermotherapy, and often the host plants are sensitive to high temperatures (BHOJWANI; DANTU, 2013; HU et al., 2015).

Chemotherapy is an alternative method for virus eradication. Chemotherapy protocols associated with meristem culture or thermotherapy were successfully used to remove ACLSV, ASGV and ASPV in apple trees (JAMES, 2010; HU et al., 2015). In chemotherapy, antiviral compounds (such as ribavirin and quercitin) prevent viral replication by inhibiting nucleic acid synthesis (PARKER, 2005). Although effective in many cases, there are reports of phytotoxicity in *in vitro* cultures. In addition, antiviral substances may cause mutagenic changes in plant tissues. As with the other methods, chemotherapy must be optimized according to the plant species and/or cultivar (SKIADA et al., 2013).

In view of the limitations of the traditional methods for obtaining virus-free parent plants, it is useful to develop efficient virus eradication technologies for fruit crops. A new tool - cryotherapy - has been shown to be highly efficient for virus eradication in species of economic importance, including *Prunus* (plum rootstock, BRISON et al., 1997), *Musa* spp. (banana, HELLIOT et al., 2002), *Vitis vinifera* (grape, WANG et al., 2003b; BAYATI; SHAMS-BAKHSH; MOIENI, 2011; MARKOVIĆ et al., 2015; PATHIRANA et al., 2015), *Fragaria ananassa* (strawberry, CAI et al., 2008), *Solanum tuberosum* (potato, YI et al., 2014), *Rubus idaeus* (raspberry, WANG; VALKONEN, 2009a), *Ipomeas batatas* (sweat potato, WANG; VALKONEN, 2008a), *Dioscorea opposite* (yam, HEE; KYOON; KEUN, 2013), *Allium sativum* (garlic, VIEIRA et al., 2015) and *Malus* spp. (apple, ROMADANOVA et al., 2016; LI et al., 2016).

Cryotherapy is dependent upon the availability of cryopreservation techniques for the desired species; in which the biological material is exposed to liquid nitrogen (LN) at -196 ºC for a short period, normally 1 to 24 hours. Under these conditions, infected vacuolated and differentiated cells are eliminated by the ultralow temperature effect, leaving only highly cytoplasmic meristematic cells (BETTONI et al., 2016; WANG; VALKONEN, 2009 a, b; WANG et al., 2003b). Among the available cryopreservation techniques, encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet vitrification are the most commonly employed (WANG et al., 2018a; ROMADANOVA et al., 2016; WANG et al., 2016; LI et al., 2016; 2015; KAYA et al., 2013; WU et al., 1999; PAUL et al., 2000).

Encapsulation dehydration is based on techniques proposed for synthetic seed production (BENELLI; DE CARLO; ENGELMANN, 2013). The protocol involves the following stages: excising of *in vitro* shoot tips; encapsulating shoot tips; incubation of encapsulated beads in cumulative sucrose concentrations; dehydrating beads; freezing at -196 °C; warming in a water bath at 40°C; and culturing on regeneration medium (WANG et al., 2018a; BETTONI et al., 2016; KAYA et al., 2013). In some cases, its application has resulted in the production of virus-free plants more quickly and economically than through the use of traditional methods (BETTONI et al., 2016). In recent years, studies have shown the effectiveness of cryopreservation in the elimination of ASPV, ACLSV, ASGV and *Apple mosaic virus* (ApMV) in apple trees (LI et al., 2016; ROMADANOVA et al., 2016). To the best of our knowledge, the present study is the first to report the application of cryopreservation techniques in Brazil for the elimination of viruses in apple trees.

The objective of the present study was to evaluate the efficiency of encapsulation-dehydration based cryopreservation method to eradicate ACLSV, ASPV and ASGV in ‘Marubakaido’ apple rootstock (*Malus prunifolia*).

6.3 MATERIALS AND METHODS

6.3.1 Plant Material

‘Marubakaido’ apple rootstocks are widely used in Brazilian apple production regions because the root system is very vigorous. Due to its value to the Brazilian apple industry, ‘Marubakaido’ was selected for cryopreservation protocol optimization and cryotherapy experimentation (FENG et al., 2013; 2014). Cryotherapy efforts focused on the development of efficient methods to eliminate apple latent viruses ACLSV, ASPV and ASGV.

6.3.2 Stock cultures

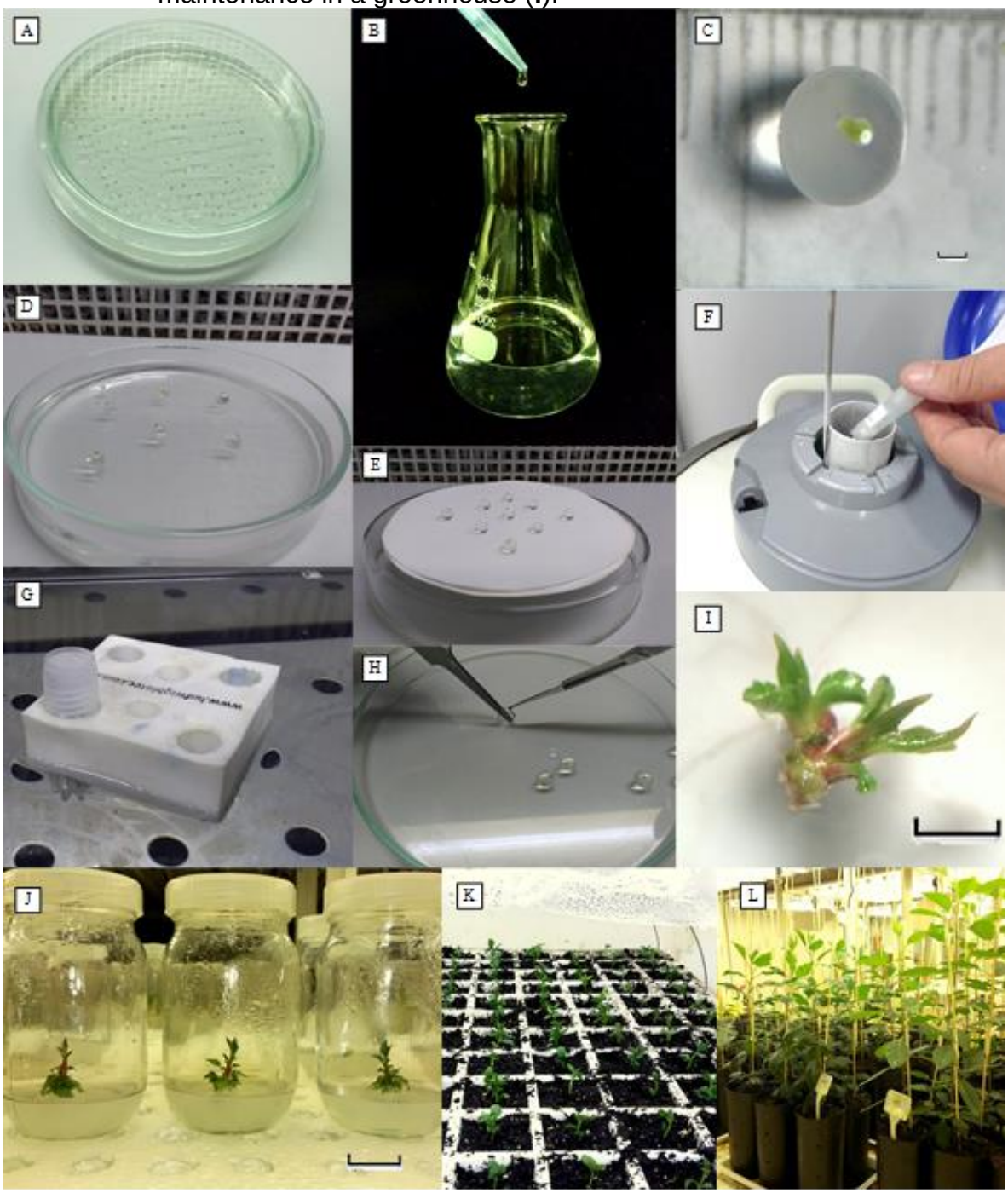
Host ‘Marubakaido’ plants infected with the viral species complex ACLSV, ASGV and ASPV were kept in a greenhouse and used to initiate *in vitro* cultures. Prior to the collection of explants, the greenhouse host plants were placed in the dark for at least 15 days to reduce the oxidation of the culture media (DOBRÁNSKI; DA SILVA, 2010). Nodal segments (2 cm) were collected from the greenhouse plants, were surface sterilized, and then introduced onto medium according to Bettoni et al., (2015c).

Virus-infected ‘Marubakaido’ cultures were maintained on MS growth medium (MURASHIGE; SKOOG, 1962) supplemented with 40 g L^{-1} sucrose, 1 mg L^{-1} 6-benzylaminopurine and 2.6 g L^{-1} Phytagel™, and the medium pH was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. Cultures were kept in a growth room at 25 ± 2 °C, under a photoperiod of 16 h light day^{-1} with a light intensity of 50 μmol m^{-2} s^{-1}. Subcultures were performed every 4 weeks.

6.3.3 Encapsulation and preculture

The encapsulation-dehydration procedures were performed as described by Feng et al. (2013; 2014), with modifications. Axillary shoot tips 1.5 mm long containing 3-4 leaf primordia (Figure 11a) were excised from *in vitro* stock cultures after four weeks of culture and were then placed in the dark for one day on basal medium (BM), composed of the MS salts supplemented with 30 g L^{-1} sucrose, 0.25 mg L^{-1} 6-benzylaminopurine, 0.01 mg L^{-1} indole-3-butyric acid and 2.6 g L^{-1} of Phytagel™, pH 5.8. Subsequently, shoot tips were suspended in liquid MS medium, without calcium, supplemented with 2.5% sodium alginate, 2 M glycerol and 0.4 M sucrose. Shoot tips were individually collected and dropped into 0.1 M $CaCl_2$ solution with 2 M glycerol and 0.4 M sucrose in liquid MS medium for 20 min at 25 ± 2 °C (Figure 11b). Encapsulated shoot tips (4 mm diameter) (Figure 11c) were pre-cultured in the dark for seven days in MS medium supplemented with 0.5 M sucrose and 2.6 g L^{-1} of Phytagel™ (Figure 11D), kept in a growth room at 25 ± 2 °C.

Figure 11 – Excised shoot tips (1.5 mm) from axillary buds of ‘Marubakaido’ apple rootstock (**a**), shoot tips individually collected of the sodium alginate solution and dropped in $CaCl_2$ solution (**b**), encapsulated shoot tips (**c**), encapsulated shoot tips pre-cultured in MS medium supplemented with 0.5 M of sucrose (**d**), encapsulated and pre-cultured shoot tips dehydrated in a laminar flow hood (**e**), freezing in liquid nitrogen for 1 h (**f**), warming in 40 ºC water bath for 3 minutes (**g**), cryopreserved shoot tips were extracted from the capsules and transferred on recovery medium (**h**), recovered ‘Marubakaido’ shoot tips after liquid nitrogen at 45 (**i**) and 60 (**j**) days of cultivation on recovery medium, acclimation (**k**) and plant maintenance in a greenhouse (**l**).



Source: Prepared by the author, 2018.
Scale Bars: **a=** 1 mm; **i=** 5mm; **j=** 2.5 cm.

6.3.4 Dehydration, cryopreservation and warming

Precultured encapsulated shoot tips were transferred to sterile filter paper in Petri dishes and dehydrated by air flow in a laminar flow hood (Figure 11e) for 0, 4, 5, 6, 7, 8 and 9 hours, at 25 ± 2 °C and 29 % humidity. The water content of the encapsulated shoot tips was determined by drying 10 beads from each treatment at 60 °C for 72 h after each time interval. Thirty of the dehydrated beads in each treatment were placed directly into BM (-LN), the remaining were transferred to 10 mL cryotubes (10 beads per cryotube) and immersed in liquid nitrogen (+LN) for at least 1 h (Figure 11f). To warm, cryotubes were immersed in a 40 °C water bath for 3 min (Figure 11g).

6.3.5 Shoot tip recovery

Encapsulated shoot tips that were either exposed or not exposed to LN were post-cultured on solid BM in Petri dishes. After 24 h, the +LN beads were transferred to fresh BM. Cultures were maintained seven days in the dark at 25 ± 2 °C, and then the shoot tips were removed from the alginate beads (Figure 11h). Shoot tips were then transferred to new BM and kept under the conditions described for stock cultures (LI et al., 2015). Cultures were transferred to fresh BM at 5-6 week intervals.

6.3.6 Assessment of survival and regrowth and experimental design

Shoot tip survival was evaluated five weeks after plating onto regrowth medium by counting the beads that exhibited green cell mass growth. Regrowth was evaluated weekly for eight weeks after cryopreservation, with regenerated cultures being those that exhibited organized plant growth (Figure 11i, 11j).

The experimental design was a randomized block design in a 7 x 2 factorial arrangement, consisting of seven dehydration times and +/- exposure to LN, with 20 shoot tips per replicate. There were 10 additional beads used to determine the bead moisture content after each dehydration interval. The experiment was repeated three times, resulting in a total of 60 shoot tips per treatment. Percentage data were submitted to arc-sine transformation and analysis of variance. The averages were compared by Tukey test ($P \leq 0.05$) using R software (R DEVELOPMENT CORE TEAM, 2014).

6.3.7 *In vitro* rooting induction, acclimatization and plant maintenance

Regenerated shoot cultures were transferred to rooting medium, composed of MS supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} indoleacetic acid and 2.6 g L^{-1} Phytagel™, pH 5.8. The cultures were maintained under the same conditions as the stock cultures and after 30 days, *in vitro* rooted cultures were pruned by preserving 2 to 3 basal leaves and 2 cm root length (Figure 11k). Plants were transferred to 100 mL honeycomb trays containing particulate substrate sterilized at 121 °C for 1 hour, using the commercial substrate Tecnomax® and sand (3:2 v/v). Each tray was placed in a plastic basin covered by glass to create saturated conditions of relative humidity. The plants remained in a growth room with a 16 h photoperiod of photosynthetically active radiation 150 µmol m^{-2} s^{-1} and at 25 ± 2 °C. After 30 days the glass cover was gradually removed and acclimated plants were transferred to 1.5 L plastic pots containing the commercial substrate Tecnomax® and kept in a greenhouse (Figure 11l).

6.3.8 Virus detection by PCR

Cryopreserved plants and ‘Marubakaido’ controls were submitted to phytosanitary status verification after 8 months of growth in a greenhouse. Controls were greenhouse plants that were infected with the viral complex, introduced into tissue culture, and underwent the encapsulation-dehydration and recovery processes but were not exposed to LN (-LN). These recovered plants served as positive controls to test the impact of cryopreservation on the elimination of each virus. Twenty recovered plants (+LN) and five recovered controls (-LN) were randomly selected for molecular diagnoses. Two additional cultivars were included as positive controls: ‘Monalisa’ and ‘Maxi Gala’.

Total RNA was extracted from young leaves by using a protocol whereby nucleic acids were adsorbed on silica particles (Sigma Chemical Co., USA) (NICKEL; JELKMANN; KUHN, 1999; ROTT; JELKMANN, 2001; DING et al., 2008). Plant tissue (500 mg) was frozen in LN, ground into a fine powder, and mixed with 300 µL of extraction buffer (1:10 w/v; 4 M guanidine thiocyanate, 0.2 M sodium acetate [pH 5.2], 25 mM EDTA [pH 8.0], 1 M potassium acetate [pH 5.2], 2.5% w/v polyvinylpyrrolidone 40), followed by the addition of 100 µL *N*-lauryl sulphate (10 %). The solution was then incubated at 70°C for 10 min, and then at 0°C for 5 min. After centrifugation for 10 min

at 13 K, 300 µL supernatant was incubated at 25ºC with a 25 µL silica suspension (Rott; Jelkmann, 2001), 150 µL ethanol and 300 µL sodium iodide (6 M) for 10 minutes. The pellet was washed (10 mM Tris-HCl [pH 7.5], 0.5 mM EDTA [pH 8.0], 50 mM NaCl, 50% ethanol) and the RNA was resuspended by vortexing the silica pellet with 150 µL autoclaved water. After incubation at 70°C for 4 min and centrifugation at 13 K for 10 min, the supernatant with nuc1eic acids was used for reverse transcription.

For the synthesis of complementary DNA (cDNA), 4 µL of total RNA, 1 µL of oligo dT primer (10 µM), 0.7 µL of RNAse inhibitor (40 U/µL) and 5 µL of autoclaved deionized water were placed in a water bath at 80 °C for 2 min, followed by 3 min at 0 °C. Then the following reagents were added: 5 µL of 5X reverse transcriptase enzyme (RT) buffer, 2 µL of DTT (0.1 M) and 1 µl of dNTP (2.5 mM each), 1 µL of the MMLV-RT enzyme (Moloney Murine Leukaemia Virus Reverse Transcriptase - 200 U/µL), 1 µL of RNAse inhibitor (RNasin® Ribonuclease Inhibitors - 40 U/µL) and 8 µL of autoclaved deionized water. The 25 µL mixture was incubated for 1 h at 50 ºC, in a thermal cycler (MJ Research). PCR reaction was performed with 2.5 µL of cDNA, 15.75 µL of autoclaved deionized water, 2.5 µL of 10X Taq polymerase buffer, 0.75 µL of $MgCl_2$ (50 mM), 2 µL of 10 µL dNTP (2.5 mM each), 0.5 µL of Taq DNA polymerase (5 U/µL), and 0.5 µL of each complementary and homologous primer (10 µM). The complementary primer CTAAATGCAAAGATCAGTCGAC (genome position: 7343-7365) and the homologous ATGGCAGCAGTTCTGAATTTG (genome position: 6754-6805) (ACLSV), the complementary ATAGCCGCCCCGGTTAGGTT (genome position: 9243-9662) and homologous CTCTTGAACCAGCTGATGGC (genome position: 8993-9012) (ASPV), the complementary CTGCAAGACCGCGACCAAGTTT (genome position: 6373-6396) and homologous ATGAGTTTGGAAGACGTGCTTC (genome position: 5641-5663) (ASGV), were used to amplify, respectively, 581, 269 and 755 bp fragments. PCR reactions were conducted using the following cycles: ACLSV at 95 °C for 2 min, 94 °C for 40 s, 60 °C for 40 s, 72 °C for 1 min; ASPV at 95 °C for 10 min, 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min; ASGV at 95 °C for 2 min, 94 °C for 40 s, 54 °C for 40 s, 72 °C for 1 min, followed by 34 cycles of amplification and a final extension of 5, 10 and 5 min, respectively. PCR products were analysed by electrophoresis in 1.2% agarose gel dissolved in TBE buffer (Tris-borate-EDTA), stained with ethidium bromide. The gel was visualised and photographed.

6.4 RESULTS

6.4.1 Effect of dehydration time on survival and regrowth from cryopreserved shoots

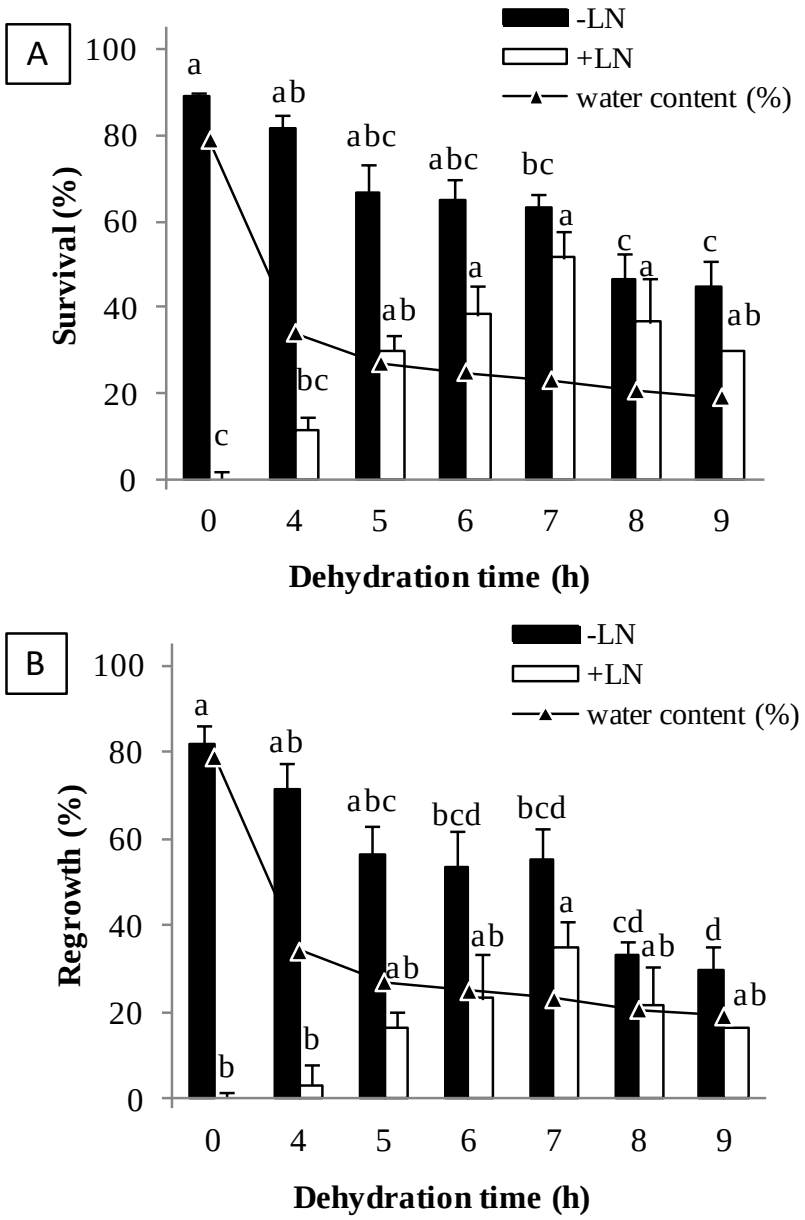
The success of the cryotherapy/cryopreservation protocols was dependent upon the extent of dehydration of the plant material before freezing. The water content of encapsulated beads was dependent upon the length of time they were air-dried in the laminar flow hood (Figure 12). The water content of the encapsulated beads significantly affected the survival and regrowth of 'Marubakaido' shoot tips (Figure 12). Encapsulated beads had initial water contents of 79% (FWB), which decreased to 34% after 4 hours of dehydration and then to 19% after 5 additional hours of dehydration. For the controls (-LN), the highest levels of survival (89%) and regrowth (82%) were observed when the beads were not dehydrated. The survival and recovery of shoot tips (-LN) within dehydrated beads decreased from 82% survival and 72% regrowth after 4 h of dehydration to 45% survival and 30% after 9 hours of dehydration (bead moisture content of 19%, FWB).

The survival and regrowth of LN-exposed (+LN) encapsulated shoot tips increased as the drying time was increased from 4 to 7 hours. Encapsulated 'Marubakaido' shoot tips dehydrated to 23% after 7 h of air drying in a laminar flow hood had the highest level of survival and regrowth after LN exposure. In our experiment, 21 plants were regenerated without callus formation from 60 initial explants.

After cryopreservation, only the shoot tips within the encapsulated beads that were dehydrated for between 6 and 9 h had normal *in vitro* development, without callus (Figure 11i, 11j). Dehydration lengths of fewer than 6 hours resulted in explants with green, unorganized tissues, callus, or hyperhydric leaf tissue.

All of the 'Marubakaido' plants that were transferred to the greenhouse survived the acclimation process (Figure 11k). No visible morphological differences were apparent in the –LN and +LN plants that were transferred to the greenhouse.

Figure 12 – Effect of dehydration time and encapsulated bead water content on survival (A) and regrowth (B) percentages of apple rootstock 'Marubakaido' with (+LN) and without (-LN) LN exposure during the encapsulation-dehydration procedure.



Source: Prepared by the author, 2018.
Vertical bars indicate confidence intervals; Letters denote significant differences among results of each treatment at α=0.05 (Tukey's test).

6.3.2 Virus eradication

After 8 months of growth in the greenhouse, young leaves from the resulting +LN and –LN 'Marubakaido' plants were sampled to test the efficiency of the encapsulation-dehydration method in eradicating the viral complex ASGV, ASPV and

ACLSV. PCR analysis showed a 90% complex virus eradication frequency. The ACLSV and ASPV viruses were not detected in any of the tested +LN plants. In 18 of the 20 +LN samples tested, there was no amplification of ASGV, indicating that the cryotherapy method, based on the encapsulation-dehydration cryopreservation protocol, successfully removed ASGV, ASPV and ACLSV viruses from most of the 'Marubakaido' shoot tips (Table 20). The plants regenerated from control (–LN) were positive for all three viruses in 5 of the 5 samples tested, indicating that the LN step was critical to the success of the cryotherapy procedure.

Table 20 – Frequency of ACLSV-, ASGV- and ASPV- virus free shoots regenerated from cryotherapy (+LN) by encapsulation-dehydration and control (–LN) of Marubakaido apple rootstock (*M. prunifolia*) after 8 months of greenhouse cultivation.

Treatments	Frequency of virus-free (%)			Virus complex-free plants (%)
	ACLSV	ASGV	ASPV	
+LN	100 (20/20)	90 (18/20)	100 (20/20)	90 (18/20)
–LN	0 (5/5)	0 (5/5)	0 (5/5)	0 (5/5)

Source: Prepared by the author, 2018.
The ratio between virus-free/total number of regenerated plants and used for detection by PCR is presented in parentheses.

6.5 DISCUSSION

In this research, an efficient cryotherapy method based on a cryopreservation protocol for the eradication of *Malus* latent viruses was tested using shoot tips excised from *in vitro* cultures. We found that the encapsulation-dehydration method previously developed for *Malus domestica* (36% regrowth for 'Wangshanhong' and 75% regrowth for 'Gala') was applicable to *Malus prunifolia* (FENG et al., 2013). To date, there have only been two published studies focused on using cryopreservation methods to eradicate apple viruses (ROMADANOVA et al., 2016; LI et al., 2016). In addition, there have been successful cryopreservation protocols for *Malus* using encapsulation-dehydration (ZHAO et al., 1999; WU et al., 1999; PAUL et al., 2000; HAO, LIU and DENG, 2001; FENG et al., 2013; 2014; LI et al., 2014; 2015), vitrification (NIINO et al., 1992a,b; WU et al., 1999), encapsulation-vitrification (PAUL et al., 2000) and droplet vitrification (HALMAGYI et al., 2010; HALMAGYI; DELIU; ISAC, 2010; CONDELO et al., 2011; FENG et al., 2014; LI et al., 2015). In these works, the propagules were

obtained from *in vitro* cultures. Cryopreservation is also possible with material collected in the field, using the technique of cryopreservation of dormant buds (JENDEREK; REED, 2017; VOLK et al., 2008).

Cryopreservation techniques have been applied to the eradication of viruses in several species of economic importance (BETTONI et al., 2016; WANG et al., 2009). Recently, Romadanova et al. (2016) demonstrated the efficiency of the vitrification cryopreservation method for the removal of the viral complex ACLSV, ASGV, ASPV and ApMV, with an eradication average of 37.5%, and the virus-free plant frequencies varied according to the genotype and type of virus. Li et al. (2016), using the encapsulation-dehydration protocol, similar to the one applied in this study, observed that after cryopreservation, 85% of the plants of the 'M9' and 'M26' apple rootstocks were free of ASPV virus. In our work, ASGV, ASPV, and ACLSV appeared to be eradicated from 'Marubakaido' rootstock shoot tips after cryopreservation by encapsulation-dehydration. Two samples, out of 20, remained infected with ASGV after LN exposure.

Wang et al. (2003b) were the first to demonstrate the application of the encapsulation-dehydration technique resulted in the eradication of *Grapevine virus A* (GVA) in 97% of the *Vitis vinifera* plants recovered after cryopreservation. In addition, these authors observed that the size of the propagule (in the range of 0.5 – 2 mm) used in the cryogenic process was not a determinant in virus eradication when encapsulation-dehydration techniques were employed, whereas, in traditional virus eradication methods such as meristem isolation or thermotherapy, the size of the excised tissue is a limiting regrowth factor. The frequency of virus elimination in the traditional shoot tip excision method is inversely proportional to the size of the excised tissue hence, the excision of fragments with relatively small dimensions ranging from 0.2 to 0.4 mm is recommended (WANG et al., 2003b; 2016). On the other hand, the viability of the culture recovery is proportional to the size of the excised tissue. Li et al. (2015) reported that 0% of the meristems that were 0.2 mm could be recovered, whereas 92.3% of the 1.0 mm shoot tips could be recovered, using 'M9'and 'M26' apple rootstocks. Despite the high regrowth level of 1.0 mm meristems, no plant was free from ASGV and ASPV. When the authors excised tissues that were 0.5 mm and had two leaf primordia, 100% of the ASPV was eradicated, but the process was inefficient for ASGV. Nevertheless, with the excision of tissues with the same 0.5 mm and containing three leaf primordia, the frequency of ASPV eradication was only 10% (LI

et al., 2015). Wang et al. (2003) found that the eradication of GVA in *Vitis vinifera* was possible when meristems were excised with 0.2 mm; in addition to the challenge of excising tiny shoot tips, only 12% of the regenerated vines were free from the GVA. According to the same authors, recovery of 100% occurred in cultures with 0.4 mm, but the virus was not eliminated.

In addition to the simplicity of excising larger shoot tips, cryotherapy by encapsulation-dehydration resulted in high levels of eradication of the tested viral complex. Traditional methods such as meristem culture may be effective in eradicating a specific virus, but may fail when working with viral complexes (BARBA; ILARDI; PASQUINI, 2015; WANG et al., 2016).

In addition to maintaining the viability of cryopreserved materials, cryopreservation/cryotherapy protocols should preserve the genetic integrity of the culture during LN storage (HALMAGYI et al., 2010). Under cryogenic conditions, plant tissue is preserved in a state where cell division does not occur and the metabolic processes are virtually suspended. Furthermore, the risk of contamination is minimized, and many samples can be easily stored for extended lengths of time (BENSON, 2008; LI et al., 2014; 2015; ZHANG et al., 2015; 2016; WANG et al., 2017). Previously, studies using flow cytometry and molecular markers have indicated that the genetic integrity of cryopreserved *Malus* species was not affected by cryogenic procedures (HAO; LIU; DENG, 2001; LI et al., 2014; 2015). Plants regrown from callus may not maintain genetic fidelity after cryoprocedures; thus, cryo- protocols should be optimised to avoiding plant recovery via callus (HARDING, 2004; MATSUMOTO, 2017a).

The results presented in this study demonstrate that the cryotherapy technique of encapsulation-dehydration, based on the cryopreservation protocol reported by Feng et al. (2013; 2014), efficiently eradicates the ASPV, ACLSV and ASGV viral complex in ‘Marubakaido’ apple rootstocks. In this protocol, 1.5 mm axillary shoot tips were excised from 4-week *in vitro* stock cultures and cultured for one day in BM, followed by the encapsulation of shoot tips, and precultured in the dark, using MS medium supplemented with 0.5 M sucrose for 7 days. Subsequently, the encapsulated beads were dehydrated in a laminar flow hood for 7 h to a moisture content of 23.1% (FWB), before immersion in LN for 1 h. Encapsulated beads were warmed in a water bath at 40 °C for 3 min. The beads were cultured in regrowth medium for 24 h, and then transferred onto fresh medium, and kept in the dark for seven days. After this

period, the shoot tips were extracted from the beads and transferred to the fresh growth medium. This protocol resulted in plant regrowth levels of 35% without callus formation. After 8 months of acclimatization, all evaluated plants were free of ACLSV and ASPV viruses and only 10% of the plants had ASGV. This is the first report of the use of cryopreservation for the eradication of viral complexes of *Malus* in Brazil. The practicality and effectiveness of this method may facilitate the production of plants free of viral pathogens on a commercial scale, generating high-quality plant material.

7 ERADICATION OF LATENT VIRUSES FROM APPLE CULTIVAR 'MONALISA' SHOOT TIPS USING DROPLET-VITRIFICATION CRYOTHERAPY

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7.1 ABSTRACT

Virally transmitted diseases in apple cause economic losses as a result of their direct effect on production and yield and by causing an increased susceptibility to other phytopathogenic agents. This study evaluated the effectiveness of a droplet-vitrification cryopreservation technique in eradicating latent viruses *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) from *in vitro* axillary shoot tips excised from 'SC417 Monalisa' apple cultivar shoot tips. *In vitro* stock cultures, four weeks after transfer, infected with ASGV, ASPV and ACLSV were used as source material. Axillary shoot tips were excised from stock cultures, precultured in MS + 2 M glycerol + 0.8 M sucrose, and then exposed to plant vitrification solution 2 (PVS2) for between 0 and 80 min at 0ºC or 22ºC prior to liquid nitrogen (LN) exposure. Shoot tips were then warmed and recovered on growth medium. The survival and regrowth of cryopreserved (+LN) and non-cryopreserved (-LN) 'Monalisa' shoot tips were evaluated and the efficiency of virus eradication was determined by RT-PCR after recovered plants were grown in a greenhouse for 6 months. With this protocol, the highest levels of regrowth (45%) were obtained after 20 to 40 min PVS2 exposure duration at 22ºC. After 6 months grown in a greenhouse, all of the evaluated cryopreserved plants that were tested were free of ASPV, 95% were free of ACLSV, and 35% were free of ASGV. This promising cryotherapy procedure may facilitate the production of virus-free plants.

Keywords*:* Malus. ASGV. ASPV. ACLSV. Eradication. Cryopreservation.

7.2 INTRODUCTION

Apples (*Malus* spp.) are among the most important fruit crops cultivated and consumed in the world (VOLK et al., 2015). Brazil is a primary fruit-producing country. In 2014, Brazil produced 1,38 million tons of apple, representing 1.63% of the world apple production (FAOSTAT, 2017). In the 1970s, Brazil quarantine procedures required that imported apple cultivars be either virus free or cleaned-up prior to entry into the country. This increased the productivity and fruit quality of Brazilian apple orchards (PETRI et al., 2011). Unfortunately, Brazilian apple orchards were already widely infected with viruses prior to the implementation of these regulations and almost five decades later there are still high viral infection levels in Brazilian apple orchards (NICKEL et al., 2001; NICKEL; FAJARDO, 2014). These viruses are believed to be spread only by using infected propagation material, transmitted by grafting (HU et al., 2017; BARBA; ILARDI; PASQUINI, 2015; NICKEL; FAJARDO, 2014; MARTELLI et al., 2007).

Apple trees infected with viruses exhibit reduced vigour, productivity, and fruit quality, as well as an increased susceptibility to other phytopathogenic agents (NICKEL et al., 2001; CIEŚLIŃSKA; RUTKOWSKI, 2008; HADIDI; BARBA, 2011; GUERRA et al., 2012; KUMAR et al., 2014). *Apple stem grooving virus* (ASGV, genus *Capillovirus*), *Apple stem pitting virus* (ASPV, genus *Foveavirus*) and *Apple chlorotic leaf spot virus* (ACLSV, genus *Trichovirus*) are several major viral pathogens of apple trees that are responsible for substantial damage to the world's apple industry (LÉMOINE, 1990; FAJARDO; NICKEL, 2014). Plants infected with these viral species that commonly occur as mixed infections are generally asymptomatic, but symptoms may become apparent when the host plants are propagated onto alternative rootstocks (BARBA; ILARDI; PASQUINI, 2015). It is difficult to control diseases within infected trees once they are planted in the field; therefore, a supply of virus-free planting materials is essential for sustainable production (WANG et al., 2014).

Faced with this scenario, there is a need for the development of simple and efficient techniques to eradicate viruses from stock plants (BI et al., 2017; WANG et al., 2009; WANG et al., 2014). Based on cryopreservation techniques, cryotherapy is a new tool that has proven to be helpful to eradicate viruses from infected plant material in multiple species of economic importance (BRISON et al., 1997; WANG et al., 2008, 2009; WANG; VALKONEN, 2008, 2009a; CAI et al., 2008; BAYATI; SHAMS-BAKHSH;

MOIENI, 2011; YI et al., 2014; MARKOVIĆ et al., 2015; PATHIRANA et al., 2015; VIEIRA et al., 2015; ROMADANOVA et al., 2016; BETTONI et al., 2018b) and may complement traditional clean-up procedures (BETTONI et al., 2016; WANG; VALKONEN, 2009b). Cryotherapy makes use of liquid nitrogen (LN) exposure (−196 °C) to selectively kill infected cells within shoot tips. Vacuolated and differentiated cells that harbor viruses are eliminated because they do not survive LN exposure, allowing the highly cytoplasmic meristematic cells to regenerate healthy, disease-free plants (WANG; VALKONEN, 2009b; CUI et al., 2015; BETTONI et al., 2016). The shoot tips used for cryotherapy may be relatively large (0.5-2 mm), compared to traditional methods for virus eradication such as meristem isolation (0.1-0.3 mm) (WANG et al., 2003b). Meristem isolation has higher levels of virus elimination when very small shoot tips are used, but it is difficult to regenerate plants from these meristems (WANG et al., 2016; WANG; VALKONEN, 2007).

To date, cryotherapy alone or in combination with thermotherapy has been shown to eradicate some apple viruses (WANG et al., 2018b; BETTONI et al., 2018b; ROMADANOVA et al., 2016). We previously published an encapsulation-dehydration cryotherapy method that eradicated ACLSV, ASGV, and ASPV from *Malus prunifolia* (BETTONI et al., 2018b). We found this encapsulation-dehydration method to be labor intensive and time consuming. Herein, we identify a vitrification-based cryotherapy procedure that appears to be both efficient and practical for eradicating the ACLSV, ASGV, and ASPV from *in vitro* ‘SC417 Monalisa’ apple (*Malus domestica* Borkh, Gala × *Malus* 4) cultures.

7.3 MATERIALS AND METHODS

7.3.1 Plant Material and stock cultures

Apple ‘SC417 Monalisa’ (Gala × *Malus* 4) was selected for this study due to its importance to the Braziliam apple industry; ‘Monalisa’ has a high level of resistance to apple scab (*Venturia inaequalis*) and to glomerella leaf spot (*Colletotrichum gloeosporioides*), as well as red spider mites (*Panonychus ulmi*); has a low chilling requirement, and a uniform red-purple skin color (CAMILO; DENARDI, 2012).

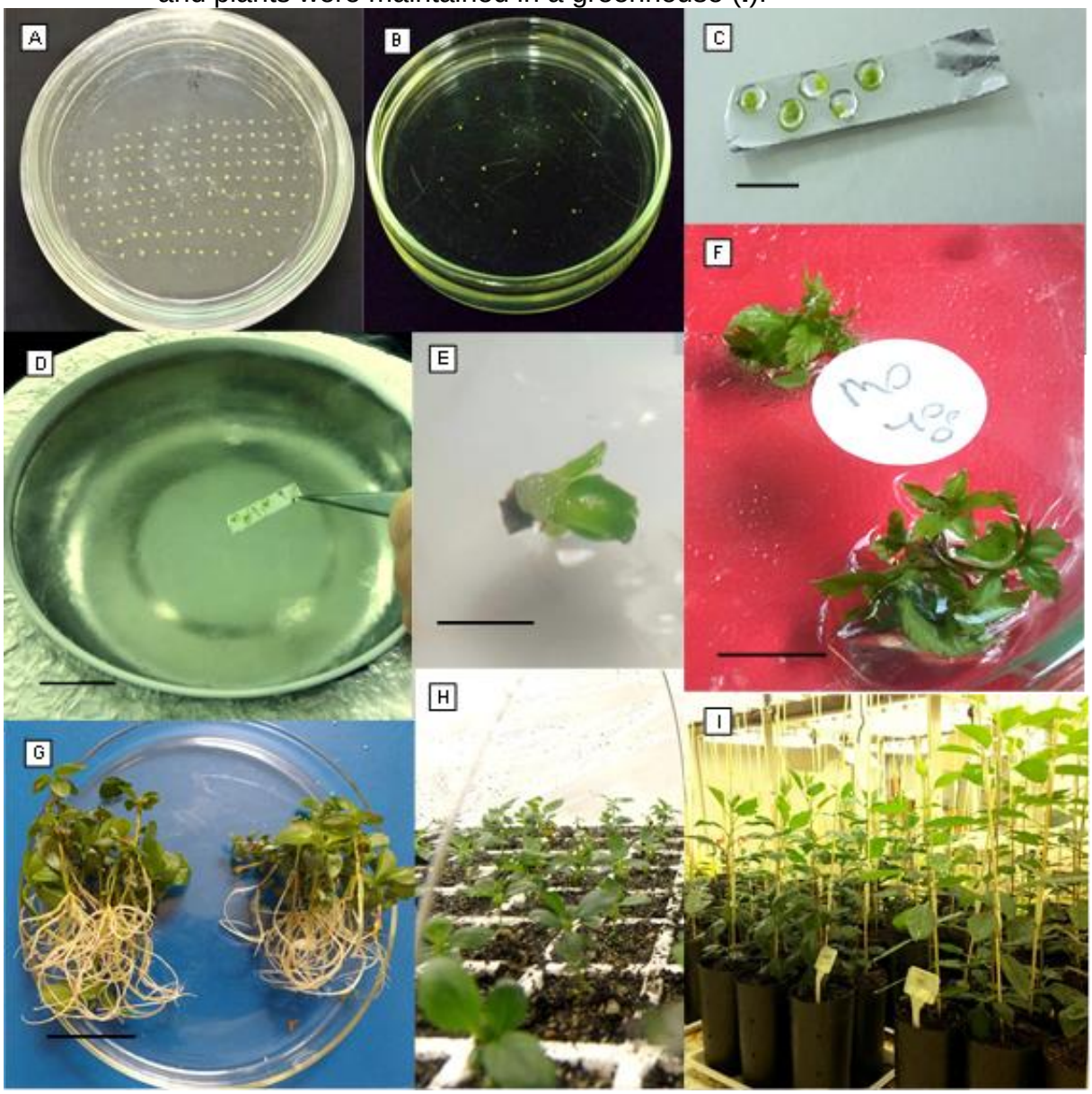
Host ‘Monalisa’ plants infected with ACLSV, ASGV and ASPV were originally received from the Epagri – Santa Catarina State Agricultural Research and Rural

Extension Agency (Brazil) and were kept in a greenhouse before the *in vitro* cultures were initiated. Prior to culture initiation, greenhouse host plants were placed in the dark for at least 15 days to reduce production of phenolics (DOBRÁNSKI; DA SILVA, 2010). Nodal segments (2 cm) were collected from the greenhouse plants, were surface sterilized with 70% alcohol (v/v) for 15 seconds and were rinsed twice for 1 min with sterile distilled water. They were then treated with 80% sodium hypochlorite solution (v/v) and 0.1% Tween 20 (v/v) for 15 min and rinsed three times in sterile distilled water, and introduced onto medium according to Bettoni et al. (2015c). Virus-infected 'Monalisa' cultures were maintained in an actively growing state on MS medium (MURASHIGE; SKOOG, 1962), containing 40 g L^{-1} sucrose, 1 mg L^{-1} 6-benzylaminopurine and 2.6 g L^{-1} Phytagel™, and the medium pH was adjusted to 5.8 prior to autoclaving. Cultures were grown at 25°C ± 2°C, under a photoperiod of 16 h light day^{-1} with a light intensity of 50 μmol m^{-2} s^{-1}. Subcultures were performed once every 4 weeks.

7.3.2 Preculture and cryopreservation

Droplet-vitrification procedures were performed as described by Li et al. (2015) using the virus-infected *in vitro* plants, with the following modifications. Axillary shoot tips 1 mm in length containing 2-3 leaf primordia (Figure 13A) were excised from 4-week *in vitro* stock cultures, and then placed on basal medium (BM; MS medium containing 30 g L^{-1} sucrose, 0.25 mg L^{-1} 6-benzylaminopurine, 0.01 mg L^{-1} indole-3-butyric acid and 2.6 g L^{-1} of Phytagel™ at pH 5.8) for 1 day at 25 ^{o}C± 2 °C in darkness. Shoot tips were then incubated on preculture medium (MS + 2 M glycerol + 0.8 M sucrose at pH 5.8) for 1 day at 25 °C± 2 °C in darkness. After incubation, shoot tips were placed in PVS2 (filter sterilized solution, MS + 30% (w/v) glycerol + 15% (w/v) ethylene glycol +15% (w/v) dimethyl sulfoxide + 0.4 M sucrose at pH 5.8, SAKAI; KOBAYASHI; OIYAMA, 1990) at 22^{o}C or 0^{o}C for 0, 20, 40, 50, 60 or 80 min. Two minutes before the end of the each treatment, PVS2-treated shoot tips were placed into 2.5 µL PVS2 droplets on sterile aluminum foil strips (6 x 25 mm, 5 shoot tips per aluminum foil strips) (Figure 13C) and then plunged into liquid nitrogen (LN) (Figure 13D). After LN exposure for a few minutes, the foils with shoot tips were transferred into a 2-ml cryotube filled with LN and kept in LN for 1 h.

Figure 13 – Selected steps in the droplet-vitrification procedure for *in vitro* apple cv. 'Monalisa'. Axillary shoot tips (1 mm) (**A**) were excised from 4-week old *in vitro* stock plants and incubated on the preculture medium for 1 day (**B**). Shoot tips placed into PVS2 droplets on sterile aluminum foil strips (**C**) prior to LN exposure (**D**). 'Monalisa' shoot tips grown for 20 (**E**) and 60 (**F**) days after cryoexposure. Cultures were *in vitro* rooted (**G**), acclimated (**H**) and plants were maintained in a greenhouse (**I**).



Source: Prepared by the author, 2018
Scale Bars: **C=** 5 mm; **D=** 25 mm; **E=** 2 mm; **F=** 10 mm; **G=** 30 mm.

7.3.3 Regrowth and cryoexposure

After one hour of LN exposure, the aluminum foil strips with shoot tips were warmed quickly by inverting the strips into unloading solution (MS + 1.2 M sucrose at pH 5.8) at 22ºC for 20 min.

For recovery, shoot tips were placed onto solid BM overnight in the dark and then transferred to fresh BM. Cultures were maintained seven days in the dark at 25 ± 2 °C, and then the shoot tips were kept under the conditions described for stock cultures. Cultures were transferred to fresh BM at 5-6 week intervals.

7.3.4 Assessment of survival and regrowth and data analyses

Shoot tip survival (green cell mass or leaf tissue) and regrowth (organized shoots with at least one leaf) were measured 8 weeks after plating on regrowth medium.

The experimental design was a randomized block design in a 6 x 2 factorial arrangement, consisting of six PVS2 exposure times and +/- exposure to LN. Each experiment was performed with three replicates of 30 shoot tips for each treatment. Percentage data were submitted to arc-sine transformation and analysis of variance. Means were calculated across experimental replicates, and ANOVA/means separation tests were performed the Tukey's mean separation test ($P \leq 0.05$) using R software (R DEVELOPMENT CORE TEAM, 2014).

7.3.5 *In vitro* rooting induction, acclimatization and plant maintenance

In vitro rooting induction, acclimatization and plant maintenance were performed according to Bettoni et al. (2018b). Regenerated shoot cultures were transferred to rooting medium, composed of MS supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} indoleacetic acid and 2.6 g L^{-1} Phytagel™, pH 5.8. The cultures were maintained under the same conditions as the stock cultures and after 30 days, *in vitro* rooted cultures were pruned by preserving 2 to 3 basal leaves and 2 cm root length (Figure 13K). Plants were transferred to 100 mL honeycomb trays containing particulate substrate sterilized at 121 °C for 1 hour, using the commercial substrate Tecnomax® (Tecnomax,

Vargem Bonita, Santa Catarina, Brazil) and sand (3:2 v/v). Each tray was placed in a plastic basin covered by glass to create saturated conditions of relative humidity. The plants remained in a growth room with a 16 h photoperiod of photosynthetically active radiation 150 µmol m^{-2} s^{-1} and at 25 ± 2°C. After 30 days the glass cover was gradually removed and acclimated plants were transferred to 1.5 L plastic pots containing the commercial substrate Tecnomax® and grown in a greenhouse (Figure 13L).

7.3.6 Virus detection by RT-PCR

The sanitary status of ‘Monalisa’ control and cryopreserved plants was checked after 6 months of growth in a greenhouse. 'Monalisa' *in vitro* stock plants infected with ASGV, ASPV, and ACLSV (control 1) and plants recovered from shoot tips that underwent the droplet-vitrification and recovery processes but were not exposed to LN (control 2) served as experimental positive controls to test the impact of cryopreservation on the elimination of each virus. ASGV, ASPV, and ACLSV were detected in the control 1 and control 2 plants and ensured that the source materials were virus infected before cryotherapy. Infected greenhouse plants were also used as a general positive control (C+). To assess virus eradication frequency, all plants cryopreserved (+LN) from all PVS2 exposure times were put together and grown in a greenhouse for 6 months, then twenty of these were randomly selected for molecular diagnosis.

Total RNA was extracted from 100 mg young leaves using an RNeasy® Plant Mini Kit according to the manual (QIAGEN, Hilden, Germany). For the synthesis of complementary DNA (cDNA), 1 µL of total RNA, 1 µL of oligo dT primer (10 µM), 1 µl of dNTP (2.5 mM each) and 12 µL of RNAse-free water were placed in a water bath at 70°C for 5 min, followed by 5 min at 0°C. Then the following reagents were added: 4 µL of 5X reverse transcriptase enzyme (RT) buffer and 1 µL of the MMLV-RT enzyme (Moloney Murine Leukaemia Virus Reverse Transcriptase - 200 U/µL). The 20 µL mixture was incubated for 5 min at 25ºC, 1 h at 42ºC and 10 min at 70ºC in a thermal cycler (MJ Research). PCR reaction was performed with 1 µL of cDNA, 19 µL of RNAse-free water, 2.5 µL of 10X Taq polymerase buffer, 0.75 µL of $MgCl_2$ (50 mM), 0.5 µL of 10 µL dNTP (2.5 mM each), 0.25 µL of *Taq* DNA polymerase (5 U/µL, Ludwig), and 0.5 µL of each complementary and homologous primer (10 µM). The complementary primer AAGTCTACAGGCTATTTATTATAAGTCTAA (genome

position: 7507-7536) and the homologous TTCATGGAAAGACAGGGGCAA (genome position: 6860-6880) (ACLSV), the complementary ATAGCCGCCCCGGTTAGGTT (genome position: 9243-9662) and homologous CTCTTGAACCAGCTGATGGC (genome position: 8993-9012) (ASPV), the complementary CTGCAAGACCGCGACCAAGTTT (genome position: 6373-6396) and homologous ATGAGTTTGGAAGACGTGCTTC (genome position: 5641-5663) (ASGV), were used to amplify, respectively, 676, 269 and 755 bp fragments. PCR reactions were conducted using the following cycles: ACLSV at 94 °C for 2 min, 94 °C for 45 s, 60 °C for 40 s, 72 °C for 1 min; ASPV at 94 °C for 10 min, 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min; ASGV at 94 °C for 2 min, 94 °C for 40 s, 54 °C for 40 s, 72 °C for 1 min, followed by 34 cycles of amplification and a final extension of 5 min for ACLSV, 10 min for ASPV and 5 min for ASGV. PCR products were analysed by electrophoresis using a 1% agarose gel in TBE buffer (Tris-borate-EDTA), stained with GelRed^{TM}. The gel was visualised and photographed under ultraviolet light.

7.4 RESULTS

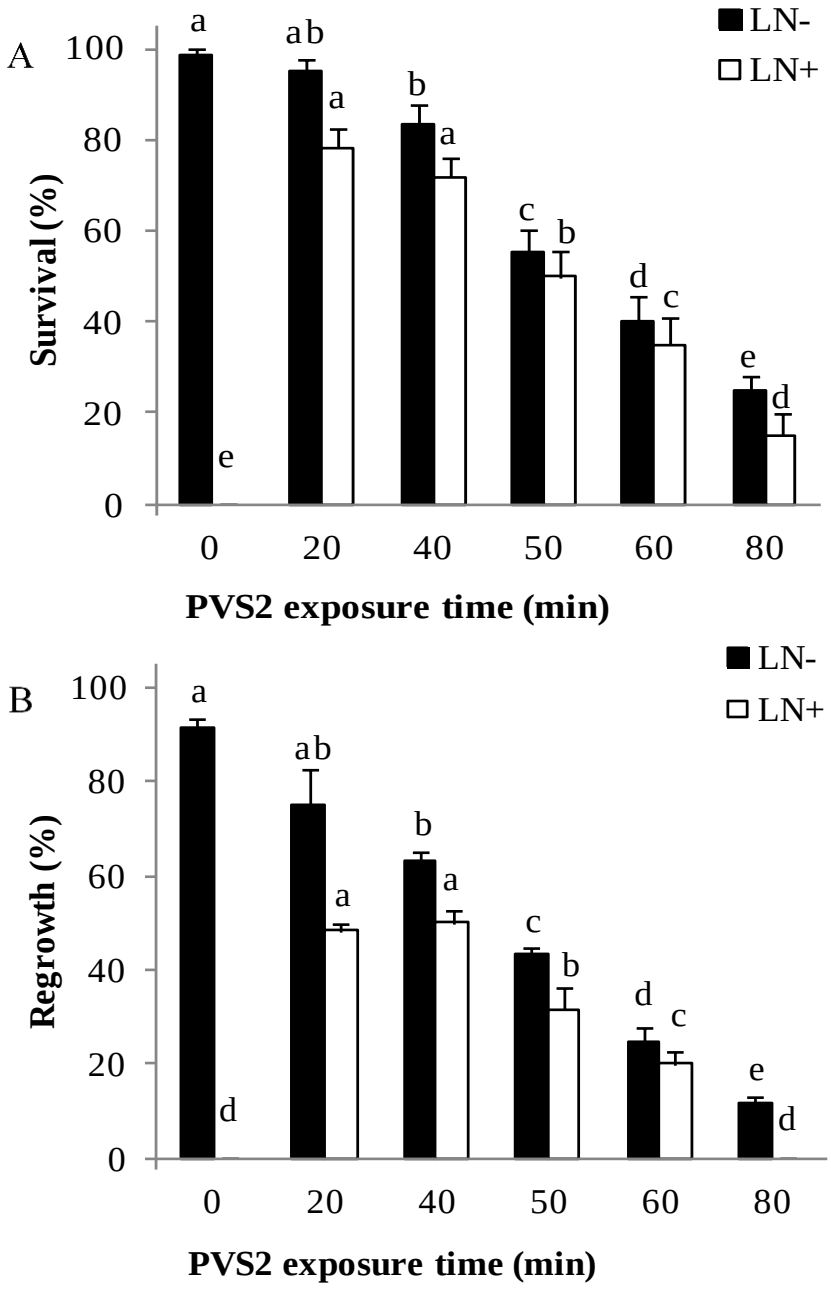
7.4.1 Effect of 22^{o}C PVS2 exposure duration on survival and regrowth of cryopreserved (+LN) and non-cryopreserved (-LN) virus-infected shoot tips

The control 2 plants, ‘Monalisa’ shoot tips from in vitro plants that were infected with the viral complex (ASPV, ACLSV, and ASGV) that were not exposed to LN (-LN) had higher levels of survival and regrowth compared to the infected plants that underwent the corresponding LN treatment (+LN) (Figure 14A, 14B). Overall regrowth levels were lower than the survival levels across all the PVS2 exposure durations (Figure 14A, 14B). Extended PVS2 exposure durations (80 min) were lethal to shoot tips, even without LN exposure (Figure 14A, 14B). Shoot tips survival levels were between 20 and 100% for the six PVS2 exposure durations for the non-LN treated shoot tips (Figure 14A). With LN exposure, shoot tip survival percentages averaged 0, 70, 65, 40, 25 and 10% for 0, 20, 40, 50, 60 and 80 min PVS2 exposure durations, respectively (Figure 14A).

Regrowth levels in control 2 non-cryopreserved shoot tips (-LN) decreased from 90% to 15% according to the increase of the exposure time to PVS2. In +LN, the maximum regrowth was 45% in 20 and 40 minutes of PVS2 exposure. (Figure 14B).

Shoot tips exposed to more than 40 min PVS2 at 22ºC had lower survival and regrowth levels, and 80 min PVS2 exposures were lethal when plants were exposed to LN.

Figure 14 – Survival (A) and regrowth (B) levels (%) of shoot tips excised from infected *in vitro* stock cultures from apple cultivar 'Monalisa' grown *in vitro* with 0, 20, 40, 50, 60 and 80 min of PVS2 at 22ºC, with (+LN) and without (-LN) LN exposure using a droplet-vitrification procedure.



Source: Prepared by the author, 2018.
Vertical bars indicate confidence intervals; letters denote significant differences among results of each LN treatment at α=0.05 (Tukey's test).

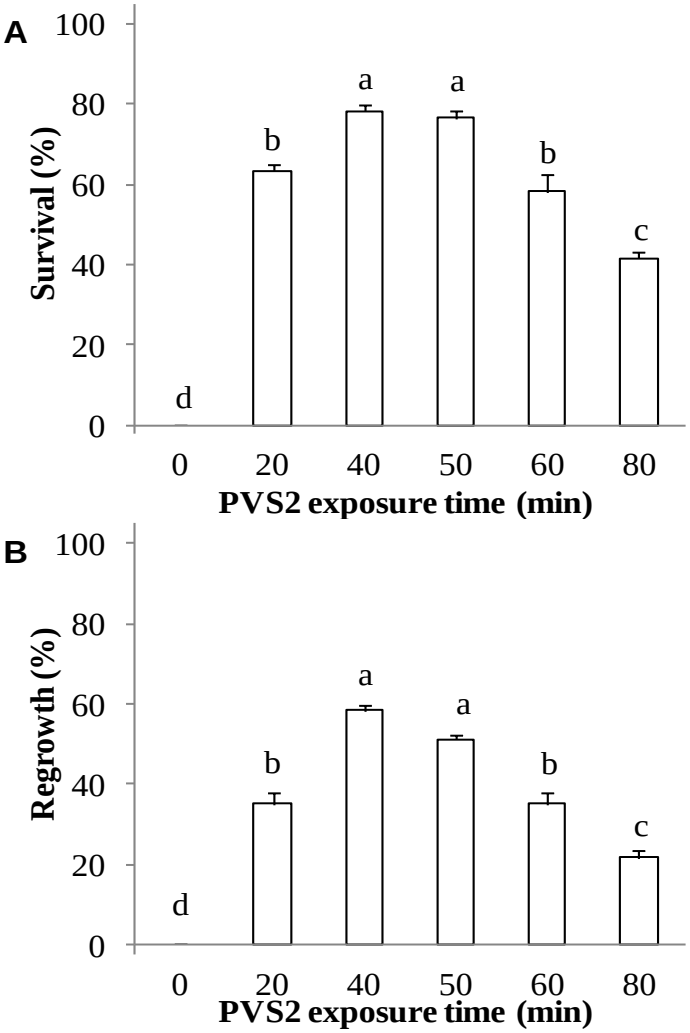
The optimal PVS2 treatment duration was determined to be 20 to 40 min at 22ºC, which resulted in an average regrowth of 45% in 1 mm cryopreserved axillary shoot tips excised from 4-weeks old *in vitro* stock cultures and incubated for 1 day on the preculture medium.

All of the 'Monalisa' cultures were successfully rooted *in vitro* and 90% survived the acclimation process (data not shown). No visible morphological differences were apparent in the –LN and +LN plants were transferred to the greenhouse. No morphological disease symptoms in either the controls or the cryo-treated plants were observed.

7.4.2 Effect of 0ºC PVS2 exposure on survival and regrowth of cryopreserved (+LN) virus-infected shoot tips

The highest survival (78% and 77%) and regrowth (58% and 51%) levels of cryopreserved (+LN) shoot tips were obtained with 40 and 50 min PVS2 exposure durations at 0ºC. Survival and regrowth levels after cryoexposure were lower when PVS2 exposures were 0, 20, 60, or 80 min at 0ºC (Figure 15A, 15B). Shoot tips tolerated longer PVS2 incubation durations at 0ºC than at 22ºC (Figure 14 and 15).

Figure 15 – Survival (A) and regrowth (B) levels (%) of shoot tips excised from *in vitro* stock cultures from apple 'Monalisa' grown *in vitro* with 0, 20, 40, 50, 60 and 80 min of PVS2 exposure at 0ºC, with (+LN) LN treatment during the droplet-vitrification procedure.



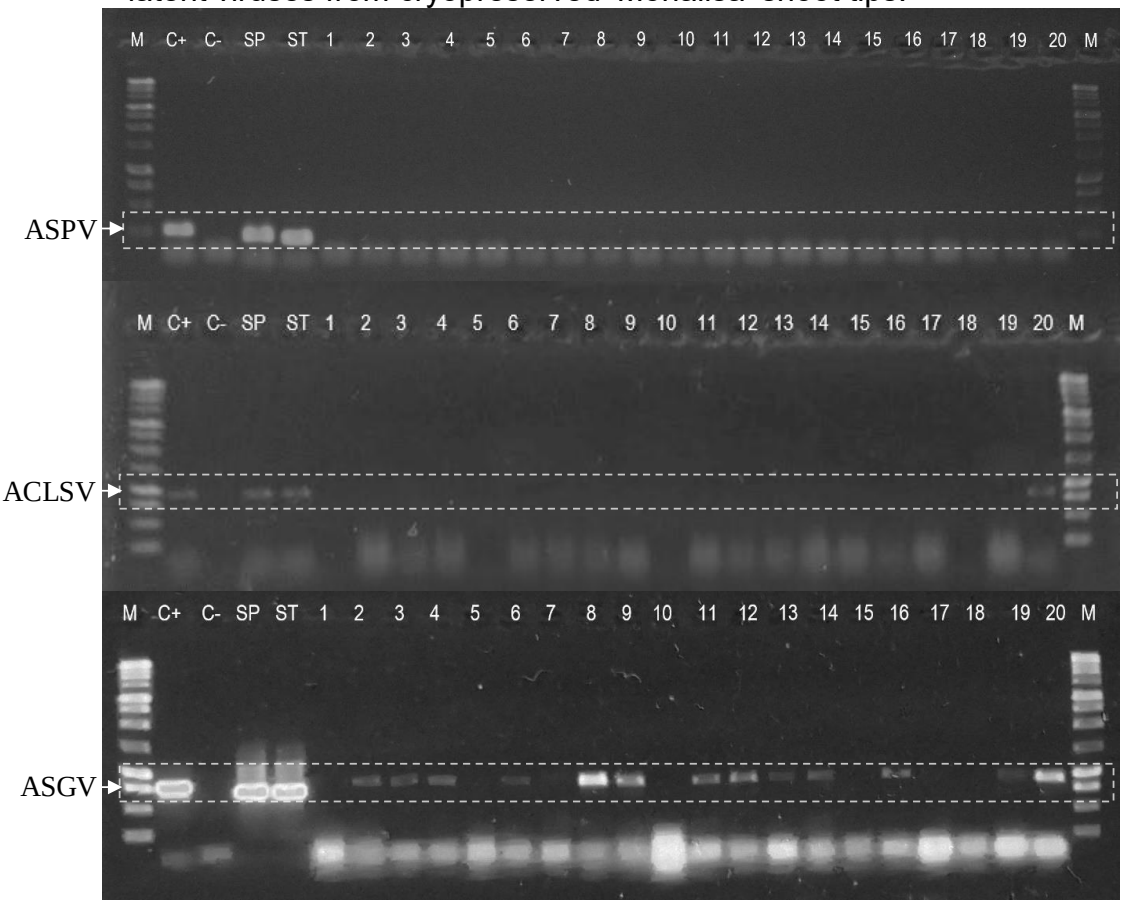
Source: Prepared by the author, 2018.
Vertical bars indicate confidence intervals; Letters denote significant differences among results of each treatment at α=0.05 (Tukey's test).

7.4.3 Virus eradication

After 6 months of growth in the greenhouse, young leaves from the resulting +LN and –LN 'Monalisa' plants were sampled to test the efficiency of the droplet-vitrification method in eradicating ASGV, ASPV and ACLSV viruses.

Specific bands were amplified by PCR when the viruses were present in the sample, and samples that did not exhibit the specific bands were considered to be virus-free. The efficiency of virus elimination differed among the three apple viruses (Figure 16).

Figure 16 – Representative electrophoretic analysis of PCR products of leaves from apple plants recovered after cryotherapy to test for the eradication of latent viruses from cryopreserved ‘Monalisa’ shoot tips.



Source: Prepared by the author, 2018.
Molecular marker (M); infected positive control ‘Monalisa’ greenhouse plants (C+), uninfected ‘Monalisa’ greenhouse plants (C-). Experimental positive controls, "Monalisa' in vitro stock plants (SP, control 1) infected with apple latent virus complex and shoot tips (ST, control 2) underwent the droplet-vitrification and recovery processes but were not exposed to LN (-LN); ‘Monalisa’ plants regenerated after droplet-vitrification cryotherapy: 1-20.

ASPV and ACLSV were more frequently eradicated than ASGV in plants recovered from cryopreserved ‘Monalisa’ shoot tips (+LN). The ASPV virus was not detected in any of the tested +LN plants, the ACLSV elimination level was 95% and the ASGV elimination level was 35%, i.e., seven out of twenty plants (35%) evaluated were free of ACLSV, ASGV and ASPV viruses. Thus, the droplet-vitrification cryopreservation protocol removed ASGV, ASPV and ACLSV viruses from ‘Monalisa’ plant shoot tips. 'Monalisa' i*n vitro* stock cultures and plants resulting from shoot tips that underwent the droplet-vitrification and recovery processes but were not exposed to LN were positive for all three viruses, indicating that the LN step was critical to the success of the cryotherapy procedure (Figure 16).

7.5 DISCUSSION

In this paper, we present a droplet-vitrification cryotherapy method based on the cryopreservation protocol reported by Li et al. (2015). This method efficiently eradicates the ASPV, ACLSV and ASGV viral complex from *in vitro* apple 'SC417 Monalisa'. After 6 months of greenhouse growth, 'Monalisa' plants recovered after cryoexposure were 100% free of ASPV, 95% free of ACLSV, and 35% free of ASGV. ASPV and ACLSV were more readily eliminated than ASGV. Thirty-five percent of the PCR tested 'Monalisa' plants were completely free of the viral complex ASPV, ACLSV and ASGV after cryotherapy using droplet-vitrification.

Successful cryopreservation protocols must have optimized PVS2 exposure durations to achieve a high degree of cellular preservation and regrowth levels after cryoexposure (LI et al., 2015; CONDELLO et al., 2011; HALMAGI et al., 2010b). Cryopreservation methods that result in high levels of viability, i.e., ≥40% after LN exposure, are often needed before they can be routinely implemented to secure genebank collections (VOLK et al., 2016). In this study, the highest levels of regrowth (45%) were obtained after 20-40 min and 40-50 min of exposure to PVS2 at 22^{o}C and 0°C, respectively, using axillary shoot tips (1 mm) excised from 4-week old *in vitro* stock cultures and incubated for 1 day on the preculture medium. Although some survival/regrowth is needed, high regrowth levels are not key to the success of cryotherapy applications. Only one or a few recovered virus-free plants are required and additional healthy plants can be propagated from those clean plants. In the current work, 35% of all the evaluated plants that were cryo-exposed, recovered, and then grown in the greenhouse for six months were free of viral species including ACLSV, ASGV, and ASPV. Further research is needed to determine the effects of shorter or longer PVS2 exposure durations on the survival of infected cells after LN exposure. Virus-monitoring and field evaluations of the virus-free plants is required before they can enter commercial distribution and field production pipelines (BI et al., 2017).

The PVS2 temperature and exposure duration are key parameters that affect the regrowth of cryopreserved shoot tips (VOLK et al., 2014b, 2006; KIM et al., 2009; KIM, 2018). We found that *Malus* shoot tips tolerated longer PVS2 incubation durations at 0^{o}C compared to those at 22^{o}C, but the shorter PVS2 incubation times at 22^{o}C were also sufficient for successful cryopreservation. These results were consistent with those reported by Condello et al. (2011) and Li et al. (2015). Li et al. (2015) found that

when the PVS2 exposure was at room temperature, 'Gala' shoot tip regrowth levels were highest after 30-50 min PVS2 exposure, and then declined with longer PVS2 exposure durations. Regrowth levels for apple cultivar 'Pinova' increased with increasing PVS2 exposure time from 15 (3%) - 60 (47%) min and remained stable up with durations of up to 100 min (43%) at 0ºC (CONDELLO et al., 2011). In mint, the permeability of the PVS2 cryoprotectant to tissue was higher and more damaging at 22 ºC than at 0 ºC (VOLK; HARRIS; ROTINDO, 2006). Apparently the longer PVS2 incubation duration at 22 ºC was toxic to *Malus* shoot tips.

Cryotherapy has proven to be an effective strategy to produce virus-free apple plants and the results obtained here were consistent with those reported by Bettoni et al. (2018b) and Romadanova et al. (2016). Studies showed that the percentage of virus-free plants after cryoexposure vary according to the genotype and type of virus. It is likely dependent upon the cellular infection patterns that may be virus-specific (SAREILA et al., 2004). ASGV was more difficult to eradicate from apple *in vitro* plants using cryotherapy than ACLSV, ASPV, and *Apple mosaic virus* (ApMV) (BETTONI et al., 2018b; ROMADANOVA et al., 2016; LI et al., 2016). We (BETTONI et al., 2018b) and Romadanova et al. (2016) previously reported the efficiency of encapsulation-dehydration and vitrification techniques to eradicate ASGV in ‘Marubakaido’ apple rootstock (*Malus prunifolia*) and in 'Sinap Almatinskyi' (*M. domestica*). In contrast, Li et al. (2016) showed that ‘M9’ and ‘M26’ apple rootstocks cryopreserved by encapsulation-dehydration were 85% ASPV-virus free, but that the technique did not produce ASGV-free plants. Recently, Wang et al. (2018b) showed that 'Gala' (*Malus* × *domestica*) apple plants regenerated from cryo-treated shoot tips by droplet-vitrification were still ASGV infected. Li et al. (2016) used immunolocalization to determine that ASGV was present in some parts of apical dome and leaf primordia and that only the top layers of cells in apical dome were free of the virus. Although cryotherapy techniques are based on cryopreservation, preferred parameters for high levels of shoot tip regrowth after cryopreservation are often not optimized for cryotherapy when the goal is to produce virus-free plants. For successful cryotherapy, the surviving cells after liquid nitrogen exposure should be limited to those within the meristem and undifferentiated leaf primordia. Wang et al. (2018b) performed the droplet-vitrification protocol described by Li et al. (2015), the same used in the present study. However, they used only the optimized 40 min room temperature PVS2 exposure duration reported by Li et al. (2015). Wang et al. (2018b) obtained high regrowth levels (62%)

of 'Gala' shoot tips after cryoexposure and observed that most of the cells in the apical dome and some leaf primordia cells survived to LN exposure. A strategy to improve virus eradication could be to either use shorter or longer PVS2 exposure durations than what are optimal, which may result in fewer cells that recover after LN exposure. In our study, recovered plants (+LN) from PVS2 times were randomly selected for PCR diagnosis, therefore a range of PVS2 treatments were represented.

Improved virus eradication by cryotherapy can be also achieved by combining thermotherapy and cryotherapy. This treatment combination was shown to be effective in ASGV-infected apple (WANG et al., 2018b), in *Raspberry bushy dwarf virus* (RBDV) infected raspberry (WANG et al., 2008) and in viruses *Onion yellow dwarf virus* (OYDV), *Leek yellow strip virus* (LYSV) and *Garlic common latent virus* (GCLV) infected garlic (VIEIRA et al., 2015). In combination, thermotherapy and cryotherapy work synergistically, and it could be effective for viruses that have the capacity to invade regions of shoot tips. Heat treatments reduce the movement of viral particles to the apical meristem by inhibition of the viral replication (WANG et al., 2006c), which makes the virus-free region larger than in shoot tips resulting from plants without heat treatment, thereby improving the chance of virus eradication by cryo-injury in infected differentiated cells (WANG et al., 2008).

The droplet-vitrification procedure tested herein could be used as a tool to eradicate ASPV, ACLSV and ASGV viruses from *in vitro* apple ‘SC417 Monalisa’ is based on method that was previously developed for cryopreserving *Malus domestica* ('Gala'), which was successfully repeated to six apple other genotypes (LI et al., 2015). This method simplifies previous methods that made use of precultured shoot tips excised from 2-4 month-old *in vitro* stock plants (HALMAGYI et al., 2010; HALMAGYI; DELIU; ISAC, 2010; CONDELLO et al., 2011). In the droplet vitrification method described by Li et al. (2015), shoot tips were excised from 4-week old *in vitro* stock cultures, incubated on a medium that combines the preculture and the loading treatments into 1-day step, followed by PVS2 exposure and LN treatment. This method simplifies the cryogenic procedure and is less time-consuming than those based on encapsulation-dehydration.

The practical and promising cryotherapy procedure demonstrated herein provides a method by which viruses were eliminated from apple cultivar ‘Monalisa’ grown *in vitro*. It may be applicable on a commercial scale as a method to generate

high-quality plant material with low costs than traditional meristem culture eradication methods.

8 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A criopreservação é um valioso meio para a conservação segura de recursos genéticos vegetais por longos períodos de tempo e a crioterapia é uma ferramenta promissora para a produção de plantas livres de vírus.

Atualmente, bancos criogênicos tem sido estabelecidos para diversas culturas de importância econômica em todo o mundo; explantes, em especial meristemas *in vitro* ou gemas dormentes, são preservados em nitrogênio líquido ou vapor de nitrogênio líquido e, então, regenerados quando há demanda pelo acesso vegetal.

A macieira está entre as culturas mais estudadas em relação à criopreservação. Atualmente grandes criobancos de *Malus* spp. estão estabelecidos e fazem o uso principalmente da técnica de gemas dormentes. A técnica de gemas dormentes proporciona um menor custo com mais rápida recuperação de explantes em relação as técnicas que utilizam como fonte de explantes materiais *in vitro*. Portanto, a escolha da técnica/fonte de propágulo para o ensaio criogênico deve estar estreitamente relacionado ao objetivo do trabalho.

Para a videira, apesar de vários trabalhos publicados com diferentes protocolos de criopreservação, até o presente, não existem criobancos estabelecidos. Como fonte de explantes para ensaios de criopreservação de videira, técnicas criogênicas que utilizam explantes *in vitro* têm sido mais promissoras que a técnica de gemas dormentes. Protocolos funcionais para pequeno número de espécies, especificidade de genótipos/espécies para um determinado protocolo e a não reprodutibilidade de protocolos entre laboratórios/técnicos ainda limitam a implantação de coleção de *Vitis* criopreservada.

O uso de crioterapia como ferramenta para eliminação de vírus têm sido demonstrada em diversas espécies de importância econômica. Embora as técnicas de crioterapia sejam baseadas em protocolos de criopreservação, os parâmetros ideais para a criopreservação podem não ser os melhores para a crioterapia; quando o objetivo é produzir plantas livres de vírus, é necessário que ocorra alguma sobrevivência/regeneração, mas os níveis altos de regeneração não são fundamentais para o sucesso das aplicações de crioterapia. Por outro lado, métodos de criopreservação que resultam em níveis altos de viabilidade após a exposição ao nitrogênio líquido são necessários para garantir que coleções de banco de genes possam ser implementados com sucesso.

Este trabalho de tese foi fundamentado nas necessidades de elaboração de um protocolo simples e eficiente para a preservação segura e a longo prazo de recursos genéticos de videira, bem como no desenvolvimento de uma ferramenta prática e efetiva para a erradicação de espécies de vírus em macieira. Os resultados apresentados neste documento representam um avanço tecnológico para a criopreservação e crioterapia de videira e macieira, respectivamente, e servirão como base para a criação de criobancos de germoplasma de videira e, irão facilitar a produção de plantas de macieira livres de vírus em escala comercial, gerando material vegetal de alta qualidade para distribuição em viveiros.

É importante relatar que a avaliação do desempenho no campo e o monitoramento de possíveis infecções residuais de vírus de plantas matrizes que passaram por crioterapia é essencial e, devem ser realizados antes da distribuição comercial e a produção em escala.

É importante destacar a necessidade de órgãos públicos ligados à fruticultura fomentarem pesquisas aplicadas em biotecnologias de criopreservação e crioterapia, visando a manutenção de bancos de germoplasmas e a produção de material propagativo de alta qualidade fitossanitária.

Todo o trabalho desenvolvido nesta tese por ser ampliado a outras culturas propagadas vegetativamente. Cabe também destacar que trabalhos nesta temática é uma forma de pesquisa aplicada que tem como beneficiários principais os fruticultores/agricultores.

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ANEXOS

ANEXO A – Treinamento pessoal em criopreservação de plantas: "*II International Workshop on in Vitro Conservation and Cryopreservation of Plant Genetic Resources"*, promovido pela Universidade Federal de Lavras, Lavras, Minas Gerais, novembro de 2015.

UNIVERSIDADE FEDERAL DE LAVRAS

A Pró-Reitoria de Extensão e Cultura da Universidade Federal de Lavras confere o presente

CERTIFICADO

a

Jean Carlos Bettoni

como *Participante* **no(a)** *II International Workshop on in vitro Conservation and Cryopreservation of Plant Genetic Resources* **promovido pelo(a)** *Programa de Pós-Graduação em Fisiologia Vegetal - Departamento de Biologia* **realizado no período de** *11/11/2015* **a** *13/11/2015* **com duração de** *15 horas.*

Lavras (MG), 27 de julho de 2018

JOAO JOSE GRANATE SA E MELO MARQUES
Pró-Reitor de Extensão e Cultura

RENATO PAIVA
Coordenador Geral

Protocolo: 2015.432239.8
Este certificado dispensa assinaturas
Verifique a autenticidade deste certificado informando o protocolo no site https://sig.ufla.br/comprovar/certificado_evento

ANEXO B – Treinamento pessoal em criopreservação de plantas: “1º Curso de criopreservação de plantas”, promovido pela Embrapa Mnadioca e Fruticultura, Cruz das Almas, Bahia, julho de 2016.

Embrapa
Mandioca e Fruticultura

Certificado

Conferimos este certificado a ***JEAN CARLOS BETTONI*** pela participação no **“1° Curso de criopreservação de plantas”**, realizado pela Embrapa Mandioca e Fruticultura, no período de 11 a 15 de julho de 2016, com carga horária de 32 horas.

Cruz das Almas, Bahia, 15 de julho de 2016

Alberto Duarte Vilarinhos
Chefe-Geral
Embrapa Mandioca e Fruticultura

ANEXO C – Artigo publicado na Revista Brasileira de Fruticultura:Cryotherapy: a new technique to obtain grapevine plants free of viroses. Bettoni et al. (2016) http://dx.doi.org/10.1590/0100-29452016833

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REVISION

CRYOTHERAPY: A NEW TECHNIQUE TO OBTAIN GRAPEVINE PLANTS FREE OF VIRUSES[1]

JEAN CARLOS BETTONI[2], MURILO DALLA COSTA[3], JOÃO PETERSON PEREIRA GARDIN[3], AIKE ANNELIESE KRETZSCHMAR[4], RANJITH PATHIRANA[5]

ABSTRACT – Through *in vitro* tissue culture techniques it is possible to propagate high quality nursery plants faster. Cryotherapy is a promising tool, based on *in vitro* culture techniques, for achieving in a short time, high frequency of regenerating plants free of viruses. The objective of this review is to present and analyze the results of research conducted in cryotherapy methods based on cryopreservation protocols for recovery of cultivars free of micro-organisms with potential agronomic interest. The main methods employed in cryotherapy are encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet vitrification, which are based on the immersion of preconditioned shoot tips in liquid nitrogen, followed by their recovery *in vitro* on to culture media for regeneration of healthy plantlets. Improvements to cryotherapy protocols used for grapevine are still needed, since there are variations in response according to the genotype. The published research mostly relates to *Vitis vinifera* and the few studies applied to other species show that the protocols need to be improved. This specificity goes beyond species, with different responses among cultivars, limiting the broader application of the technology. On the other hand, traditional methods used for virus removal from infected plant materials also have limitations and therefore investment in research for the development and application of cryopreservation techniques is highly justified, considering its efficiency and low-cost, once the protocols are developed. High frequency of virus-free plants among regenerants within a short time frame is the most desirable aspect of cryotherapy. Therefore, these advantages make the technique a promising tool for institutions mandated to the development of high-health planting materials with high genetic and agronomic potential for viticulture.

Index Terms: *Vitis*, encapsulation-dehydration, encapsulation-vitrification, vitrification, droplet-vitrification, plant disease.

CRIOTERAPIA: UMA NOVA TÉCNICA PARA OBTENÇÃO DE PLANTAS DE VIDEIRA LIVRES DE VIROSES

RESUMO – Por meio de técnicas de cultura de tecidos *in vitro* é possível propagar plantas matrizes de alta qualidade, mais rápido. A crioterapia é uma ferramenta promissora, baseada em técnicas de cultura *in vitro* para obtenção em curto espaço de tempo de alta frequência de plantas regenerantes livres de viroses. O objetivo desta revisão é apresentar e analisar os resultados de pesquisas realizadas em métodos de crioterapia, baseados em protocolos de criopreservação, para recuperação de cultivares de videiras livres de micro-organismos e com potencial de interesse agronômico. Os principais métodos utilizados para crioterapia são encapsulamento-desidratação, encapsulamento-vitrificação, vitrificação e vitrificação de gota, os quais se fundamentam na imersão de ápices meristemáticos pré-condicionados em nitrogênio líquido, seguido por sua recuperação *in vitro* em meios de cultura para regeneração de plântulas saudáveis. Melhorias para protocolos de crioterapia empregados para a videira ainda são necessários, uma vez que existem variações na resposta de acordo com o genótipo. As investigações publicadas na sua grande maioria estão relacionadas a cultivares de *Vitis vinifera* e os poucos estudos aplicados a outras espécies mostram que os protocolos precisam ser melhorados. Essa especificidade, que vai além da espécie, com respostas diferenciadas entre cultivares, por enquanto limita a aplicação mais ampla da tecnologia. Por outro lado, os métodos tradicionais utilizados para eliminação de viroses a partir de materiais de plantas infectadas também possuem limitações e, portanto, investimentos em pesquisas para o desenvolvimento e aplicação de técnicas de criopreservação são altamente justificáveis, considerando a sua eficiência e o baixo custo, uma vez que os protocolos são desenvolvidos. Essas limitações deverão ser superadas. Alta frequência de plantas livres de vírus entre os regenerantes dentro de um curto espaço de tempo é o aspecto mais desejável da crioterapia. Portanto, essas vantagens fazem da técnica de crioterapia uma ferramenta promissora para instituições especializadas para o desenvolvimento de materiais de plantio de alta qualidade fitossanitária com alto potencial genético e agronômico para a viticultura.

Termos para indexação: *Vitis,* encapsulamento-desidratação, encapsulamento-vitrificação, vitrificação, doenças de plantas.

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INTRODUCTION

In Brazil, grapevine occupies an area of 81.286 hectares, and 71.15 % of this area is in the temperate zone in Rio Grande do Sul, Santa Catarina and Paraná States (SIDRA, 2013). Although there is a favorable commercial scenario, area planted with grapevine and the harvest in the south of Brazil have been declining, it is observed that new vineyards face low productivity and poor quality of grapes due to several complex issues. In relation to plant health, one of the most important obstacles is the infection of vines with viruses, resulting in vine decline and death (BETTONI et al., 2015; GARRIDO et al., 2004).

Viruses have become a barrier to productive development of a profitable viticulture. Infected plants show growth reduction, low productivity and poor quality of grapes which can decline and die in few years (WANG et al., 2003a; FAJARDO et al., 2007). A worrying factor is that the viruses are disseminated by the use of infected propagating material contributing to the spread of virus in new areas. According to Shatnawi et al. (2011), some species show symptoms such as leaf yellowing, bright yellow stripes near to the nerves and nerve bifurcation. In contrast, some American and hybrid cultivars do not show typical virus infection symptoms, remaining latent, making the diagnosis difficult and misleading, so further facilitating virus dissemination.

The acquisition of plant material with origin and guarantee of high-health status is important for the success in vineyard establishment and management. In practice, it is often difficult to maintain such standards, and high-health planting material is not available in the market. Therefore, it is necessary to identify and implement functional and robust methods in order to eliminate microorganisms, using efficient protocols, able to generate high-health materials for promoting efficient vineyard practices (PATHIRANA et al., 2012; 2013). It is necessary to follow this with stringent regulatory measures for clonal grapevine nurseries, in relation to maintenance, propagation and commercialization of plant materials. Considering the limitation of traditional methods in obtaining mother plants free of viruses, the development of new and more efficient technologies is important, with practical and efficient protocols, to produce healthy plants (WANG et al., 2009). *In vitro* techniques can have a positive impact in overcoming the problems associated with production of high-health nursery material in order to optimize, stabilize and maximize the grape industry.

Cryotherapy is a biotechnological method used to eliminate pathogens from infected clonal plant materials and it is based on cryopreservation using vitrification techniques, in which the biological material is exposed to liquid nitrogen (LN) at -196 ºC for a period. Under these conditions, infected vacuolated and differentiated cells are eliminated by the ultra-low temperature effect, leaving only highly cytoplasmic meristematic cells. Cryotherapy has been used successfully in eradicating virus infection from several species with economic importance as plum, banana, strawberry, potato and garlic, besides grapevine (WANG and VALKONEN, 2009a; VIEIRA et al., 2015). High efficiency of cryotherapy in several species, combined with the low operational costs and rapid recovery of plant material without virus infection at a high frequency, have made this method a promising tool for institutions aiming at the recuperation of crops with agronomic potential. Being a new method, there are still few studies in using cryotherapy in comparison with the traditional methods in eliminating viruses, and results on agronomic evaluation of plants that were subjected to cryotherapy are still not available in literature. Despite the benefits and positive results of cryotherapy technique, challenges still remain due to variable response of genotypes within and between species, restricting the applicability of cryotherapy (WANG et al., 2014; FENG et al., 2013). Hence, the next stage of progress in this field will see the development of robust protocols applicable across genotypes, in each studied species, in order to maximize the generation of plants without viral particles. Further evaluation of genetic stability and agronomic behavior of treated plant material is another aspect that will be addressed in cryotherapy research in the coming years.

Therefore, the objective of this review is to present results from research performed with cryotherapy methods based on protocols of cryopreservation, in order to recover *in vitro* grapevine plants without viruses.

METHODS OF VIRUS ERRADICATION FROM INFECTED PLANT MATERIAL

Traditional methods used to obtain plant material without pathogens include meristem culture and heat therapy followed by meristem culture (DÍAS-BARRITA et al., 2007; MALIOGKA et al., 2009).

There are successful scientific reports of techniques in several species; however, in functional terms, methodological difficulties have hampered the extensive use of these biotechnological tools (WANG et al., 2014). In meristem culture, which has been used for decades, the elimination capacity of viral particles is associated with the dimension of the excised tissue. For majority of species, the recommended size is extremely small, from 0.1 to 0.3 mm (TORRES et al., 1998). Besides the mechanical problems with meristem removal, the low regeneration capacity and, mainly, the failure to guarantee the removal of viral particles are the bottlenecks (FACCIOLI and MARANI, 1998; DÍAS-BARRITA et al., 2007). Heat therapy application with meristem culture is an expensive process, demanding proper equipment; the treatment period is dependent on the type of virus and not all viruses are eliminated by the technique and many host plants are heat sensitive (BHOJWANI and DANTU, 2013).

Other methods are noted as alternative ones for the elimination of viruses, among them are somatic embryogenesis and chemotherapy. Somatic embryogenesis, besides a time-consuming method, is difficult to be executed and, at the end, it can cause somaclonal variation in regenerated plants. However, there are reports of successful elimination of grapevine viruses using somatic embryogenesis. Gambino et al. (2009) related the efficiency of somatic embryogenesis for eradicating the virus responsible for grapevine degeneration (*Grapevine Fan Leaf Virus*) for three *Vitis vinifera* cultivars (Cari, Provinè and Roussan). Chemotherapy protocols were applied successfully to remove the virus responsible for grapevine leaf-roll disease (GLRaV-1 and 3) (PANATTONI et al., 2011), but there are also reports demonstrating high phyto-toxicity induced by antiviral compounds. Moreover, variable responses have been observed among different cultivars and there is no research proving that antiviral compounds do not induce mutagenic alteration. In general, all the above mentioned techniques have their disadvantages and each needs to be optimized (SKIADA et al., 2013).

CRYOTHERAPY

Cryotherapy is a relatively new method used to eliminate pathogens from infected plant tissue based on cryopreservation techniques. In this method, the biological material is exposed to LN at -196 ºC for a short period, normally 1 hour, (FENG et al., 2013) and under those conditions, infected cells in the vascular region and vacuolated, more differentiated cells of the apical dome are eliminated by the ultra-low temperature due to ice crystallisation, leaving only highly cytoplasmic cells in the meristematic region (WANG and VALKONEN, 2008b). Thus, the technique is based on the physico-anatomical differences among tissues, because meristematic cells are small, with smaller vacuoles and greater nuclear-cytoplasm ratio than cells which are further from the meristematic region (Figure 1) (WANG et al., 2009; WANG and VALKONEN, 2009a). Since meristematic cells are smaller and proliferating fast, away from the vasculature, it is suggested that virus may not reach that region (WANG and VALKONEN, 2009a). Cryotherapy has been used successfully in eradicating virus infections in several species with economic importance such as *Prunus* (BRISON et al., 1997), *Musa* spp. (HELLIOT et al., 2002), *Vitis vinifera* (WANG et al., 2003a; BAYATI et al., 2011; PATHIRANA et al., 2015), *Fragaria ananassa* (CAI et al., 2008), *Solanum tuberosum* (WANG et al., 2006; BAI et al., 2010; 2012; YI et al. 2014), *Rubus idaeus* (WANG and VALKONEN 2009b; WANG et al., 2008), *Ipomea batatas* (WANG and VALKONEN, 2008a), *Dioscorea opposite* (HEE et al., 2013) and *Allium sativum* (VIEIRA et al., 2015) (Table 1).

Considering the results from about a decade of research, cryotherapy can be considered as a fast developing method that helps and/or replaces traditional methods in eradicating microorganisms from infected plant tissue (WANG and WALKONEN, 2009). There are a number of advantages in cryotherapy technique in relation to traditional methods for pathogen eradication (WANG et al., 2003a); ability to treat a large number of samples quickly and simultaneously (BHOJWANI and DANTU, 2013); low cost and high frequency of plants free from viruses after recovery (FENG et al., 2013; WANG et al., 2006; WANG et al., 2003a). After the protocol establishment for the genotype in question, the technique is easy and does not require special equipment, only basic equipment available in a tissue culture laboratory (WANG et al., 2009).

The greatest challenge to the wider application of cryotherapy technique is the differentiated

response that genotypes of the same species can show under cryo-treatment, which still limits the applicability of cryotherapy (WANG et al., 2014; FENG et al., 2011; 2013; WANG and WALKONEN, 2009). Several protocols with different cryotherapy methods have been reported and results differ between laboratories, with varying levels of survival and regeneration (BENELLI et al., 2013; GRIBAUDO et al., 2012; BENSON, 2008; WANG et al., 2003a; MATSUMOTO and SAKAI, 2003).

Intracellular water content is a critical factor for the efficiency of cryotherapy protocols. It is important to achieve proper dehydration of plant material, reducing harmful damage to tissues (ENGELMANN, 2004). Wang et al. (2000), working with *Vitis* sp., reached higher indexes of survival when tissue water content was near 16% at the time of cryopreservation; in contrast, higher or lower water content in the tissue decreased the survival. Excessive dehydration causes negative effects on survival rate and, likewise, water in excess in the tissue causes irreversible damage because water crystallization inside the cell causes rupture of cell membrane system, cell collapse and death (VIEIRA et al., 2015; WANG et al., 2009). So, before the exposure of plant tissue to LN, part of the intracellular water is removed by the addition of plant vitrification solution 2 (PVS2) (SAKAI et al., 1990; MATSUMOTO and SAKAI, 2003; GRIBAUDO et al., 2012), or they are physically dehydrated using silica gel or by exposing to the air flow of the laminar hood (WANG et al., 2000).

There are still fewer studies on the use of cryotherapy in comparison with the traditional methods for eliminating viruses, and results on agronomic evaluation of plants that have undergone cryotherapy are yet to be published (WANG et al., 2014). Several different techniques have been applied for cryotherapy of *Vitis* spp. These include vitrification (MARKOVIC et al., 2013; GANINO et al., 2012; MATSUMOTO and SAKAI, 2003; WANG et al., 2003a), encapsulation-dehydration (MARKOVIC et al., 2013; BAYATI et al., 2011; WANG et al., 2003a; WANG et al., 2000), encapsulation-vitrification (GRIBAUDO et al., 2012) and droplet vitrification (PATHIRANA et al. 2015).

CRYOTHERAPY BY VITRIFICATION

Vitrification protocols are based on dehydrating material in cryoprotective glycerol based solutions such as PVS2 and PVS3 (SAKAI and ENGELMANN, 2007). Once sufficiently dehydrated, the sparsely distributed water molecules in plant tissues are unable to come together to form ice crystals during rapid freezing. Therefore they undergo a transition from liquid to a vitreous state, when the material is exposed to LN (FAHY et al., 1984). This phenomenon, known as vitrification, is facilitated by ultra-fast cooling rates achieved by direct dipping of plant material in LN. PVS2, the most efficient vitrification solution developed so far, is composed of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) in Murashige and Skoog (1962) medium (MS) with 0.4 M sucrose (MATSUMOTO and SAKAI, 2003; WANG et al., 2003a). PVS3, which has been successfully used in a limited number of species such as asparagus is composed of 50% (w/v) glycerol and 50% (w/v) sucrose in water (NISHIZAWA et al., 1993). When comparing the effect of PVS2 and PVS3 on *Vitis vinifera* dehydration, Markovic et al. (2013) observed that, 40 minutes exposure followed by LN treatment for one hour resulted in no plant recovery using PVS3, whereas 40% of survival was achieved with PVS2 dehydration. Vitrification protocols involve the following stages: excision of meristematic tissue (apical or axillary buds) from *in vitro* propagated material; pre-culture in sucrose-enriched media (0.3 M, or increasingly higher sucrose concentrations up to 1 M); osmoprotection in liquid culture medium enriched with glycerol and sucrose; dehydration with vitrification solution (PVS2 or PVS3); instant freezing in LN at -196 °C; recovery on to warm (in a water bath heated to 38-40°C) recovery solution (liquid culture medium enriched with sucrose) followed by transfer to high sucrose solid media; and regeneration on culture medium (MATSUMOTO and SAKAI, 2003; MIAJA et al., 2004; SAKAI and ANGELMANN, 2007).

Variations in vitrification protocols for *Vitis* sp. comprise of sucrose concentrations used in osmoprotection and rinsing stages, exposure time to cryo-protectants and regeneration medium (MARKOVIC et al., 2014; GANINO et al., 2012; WANG et al., 2003a; MATSUMOTO and SAKAI, 2003; NISHIZAWA et al., 1992). In any vitrification method, procedures such as pre-culture, osmoprotection, exposure time to cryo-protectants and careful handling during and after LN treatment

are essential for survival and regeneration of the tissues containing meristematic cells (SAKAI and ENGELMANN, 2007).

Using *V. vinifera* cv. Cabernet Sauvignon, Matsumoto and Sakai (2003) observed that pre-culture of shoot tips in 0.3 M sucrose concentration for 3 days was optimal, with 65.5% recovery after freezing. On the other hand, Wang et al. (2003a) working with another cultivar from the same species, reached 50% of regeneration after cryopreservation when shoot tips were pre-cultured with 0.75 M sucrose. Different responses from genotypes for cryotherapy techniques make it difficult to develop generalized protocols for *Vitis*, so demanding adjustments to protocols for different cultivars (WANG et al., 2014).

Optimising the time of exposure of explants to cryoprotective solutions is needed in order to establish vitrification protocols. Excessive exposure can cause injuries by chemical toxicity and death of meristem cells (SAKAI and ENGELMANN, 2007; SAKAI, 1995). In Kober 5BB rootstock (*Vitis berlandieri* x *Vitis riparia*), GANINO et al. (2012) observed the effect of PVS2 toxicity in grapevine shoot tips: without both cryoprotective treatment and freezing, 94% of the plants regenerated; after 30 minutes of exposure, 57% of the plants regenerated; after 60 minutes, the regeneration dropped to 14%; and after 90 minutes there was no regeneration even without subjecting tissues to freezing. Likewise, MARKOVIC et al. (2013) noted a decrease in survival and regeneration of grapevines with the increase of dehydration period by PVS2: survival decreased from 70% in 25 minutes treatment to 30% after 75 minutes of treatment. For *Vitis*, the best results in dehydration using PVS2 have been achieved when the process was performed in two stages, with initial exposure of explant material for 30 minutes to ½ PVS2 (15% glycerol, 7.5 % ethylene glycol, 7.5 % DMSO and 0.4 M of sucrose) followed by a treatment for 50 minutes in PVS2. Wang et al. (2003a), Matsumoto and Sakai (2003), using dehydration by PVS2 in two stages reached 50% and 82.5% of *Vitis* regenerated plants, respectively. Exposure time to PVS2 varies among species; in general, tissues smaller than 0.5 mm are more sensitive to treatment with PVS2, but those tissues exceeding 3 mm require greater dehydration time (SAKAI and ENGELMANN, 2007).

After dehydration, plant material is placed in cryo-tubes with cryo-protectors and subjected to NL freezing for 1 hour; after, it is thawed in recovery solution at 40°C for 3 minutes by keeping inside a pre-heated water bath (WANG et al., 2003a). Rinsing and caution in handling plant material after defrosting are essential to cryotherapy success (SAKAI and ENGELMANN, 2007).

Success in vitrification method for eliminating viruses from grapevine was demonstrated by Wang et al. (2003a) who noted 97% of regenerated plants without GVA (*Grapevinevirus A*) against only 12% after meristem culture. Recently, Pathirana et al. (2015) demonstrated the removal of three *Closteroviridae* viruses causing leafroll disease in grapevine, using vitrification-based cryotherapy of apical and axillary buds of infected clones.

CRYOTHERAPY BY ENCAPSULATION-DEHYDRATION

Cryotherapy by the method of encapsulation-dehydration is based on techniques proposed for synthetic seed production (BENELLI et al., 2013). The protocol involves the following stages: excision of *in vitro* propagating material; encapsulation; pre-cultivation under cumulative sucrose concentrations; dehydration; freezing at -196 °C; defrosting in water bath at 40°C; and culture on regeneration medium.

After excision of *in vitro* propagating material, the method consists in the formation of a artificial endosperm around the tissue composed by a gelling agent (3% sodium alginate) which cures when contacting calcium chloride solution and produces small pellets (BAYATI et al., 2011; GONZÁLEZ-BENITO et al., 2004). In sequence, they are pre-cultivated in solutions with increasing sucrose concentrations (0.25, 0.50, 0.75 and 1.0 M), during four days, a stage per day (MARKOVIC et al., 2013; BAYATI et al., 2011; WANG et al., 2000; WANG et al., 2003a). Pre-cultivation allows encapsulated propagating materials become resistant to dehydration and freezing; in turn, the gradual increase of sucrose concentration prevents harmful effects of the plant exposure to high sucrose concentrations (PLESSIS et al., 1991). Encapsulation protects the tissue from high sucrose concentrations, preventing injury in cell tissues (ENGELMANN, 2011), which could otherwise be harmful or fatal for non-encapsulated propagating material (MATSUMOTO and SAKAI, 2003).

After pre-cultivation, encapsulated tissue can be dehydrated under air flow in laminar air flow cabinet (WANG et al., 2003a) or in containers with silica-gel (MARKOVIC et al., 2013). Comparing both techniques on the survival of LN 33 grapevine hybrid (Courderc 1613 x Sultana), Wang et al. (2000) showed that results were not dependent on dehydration method but on dehydration time of

each method and the original water content in the propagating material including the beads. The best survival rate achieved was around 60% when beads were dehydrated for 7 hours on air flow and 4.5 hours in silica-gel, with a final water content of about 16%. For *Vitis vinifera*, Portan cultivar, around 10% of the plants regenerated at a range of bead moisture contents, from 16.2% to 22.3% (MARKOVIC et al., 2013). After 12 hours of dehydration under air flow and NL freezing, 59% of encapsulated shoot tips of *V. vinifera,* Black cultivar survived (BAYATI et al., 2011). For cv. Superior (*V. vinifera*), dehydration periods of more than 6 hours decreased survival rate of encapsulated shoot tips (WANG et al., 2000). Results from aforementioned research indicate that the dehydration time of encapsulated tissue to reach proper water content is variable and cultivars from the same species show different survival and regeneration rates. Thus, in order to achieve higher survival rates from a specific cultivar it is important to investigate, initially, the sole effect of dehydration before attempting cryotherapy.

After dehydration, encapsulated tissue is transferred to sterile cryo-tubes for treatment in LN for 1 hour, and afterwards, they are thawed in recovery solution maintained on a water bath set at 40°C for 3 minutes and then transferred to regeneration culture medium (BAYATI et al., 2011; WANG et al., 2003a; WANG et al., 2000).

Successful attempts on encapsulation-dehydration method to eradicate viruses from infected grapevine have been reported. Bayati et al. (2011) and Wang et al. (2003a) have registered frequencies of 42.2 and 97% of plants free from GVA, with 59 and 62% of survival after cryotherapy, respectively.

CRYOTHERAPY BY ENCAPSULATION-VITRIFICATION

Encapsulation-vitrification protocols are based upon combinations of encapsulation-dehydration and vitrification procedures (BENELLI et al., 2013; ENGELMANN, 2011). For this method, shoot tips from *in vitro* cultures are excised and encapsulated in alginate beads as already described. The encapsulated beads are then pre-cultured in solutions with increasing sucrose concentrations, followed by osmoprotection in loading solution followed by dehydration with vitrification solution, freezing at -196 °C, defrosting in water bath at 40 °C, rinsing in recovery solution and culture in regeneration media. In literature, there are few studies relating the use of encapsulation-vitrification method to eradicate viruses in crops. Gribaudo et al. (2012) investigated the efficiency of this method in cv. Nebbiolo cultivar (*V. vinifera*) infected with GVA and GLRaV-3 and, after cryotherapy, the healthy vegetative material was regenerated.

The convenience of vitrification technique allows that shoot tip explants could be subjected to cryotherapy for a short time; however, it requires extra care during handling when compared with encapsulation-dehydration method, in which the procedures are done with encapsulated shoot apices. On the other hand, the encapsulation-dehydration method demands more time to prepare material for freezing, especially in dehydration phase. So, encapsulation-vitrification method benefits from both encapsulation-dehydration and vitrification advantages (SAKAI and ENGELMANN, 2007).

CRYOTHERAPY BY DROPLET VITRIFICATION

The droplet vitrification method was first applied by Schafermenuhr et al. (1994) for cryopreserving potato meristems. It combines the application of highly concentrated vitrification solutions with ultra-fast freezing and thawing rates, which are critical for its successful results (PANIS et al. 2005).

This method involves the treatment of samples with cryoprotectants, which mainly includes exposure to the loading solution, followed by dehydrating in PVS2 vitrifying solution. The samples are then placed on an aluminum foil strip mounted with a droplet of vitrifying solution and immersed in LN for rapid cooling. During rewarming, the removal of cryoprotectant is achieved using unloading solution, and then transferred to recovery and regeneration media (BENELLI et al. 2013). It is vital that the samples are sufficiently dehydrated by the vitrification solution in order to vitrify while rapid cooling in LN. Thus the key for successful droplet vitrification is to quantify and optimize the dehydration tolerance of samples in vitrifying solution. Tolerance to vitrification solution is achieved by optimizing the preconditioning and loading treatments along with the standardization of concentration, time of exposure and temperature of the vitrification solution. Similar to other methods, the warming and recovery steps are also crucial for its success (GONZALEZ-ARNAO et al. 2014; SAKAI and ENGELMANN 2007). Droplet vitrification achieves fastest rates of cooling thanks to the high

thermal conductivity of aluminum, and researchers believe that this method will become a generic method applicable to many species in future (PANIS et al., 2009; 2011).

Droplet vitrification method has been successfully applied to removing three *Closteroviridae* viruses from infected grapevine clones (PATHIRANA et al. 2015). They used 'Chardonnay' and 'Lakemont Seedless' infected with *Grapevine leafroll associated virus-3* (GLRaV-3, an *Ampelovirus*), 'Pinot gris' and 'Sauvignon Blanc 316' infected with *Grapevine leafroll associated virus-2* (a *Closterovirus*), and another clone of 'Sauvignon Blanc' infected with both *Grapevine leafroll associated virus-1* (an *Ampelovirus*) and GLRaV-3. Plants regenerated after cryo-treatment tested negative for all three viruses, whereas untreated control plants tested positive.

GENERAL CONDITIONS FOR CRYOTHERAPY METHODS

Regardless of the cryotherapy method or protocol, it is important that conditions after cryotherapy be favorable for survival and regeneration of the cryopreserved material. Optimising the concentration of growth regulators (WANG et al., 2000) or composition of culture media (PENNYCOOKE and TOWILL, 2001) is essential for good regeneration of cryo-preserved buds. Growth regulators play important roles in regeneration medium (WANG et al., 2003b) and they can meet specific requirements in the recovery of cryo-preserved materials (BHOJWANI and RAZDAN, 1996). Markovic et al. (2014) highlighted that growth regulators such as trans-zeatin riboside (ZR) and benzyl amino purine (BAP) act in the same way in recovering grapevine cryo-preserved shoot tips and, even in small concentrations, they are essential to regenerate explant materials. The effects of culture medium supplemented with different concentrations of BAP and naphthaleneacetic acid (NAA) on the development of cryo-preserved grape buds were evaluated by Wang et al. (2000). Fastest shoot elongation without any callus formation was obtained when cryopreserved shoot tips were cultured on the post-culture medium composed of half-strength MS medium supplemented with 1 mg/L BA and 0.1 mg/L NAA. However, a further increase in BAP or NAA concentration, when used in combination, resulted in callus formation. This is undesirable for cryotherapy because it can cause somaclonal variation (WANG et al., 2003b). Optimum concentration of growth regulators in regeneration medium for cryo-preserved materials has varied among cryogenic methods (WANG et al., 2003b) and among cultivars (WANG et al., 2000). Therefore, investigative research needs to be performed on selected genotypes in each species, in combination with vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet vitrification methods, in order to establish efficient protocols which can result in high survival rates with high frequency of viruses eradication.

TABLE 1 - Efficiency of cryotherapy methods for eradication of viruses in plant species.

Pathogen	Plant species	Cryotherapy method	References
Plum pox virus	*Prunus*	VI	Brison et al. (1997)
Banana streak virus	*Musa*	VI	Helliot et al. (2002)
Cucumber mosaic virus	*Musa*	VI	Helliot et al. (2002)
Grapevine virus A	*Vitis vinifera*	EN-DE; VI	Wang et al. (2003)
Potato virus Y	*Solanum tuberosum*	EN-DE; VI; DR-VI	Wang et al. (2006)
Potato leafroll virus	*Solanum tuberosum*	EN-DE; VI; DR-VI	Wang et al. (2006)
Strawberry mild yellow-edge virus	*Fragaria ananassa*	VI	Cai et al. (2008)
Sweet potato feathery mottle virus	*Ipomoea batatas*	EN-VI	Wang and Valkonen (2008a)
Sweet potato chlorotic stunt virus	*Ipomoea batatas*	EN-VI	Wang and Valkonen (2008a)
Sweet potato little leaf phytoplasma	*Ipomoea batatas*	EN-VI	Wang and Valkonen (2008a)
*Raspberry bushy dwarf virus**	*Rubus idaeus*	EN-VI	Wang et al. (2008b)
Raspberry bushy dwarf virus	*Rubus idaeus*	EN-VI	Wang and Valkonen (2009b)
Grapevine virus A	*Vitis vinifera*	EN-DE	Bayati et al. (2011)
Potato Virus X	*Solanum tuberosum*	DR-VI	Bai et al. (2010; 2012)
Potato Spindle Tuber Viroid	*Solanum tuberosum*	DR-VI	Bai et al. (2012)
Yam mosaic virus	*Dioscorea opposita*	EN-DE	Hee et al. (2013)
Potato leafroll virus	*Solanum tuberosum*	DR-VI; EN-VI	Yi et al. (2014)
Potato virus Y	*Solanum tuberosum*	DR-VI; EN-VI	Yi et al. (2014)
Onion Yellow Dwarf Virus	*Allium sativum L.*	VI	Vieira et al (2015)
Leek Yellow Strip Virus	*Allium sativum L.*	VI	Vieira et al (2015)
Garlic Common Latent Virus	*Allium sativum L.*	VI	Vieira et al (2015)
Grapevine associated virus 1	*Vitis vinifera*	DR-VI	Pathirana et al. 2013; 2015
Grapevine associated virus 2	*Vitis vinifera*	DR-VI	Pathirana et al. 2013; 2015
Grapevine associated virus 3	*Vitis vinifera*	DR-VI	Pathirana et al. 2013; 2015

VI: Vitrification; EN-DE: Encapsulation-Dehydration; EN-VI: Encapsulation-Vitrification; DR-VI: Droplet-Vitrification.
* Thermotherapy followed by cryotherapy by Encapsulation-Vitrification

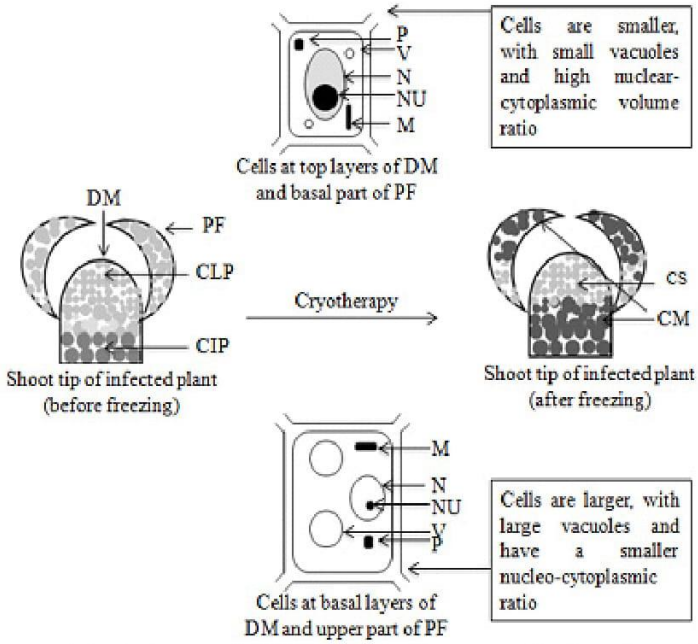

FIGURE 1 - Layout of physical-anatomic differences in cells near and far from the meristematic domein the apical zone. Cells in upper meristematic dome and basal part of leaf primordia are smaller, with small vacuoles and high nuclear-cytoplasm ratio, while cells in the lower part of meristematic dome and upper part of leaf primordia are larger, with bigger vacuoles and low nuclear-cytoplasm ratio and are more prone to injuries after cryotherapy. DM, meristematic dome; PF, leaf primordia; CLP, cells without pathogen; CIP, cells infected by pathogen; CS, surviving cell; CM, dead cells; M, mitochondria; V, vacuole; NU, nucleus; N, nucleolus; P, proplastid. Source: Wang and Valkonen (2009a).

CONCLUSION

Improvements to cryotherapy protocols applied for grapevine are still needed. Most of the published investigations are related to *Vitis vinifera* and studies applied to cultivars from other genetic backgrounds have shown that the same protocols are not suitable. For now, that specificity with different responses among cultivars, beyond the species, limits the wide applicability of cryotherapy. Conversely, traditional methods used to eliminate viruses also have limitations and, with the development and investment in research on cryo-preservation techniques, those barriers could be overcome. Some features of cryotherapy make it very attractive for institutions aiming at the development of high-health nursery material with agronomic potential. These features include its applicability across many species of plants, low running cost and the development of plants free from virus quickly and at a high frequency.

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ANEXO D – Artigo submetido na Acta Horticulturae: Successful cryopreservation of *Vitis vinifera* cv. 'Chardonnay' through a collaboration between Brazil and the United States Department of Agriculture. Bettoni et al. (2018), NO PRELO

Successful cryopreservation of *Vitis vinifera* cv. 'Chardonnay' from both *in vitro* and growth chamber source plants

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Abstract

Both the United States and Brazil maintain genebank collections of vegetatively propagated crops, including grapevine (*Vitis*). *Vitis* collections are expensive to maintain in the field and are vulnerable to abiotic and biotic threats. When robust methods are available, cryopreservation provides an opportunity to securely back-up collections with lower maintenance costs than those of duplicated field collections. We have developed a droplet-vitrification method that results in high levels of shoot tip regrowth of *Vitis vinifera* cultivar 'Chardonnay' after liquid nitrogen exposure. Uniform shoot tips were obtained from nodal sections cultured from *in vitro* or growth chamber stock plants. Pretreatments included 0.3 M sucrose, salicylic acid, ascorbic acid, and glutathione (reduced form). Half-strength PVS2 was applied for 30 minutes at 22°C, prior to full-strength PVS2 treatment at 0°C. Shoot tips derived from *in vitro* grown stock plants had the highest regrowth level of 68% with a PVS2 exposure duration of 75 min at 0°C. Shoot tips derived from growth chamber grown stock plants had the highest regrowth level of 43% with a PVS2 exposure duration of 30 min at 0°C. The high levels of regrowth using both *in vitro* and growth chamber stock plants suggest that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture.

Keywords: cryopreservation, droplet vitrification, genebanking, grapevine

INTRODUCTION

Grapes are among the most important fruit crops that are cultivated and consumed. Globally, in 2016, grapevines covered 7.52 million hectares and produced 75.8 million tons of fruit (OIV, 2017). There are more than 70 species within the *Vitis* genus, with many of the commercially important cultivars assigned to *Vitis vinifera* (Li et al., 2017). Other *Vitis* species as well as *Muscadinia rotundifolia* are important for breeding programs focused on developing new cultivars and rootstocks that are tolerant/resistance to pests and diseases and that are adapted to diverse and changing environmental conditions (Smith et al., 2016; Camargo et al., 2014).

In Brazil, grapes are grown on 85,000 hectares and have production levels of 1.1 million tons annually, 67% for fresh consumption and 33% for processing. Currently, Brazil is 16th worldwide in grape production and 19th in production area (OIV, 2017). Brazilian viticulture is characterized by the diversity of production zones, including temperate, subtropical and tropical regions (Camargo et al., 2011). The Rio Grande do Sul state is responsible for the production of 58% of the grape produced in Brazil, followed by Pernambuco, São Paulo, Paraná, Santa Catarina, Bahia and Minas Gerais (Ritschel et al., 2015).

In 2016, the United States produced 7.67 million tons of grapes on 409,947 hectares of land (USDA NASS, 2018). In 2016, grape production was 6.73 million tons in California, 488,000 tons in Washington, 171,000 tons in New York, 93,400 tons in Michigan, and 85,800 tons in Pennsylvania (USDA NASS, 2018). Within the U.S., grapes are primarily used to produce wine and for fresh table consumption.

Genebank Collections

Currently, Brazil maintains the largest *Vitis* collection in South America with 1430 accessions that include cultivars, interspecific hybrids, and wild species. The Grapevine Germplasm Bank (GGB) is maintained in Rio Grande do Sul state at Embrapa Grape and Wine (http://www.cnpuv.embrapa.br/prodserv), where there are 50 species of grapevine that include 655 accessions of *Vitis vinifera*, 64 accessions of *Vitis labrusca* and hybrids, and 651 accessions of interspecific hybrids (Maia et al., 2015). Epagri (Empresa de Pesquisa Agrícola e Extensão Rural de Santa Catarina) is a Brazilian company that maintains 51 cultivars of *Vitis vinifera*, 22 cultivars of *Vitis labrusca* and hybrids, and 17 table grape cultivars (*Vitis vinifera*, *Vitis labrusca* and hybrids) (Souza, André Luiz Kulkamp, personal communication). In addition, other collections are maintained by Agricultural Research Companies located in the Minas Gerais state (Empresa de Pesquisa Agropecuária de Minas Gerais, Epamig), São Paulo (Instituto Agronômico de Campinas, IAC), Paraná (Instituto Agronômico do Paraná, IAPAR) and in the Northeastern region of Brazil (Embrapa Tropical Semiarid) (Maia et al., 2015).

The USDA-ARS National Plant Germplasm System (NPGS) also maintains a vast collection of *Vitis* genetic resources. The NPGS collection is split between two locations. The cold-hardy grape collection is maintained by the USDA-ARS Plant Genetic Resources Unit (PGRU) in Geneva, NY and the *Vitis vinifera* cultivars and less cold hardy accessions are maintained by the USDA-ARS National Clonal Germplasm Repository for Tree Fruit and Nut Crops and Grapes in Davis, CA. The Geneva collection has 1203 *Vitis* accessions representing 26 taxa and the Davis collection has 2913 *Vitis* accessions representing 49 taxa (Postman et al., 2006). Most of the accessions are maintained as vines in the field; however, the Geneva collection also has some species materials conserved as seeds in -18°C cold storage.

As field collections, *Vitis* accessions are vulnerable to pathogens and environmental disasters (Pathirana et al., 2015; Markovic et al., 2013). *In vitro* culture back-up is an option, but it has some limitations because it is labor-intensive, has a high cost of maintenance, has a risk of microbial contamination, and may experience somaclonal variation (Hassan and Haggag, 2013).

***Vitis* Cryopreservation**

Cryopreservation, the storage of biological materials in liquid nitrogen (LN, -196 °C) or liquid nitrogen vapor (LNV, -165 °C), has been considered an ideal means for the long term preservation of plant materials within genebanks. Cryopreservation minimizes the risk of contamination and materials remain genetically unchanged. It is also cost effective to maintain collections for extended lengths of time in LN (Bi et al., 2017; Reed, 2017; Volk et al., 2014; Benelli et al., 2013).

To date, there have been several *Vitis* cryopreservation protocols reported in the literature. The first reports of the application of cryopreservation to *Vitis* shoot tips were in the 1990s using the encapsulation-dehydration technique, and in the following years other works were carried out with the same technique (Plessis et al, 1991; 1993; Wang et al., 2000; Wang et al. 2003; Bayati et al, 2011;. Markovic et al, 2013). Other protocols or techniques were also investigated: vitrification (Bettoni et al., 2015, Matsumoto; Sakai, 2003; Wang et al, 2003; Ganino et al, 2012), encapsulation-vitrification (Gribaudo et al, 2012; Wang et. al, 2004) and more recently, droplet-vitrification (Pathirana et al 2015; Markovic et al, 2013).

Reliable cryopreservation methods that result in high levels of viability (≥40% post-cryopreservation) and highly skilled staff are key to the development of successful base collections (Volk et al., 2016). Access to reliable cryopreservation methods is important for the widespread use of *Vitis* cryopreservation methods (Reed et al., 2004). Based on the characteristics of interest of cryopreservation protocols, we collaborated to identify cryopreservation methods for grapevine cryopreservation that could be implemented in our respective Brazilian and USDA genebanks.

MATERIALS AND METHODS

Plant materials

Vitis vinifera cv. 'Chardonnay' was originally received from the USDA-ARS National Clonal Germplasm Repository for Tree Fruit, Nut Crops and Grapes in Davis, CA, USA. Growth chamber plants were grown at 25 °C, under a photoperiod of 16 h light day^{-1}. *In vitro* stock cultures were maintained on MS medium (Murashige and Skoog, 1962), containing 30 g L^{-1} sucrose, 0.175 mg L^{-1} IAA[3] and 2.5 g L^{-1} gellan gum at pH 5.7 after sterilization. Cultures were placed in a growth room at 25 °C, under a photoperiod of 16 h light day^{-1} with a light intensity of 40 µM m^{-2} s^{-1}.

Cryopreservation and regrowth

Nodal sections (2 cm) containing a single bud were harvested from the growth chamber plants, surface sterilized by treatment with 70% isopropanol for 1 min, followed by 2 rinses with distilled water. They were then treated with 5% sodium hypochlorite and 0.1% Tween 20 (v/v) for 5 min, and rinsed three times in sterile water. Nodal sections were also obtained from 2- to 3-month old *in vitro* stock plants. Nodal sections from both sources were plated on pretreatment medium (MS containing 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA[3], 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form) and 3 g L^{-1} gellan gum at pH 5.7 after sterilization). Plant preservation mixture (PPM, 1.5% v/v) was included in the medium for the growth chamber nodal sections to reduce microbial contamination. Nodal sections were cultured on pretreatment medium for 2 to 3 weeks in the same conditions as the *in vitro* stock cultures.

Uniform apical shoot tips (1 mm) were excised from nodal sections that originated from either *in vitro* or growth chamber stock plants. Shoot tips were precultured on semi-solid ½ MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form) and 3 g L^{-1} gellan gum at pH 5.7 (after sterilization) for 3 days at 25 °C in darkness (Fig. 1). PPM (1.5 % v/v) was added to the medium for the growth chamber nodal sections to reduce microbial contamination.

Shoot tips were placed into loading solution (1/2 strength MS + 2M glycerol + 0.4 M sucrose at pH 5.7) for 20 min at 22°C, followed by half-strength PVS2 (Sakai et al., 1990) for 30 minutes at 22°C and full-strength PVS2 at 0°C for 60, 75, 90 or 105 min for *in vitro* derived shoot tips and 30, 40 or 50 min for growth chamber derived shoot tips (Fig. 1). *Vitis* shoot tips were then placed onto foil strips and plunged into LN for 1h (Fig. 1), and warmed in unloading solution (½ strength MS + 1.2 M sucrose at pH 5.7) at 22°C for 20 min.

For recovery, shoot tips were placed onto recovery medium 1 (1/2X MS macroelements, 1X MS microelements, and *Vitis* vitamins (100 mg L^{-1} myo inositol, 10 mg L^{-1} thiamine HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} Ca pantothenate, 0.01 mg L^{-1} biotin, 2 mg L^{-1} glycine, without NH_4, supplemented with 0.6 M sucrose and 8 g L^{-1} agar at pH 5.7) overnight in the dark and then transferred to recovery medium 2 (1/2X MS macroelements and 1X MS microelements with *Vitis* vitamins), without NH_4, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA[3] and 8 g L^{-1} agar) and cultured for 2 weeks at 25 °C in darkness. They were then transferred onto recovery medium 3 (1/2 strength MS, supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} BA[3] and 8 g L^{-1} agar at pH 5.7) and grown in the same conditions as the *in vitro* stock cultures.

Shoot tip survival (green cell mass or leaf tissue) and regrowth (organized shoots with at least one leaf) were evaluated 8 weeks after plating on regrowth medium. Each experiment was performed with two or three replicates of 20 shoot tips for each treatment.

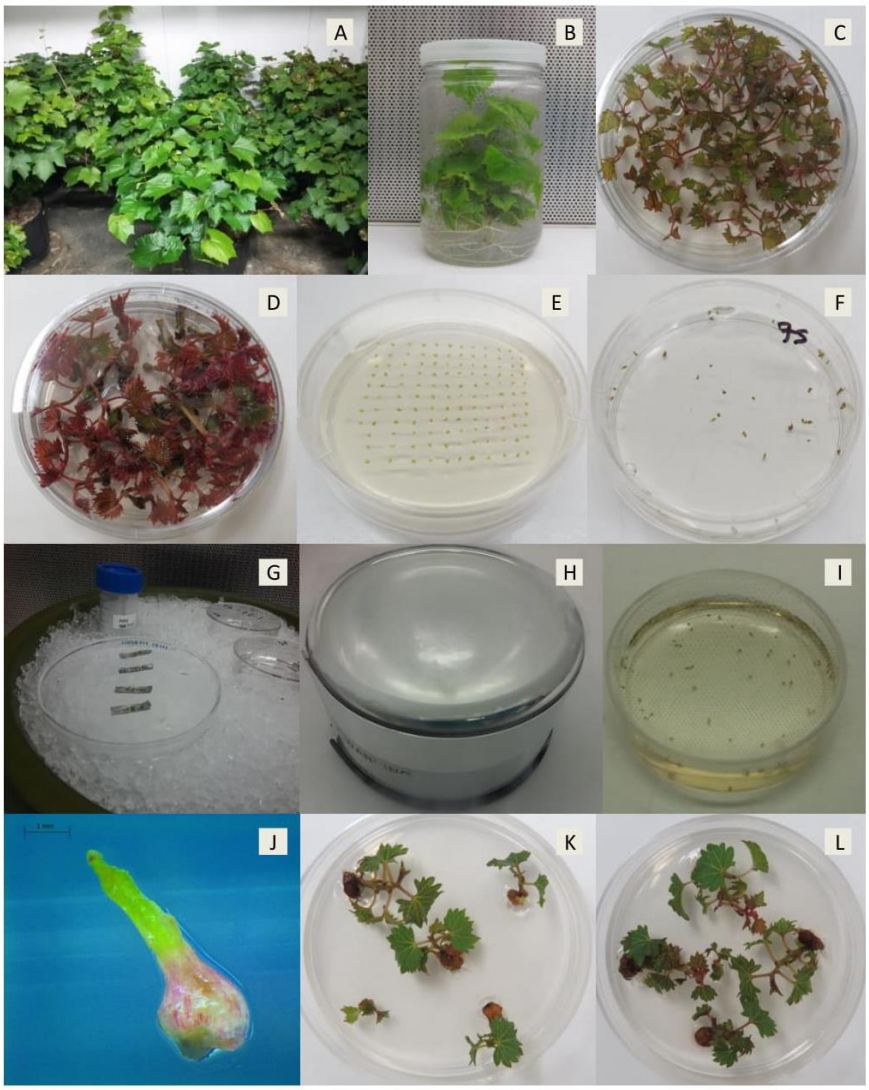

Fig. 1. Droplet vitrification for grapevine shoot tips from nodal sections excised from either growth chamber (A, C) or *in vitro* (B, D) stock plants. Shoot tips were incubated on the preculture medium (E), loading solution and ½ strength PVS2 at 22°C (F) followed by full-strength PVS2 at 0 °C prior to placement on foil strips in a thin layer of PVS2 (G). They were then plunged into LN (H). After 1 h shoot tips were warmed into unloading solution at 22°C (I) and then transferred to recovery medium. Shoot tips were grown on medium to assess regrowth. 'Chardonnay' shoot tips exposed to LN and recovered for 15 days (J) and 2 months (K) and without LN exposure after 2 months of regrowth (L).

RESULTS AND DISCUSSION

Shoot tips that were not treated with LN had higher survival and regrowth levels than those exposed to LN (Fig. 2). Regrowth levels after LN exposure of shoot tips derived from *in vitro* stock plants were 53, 68, 58, and 50% for PVS2 exposure durations of 60, 75, 90, and 105 min., respectively. In contrast, regrowth levels after LN exposure of shoot tips derived from the growth chamber stock plants were 43, 37, and 30% for PVS2 exposure durations of

30, 40 and 50 min (Fig. 2). We found that growth chamber derived shoot tips could not tolerate PVS2 incubation durations that were as long as those derived from *in vitro* source cultures; however, the shorter incubation times used for the growth chamber plants were sufficient for adequate levels of shoot tip regrowth after LN exposure (Fig. 2).

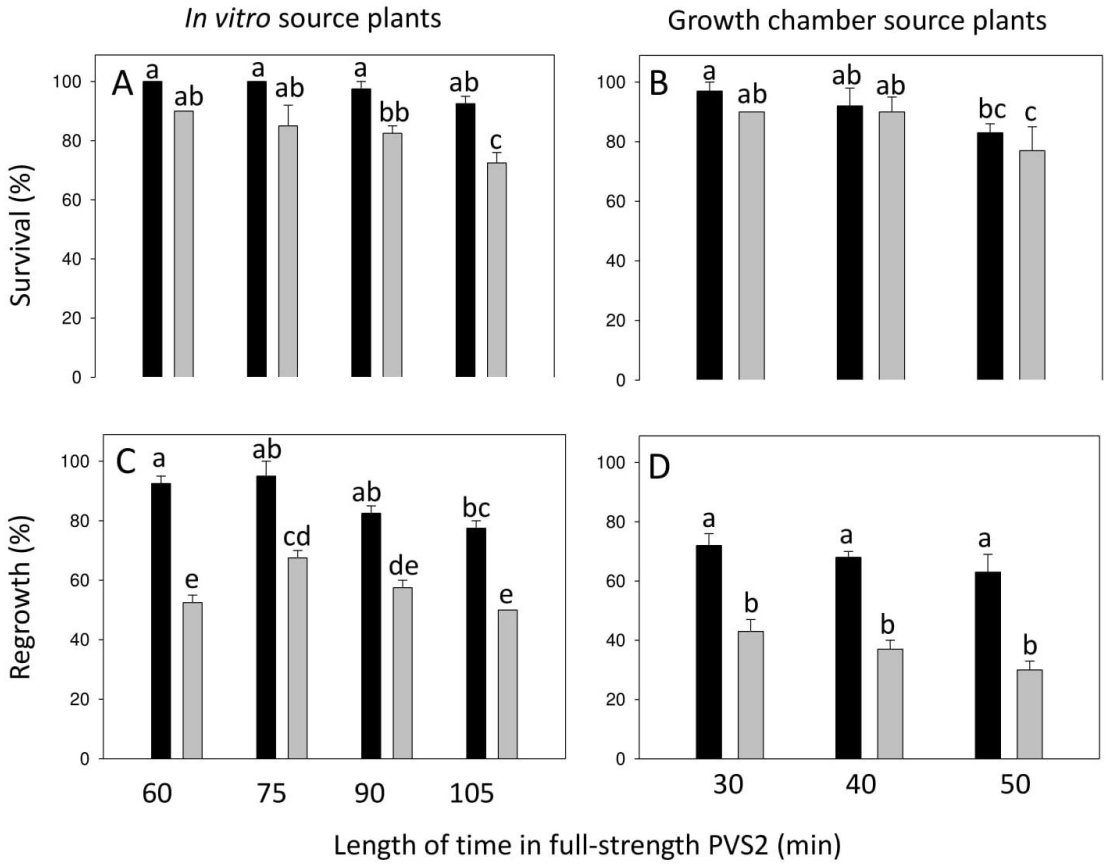

Fig. 2. Effect of full-strength PVS2 exposure length on survival and regrowth percentage of *Vitis vinifera* 'Chardonnay' either without LN (black bars) or with LN (gray bars) exposure. Survival (A, B) and regrowth (C, D) levels were measured as a percentage (with two or three replicates of each treatment of 20 shoot tips). Shoot tips were from *in vitro* (A, C) or from growth chamber (B, D) source plants. Vertical bars indicate the standard error. Significant differences among the eight or six treatments within each graph are indicated by letters.

The high levels of regrowth after shoot tip cryopreservation using shoot tips derived from both *in vitro* and growth chamber stock plants suggest that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture. *In vitro* stock plants require an introduction and multiplication step in tissue culture that increases the time and labor

required to cryopreserve each *Vitis* accession. The labor and resources required for *Vitis* cryopreservation would be reduced if shoot tips can be excised from nodal sections harvested directly from growth chamber plants. At this point, contamination poses the greatest challenge to using nodal sections from growth chamber plants. PPM was added during the pretreatment and preculture steps to reduce and/or eliminate contamination issues. Shoot tip variability and shoot tip sensitivity to preculture medium are also concerns.

Availability of a cryopreservation method for *Vitis* may facilitate the use of cryotherapy methods to eradicate viruses (Bayati et al. 2011; Bettoni et al., 2016; Pathirana et al., 2015; Wang et al., 2003). In some cases, its application has resulted in the production of virus-free plants more quickly and economically than through the use of traditional clean-up methods (Bettoni et al., 2016).

The regrowth levels that we obtained after 'Chardonnay' shoot tips were exposed to LN suggest that it may soon be possible to cryopreserve *Vitis* collections in genebanks, including those in Brazil and the USDA. Future work will focus on determining the applicability of the method to additional *V. vinifera* cultivars and evaluating the efficacy of using shoot tips derived from growth chamber and field plants.

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ANEXO E – Resumo publicado no The Third International Symposium on Plant Cryopreservation, CryoSymp 2018, Bangkok, Thailand: The development of a droplet-vitrification method to conserve *Vitis* collections in the USDA-ARS National Plant Germplasm System and UDESC-CAV Santa Catarina State University in Brazil.

International Society for Horticultural Science

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This submission belongs to: III International Symposium on Plant Cryopreservation

Below is the abstract for symposium nr 493, abstract nr 42:

Title:

The development of a droplet-vitrification method to conserve Vitis collections in the USDA-ARS National Plant Germplasm System and UDESC-CAV Santa Catarina State University in Brazil

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Preferred presentation method:

Oral

Abstract body text:

Both the United States and Brazil maintain vast collections of grape genetic resources. We share a common interest in using cryopreservation methods for the secure, long-term back-up of accessions within these field collections of the USDA-ARS National Plant Germplasm System and UDESC-CAV Santa Catarina State University in Brazil. We have developed a *Vitis* droplet-vitrification method that results in high levels of shoot tip regrowth after liquid nitrogen (LN) exposure. Uniform shoot tips were obtained from nodal sections of either in vitro or growth chamber stock plants. Pretreatments included 0.3 M sucrose, salicylic acid, ascorbic acid and glutathione. Half-strength PVS2 was applied for 30 min at 25^{o}C, prior to full-strength PVS2 treatment at 0^{o}C. The optimum PVS2 exposure duration was 30 min for growth chamber-derived shoot tips and 90 min for in vitro derived shoot tips. Shoot tips were then placed onto foil strips and plunged into LN. Regrowth levels ranged from 45 to 72 % for the seven *Vitis* species tested from in vitro plants, and from 35 to 65 % for three *Vitis vinifera* cultivars and one *Vitis* hybrid tested from growth chamber plants. The high levels of regrowth suggest that *Vitis* cryopreservation may soon be ready for implementation within genebanks.

Keywords:

Vitis, plant vitrification, genetic resources, cryopreservation, shoot tips

APÊNDICES

APÊNDICE A – Componentes da formulação de Murashige & Skoog (1962).

Componentes	
Macroelementos	**mg L^{-1}**
NH_4NO_3	1.650
$CaCl_2 2H_2O$	440
$MgSO_4 7H_2O$	370
KH_2PO_4	170
KNO_3	1.900
Microelementos	**mg L^{-1}**
$MnSO_4.H_2O$	16,9
$ZnSO_4.7H_2O$	8,6
H_3BO_3	6,2
KI	0,83
$Na_2MoO_4.2H_2O$	0,25
$CuSO_4.5H_2O$	0,025
$CoCl_2.6H_2O$	0,025
FeEDTA	**mg L^{-1}**
$FeSO_4.7H_2O$	27,8
Na2EDTA .2H2O	37,3
Vitaminas	**mg L^{-1}**
Glicina	2
Ácido nicotínico	0,5
Piridoxina-HCl	0,5
Tiamina-HCl	0,1
Mioinositol	100
Sacarose	30.000
pH	5,7 - 5,8

Fonte: Murashige e Skoog (1962).