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HIGH VIABILITY OF DORMANT *Malus* BUDS AFTER 10 YEARS OF STORAGE IN LIQUID NITROGEN VAPOUR

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Abstract

Three hundred and sixty two *Malus* accessions from the Canadian Clonal Genebank of Plant Gene Resources of Canada were cryopreserved as dormant buds at the USDA-ARS National Center for Genetic Resources Preservation in 1996. According to grafting data collected on 165 of these accessions in 1999, 80% of the accessions had at least 40% viability. A subsample of these accessions was processed for cryopreservation by either adjusting the moisture content of the budwood sections containing dormant buds to 32 or 37% moisture (fresh weight basis) or by not drying the budwood sections (46% moisture fresh weight basis) prior to cooling. Budwood sections were then slow-cooled at 1ºC h^{-1} to -30ºC, held for 24 h at -30ºC and then rapidly transferred to the vapour phase of liquid nitrogen. Cryopreserved buds from 13 accessions that were dried using the various techniques were warmed and grafted in both 1999 and 2006 to determine viability. Overall, bud viability was high at both storage times. At the 10 year timepoint, some accessions had higher bud growth when they were desiccated prior to slow-cooling when compared to those that were not.

Keywords: apple, cryopreservation, genetic resources, genebank

INTRODUCTION

National collections of apple genetic diversity are maintained primarily under field conditions. As a preventative measure against field disasters, large portions of the national Canadian and USDA *Malus* collections are also backed-up in liquid nitrogen vapour (LNV) at the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, Colorado.

Winter vegetative buds from hardy fruit trees can be held in liquid nitrogen vapour, and subsequently grafted to rootstocks to regenerate clones (4). Cryopreservation methods for *Malus* were originally reported by Sakai and Nishiyama (5), who demonstrated that buds stored in LN for 23 months remained viable. The NCGRP routinely cryopreserves *Malus* dormant buds by first desiccating single bud sections to 25-30% moisture content, slow-cooling tubes of bud sections at 1ºC h^{-1} to -3ºC, holding the samples at -30ºC for 24 h, and then quickly placing tubes into LN vapour for long term storage (2). Each accession is stored

as five tubes, each containing 12 buds, with a 40% minimum viability as determined by grafting after one month of storage (2). Cryopreservability is dependent upon cold hardiness, with the least cold hardy accessions having lower survival rates after cryoexposure (2, 11). It has been documented that there was no decline in viability (as determined by grafting) between one month and four years of storage across 64 USDA accessions and a subsequent study reported no declines in viability after four to seven years across these same 64 accessions (1, 2).

Dormant winter buds survive LN exposure when they are slowly cooled to -30ºC to allow the freezable water to move from within the cells to the extracellular spaces, thus minimizing the damage caused by ice crystallization (3). Fully acclimated dormant buds have increased levels of dehydration tolerance (14). Stushnoff (7) reported that the 50-60% field moisture of dormant buds must be reduced to 20-30% to limit ice crystal formation. This can be accomplished by either desiccating budwood prior to slow cooling (12, 9) or by slowly cooling at a rate such that water is effectively removed from the budwood during the cooling process (10). In apple, dormant bud sections that were not previously dehydrated can also be successfully cryopreserved using slow-cooling techniques (10). The rate of cooling was critical to ensure that water had sufficient time to migrate to the extracellular spaces. Even with desiccation, Tyler *et al*. (13) found that budwood cooled at rates of -10ºC h^{-1} must be held at temperatures between -30 and -40ºC for 24 h prior to immersion in LN. In some cases, cooling at -5ºC d^{-1} resulted in higher viabilities (10). Nuclear magnetic resonance spectroscopy revealed that these conditions removed the freezable water from the apple buds (8, 13).

In 1996, a restructuring of Agriculture and Agri-Food Canada necessitated that the field *Malus* collections be moved from the Canadian Clonal Genebank (CCG) located at the Smithfield Experimental Farm in Trenton, Ontario to a location in Harrow, Ontario. Budwood from the 362 *Malus* accessions that are unique to the CCG was sent to the NCGRP (formerly National Seed Storage Laboratory) to be cryopreserved as a back-up for the collection when the field materials at the Trenton site were destroyed (Towill and Waddell, unpublished). Some accessions were originally processed using both desiccated and non-desiccated scions. The goal of this study was to determine the viability of a subset of the CCG collection that was processed using both desiccated and non-desiccated scions and was subsequently maintained at the NCGRP in LNV for 10 years.

MATERIALS AND METHODS

In early 1996, dormant one-year-old apple budwood from 362 accessions was collected from trees growing at the Canadian Clonal Genebank, located at the Smithfield Experimental Farm in Trenton, Ontario and sent overnight express to the NCGRP, where plant material was kept at -5ºC until use. The budwood was cut into one-bud sections (35 to 40 mm long) and was either not dried, dried one week, or dried to an average of 30% moisture content on a fresh weight basis. The desiccation treatment was performed by placing sections on trays at -5ºC for between seven and 61 days (average of 26 days). Representative bud sections were oven-dried to determine the moisture content of the remaining bud sections. After desiccation, the budwood sections were immediately sealed in plastic bags and maintained at -5ºC. Before slow-cooling, 10 one-bud sections were placed within 280 mm long, clear heat-shrinkable flexible polyolefin tubes (3M, Austin, TX) that varied in diameter from 13 to 19 mm. Tubes were heat-sealed at both ends around wooden plugs, and placed in aluminium cryoboxes (64 mm x 64 mm x 285 mm) for slow-cooling.

Cryoboxes were cooled in an ultra-low-temperature chest freezer (Thermo Electron Corporation, Asheville, NC) into which an aluminium plate surrounded by an insulated styrofoam box was inserted. A strip heater and fans were placed underneath the aluminium plate within the styrofoam block. The chest freezer was maintained at -45ºC. Temperatures within the styrofoam box were controlled using a programmable digital temperature controller (Omega Engineering, Stamford, CT) which turned the strip heater on or off automatically depending on the temperature setpoint. The aluminium plate served as a heat sink, stabilizing temperatures within the box and preventing extreme fluctuations in temperature within the box. Cryoboxes within the styrofoam boxes were held at -5ºC for 1 h, cooled at -1ºC h^{-1} to -30ºC, held for 24 h at -30ºC, and rapidly transferred to the vapour phase of liquid nitrogen within a stainless steel cryotank.

Viability assessments in 1997 and 1999

For viability assessments in 1997 and 1999, one tube of each cultivar was removed from cryoboxes and slowly warmed at +3ºC overnight. Tubes with bud sections were placed in plastic bags in Fed-Ex boxes and shipped to the Greenhouse and Processing Crops Research Centre (PGRC), Harrow, Ontario, Canada for budding. Bud sections received at Harrow were placed in moist peat moss for two weeks to rehydrate. All 10 buds were grafted to *M. antonovka* rootstocks that had been growing in the greenhouse for 18 to 21 days. After two weeks, budding ties were removed and the tops of the rootstocks were cut back to within 5 cm of the upper bud. Viability readings were taken six to seven weeks after budding.

Viability assessments in 2006

Two replicate tubes from selected accessions were warmed for each accession and dehydration state. The first replicate set of tubes was thawed between June 28 and July 13, 2006 and the second replicate set of tubes was thawed between July 13 and August 10, 2006. Tubes of 10 accessions identified for retrieval were removed from the cryoboxes and placed at +3ºC overnight. One bud section was removed from each tube to determine moisture content. The following day, the remaining nine bud sections were placed in damp peat moss in sealed plastic sandwich bags for rehydration. Bud sections in peat moss were kept at +3ºC for two weeks prior to budding. Bud sections that were not dried prior to cooling were budded without a prior rehydration.

One-year-old bare root *Malus X domestica* seedling rootstocks (Lawyer Nursery, Plains, MT) were stored in the dark at +3ºC in damp medium-grade vermiculite. Three weeks prior to budding, individual rootstocks were placed in conical tree-seedling nursery containers (Stuewe and Sons, Corvallis, OR) containing growth medium (Metromix 200, Scotts-Sierra Horticultural Products, Marysville, OH) supplemented with slow release fertilizer (Osmocote Plus 15-9-12, Scotts-Sierra Horticultural Products, Marysville, OH) and grown in the greenhouse for three to four weeks prior to budding. Individual rootstocks were budded with two rehydrated (or undried buds that were not rehydrated) buds using a chip budding technique. Each bud was wrapped with one budding rubber band. Seventeen days after budding, budding rubber bands were removed and the growing branch of the rootstock was pruned off to enhance bud expansion and growth. Bud recovery and growth was evaluated after four weeks. Any bud which expanded and began shoot elongation was evaluated as viable.

Means and standard errors were calculated across experimental replicates. Statistical comparisons (ANOVA, means separation tests) were made with the JMP software package (SAS Institute Inc., Cary, NY, USA).

RESULTS AND DISCUSSION

Most of the PGRC dormant buds processed in 1996 were dried to an average moisture content of 30% prior to slow-cooling. Across the 13 accessions sampled in 2006, the original 1996 moisture contents were 32% for the sections dried, 37% for the sections dried one week, and 46% for the undried bud sections (Table 1). The moisture contents obtained for the samples in 2006 did not significantly change over the 10 years of storage (Table 1).

Table 1. Percent moisture and standard error (fresh weight basis) of nodal bud sections for 13 Canadian *Malus* accessions that were dried to different moisture contents. Sections from 1996 were measured before cooling. Sections from 2006 were measured from LNV storage conditions. Means separation tests were performed across the average values obtained for each treatment.
n.a.:not assessed.

Canadian		Undried		Dried 1 week		Dried	
Acc. No.	Cultivar	1996	2006	1996	2006	1996	2006
MAL 0298	Snow Red Jones	45 ± 1	46 ± 1	37 ± 1	37 ± 1	35 ± 1	32 ± 1
MAL 0326	Minnesota no.447	48 ± 1	44 ± 1	n.a.	n.a.	24 ± 1	37 ± 1
MAL 0348	Scarlet Pippen	50 ± 1	47 ± 1	n.a.	n.a.	31 ± 1	32 ± 1
MAL 0563	Geneva Crab	50 ± 1	50 ± 1	n.a.	n.a.	28 ± 1	32 ± 1
MAL 0604	Roy-A1	46 ± 1	44 ± 1	n.a.	n.a.	34 ± 1	31 ± 1
MAL 0698	Crab1	48 ± 1	46 ± 1	n.a.	n.a.	25 ± 1	24 ± 1
MAL 0699	Crab3	45 ± 1	44 ± 1	35 ± 1	37 ± 1	36 ± 1	36 ± 1
MAL 0728	Donald Wyman	47 ± 1	46 ± 1	n.a.	n.a.	36 ± 1	36 ± 1
MAL 0751	Sumac	42 ± 1	43 ± 1	n.a.	n.a.	36 ± 1	35 ± 1
MAL 0763	September Ruby	47 ± 1	44 ± 1	38 ± 1	37 ± 1	24 ± 1	30 ± 1
MAL 0770	Ottowa 5410	44 ± 1	41 ± 1	n.a.	n.a.	36 ± 1	36 ± 1
MAL 0789	SJM 50 92-1016	n.a.	n.a.	36 ± 1	37 ± 1	35 ± 1	33 ± 1
MAL 0822	SRM no.18	44 ± 2	46 ± 1	n.a.	n.a.	33 ± 1	33 ± 1
Average		46 [a]	45 [a]	37 [b]	37 [b]	32 [c]	32 [c]

Initial grafting data revealed that most of the PGRC accessions were successfully cryopreserved. Ten buds (one tube) each of a total of 44 accessions that were dried prior to slow-cooling were warmed and grafted in 1997. Of these accessions, 93% had acceptable viabilities as determined by grafting (>40%). In 1999, 165 accessions were dried prior to slow-cooling and 80% of these accessions had viabilities of greater than 40% as determined by grafting (Table 2).

Table 2. Shoot development from grafted buds of LNV-exposed sections from the Canadian *Malus* accessions cryopreserved at the NCGRP and budded at the PGRC in 1997 or 1999.

% of grafted buds showing growth	Number or percent of accessions			
	1997		1999	
	No.	%	No.	%
61-100	34	77	86	52
40-60	7	16	46	28
10-39	3	7	26	16
0-9	0	0	7	4

The 13 accessions selected for grafting in 2006 were based on those that had high percentages of bud break after grafting in 1999 (average of 82 ± 2%). Most of these accessions were slow-cooled either as undried buds or after desiccation. An intermediate moisture content was achieved by drying one week at -5ºC for accessions MAL 0298, MAL 0699, MAL 0763, and MAL 0789. Overall, the average percent bud break after grafting did not significantly differ across the three moisture content treatments (Table 3), although significant differences in viability between undried and dried were observed in three of the accessions. Twelve of the 13 accessions had bud growth percentages of greater than 40% following at least one processing method. Only one accession of the four tested (MAL 0699) tended to have higher levels of bud survival when dried for one week, compared to the other two treatments. The only accession that had no survival in 2006 when processed in the undried state was MAL 0763. Accessions MAL 0763 and MAL 0348 had significantly higher bud survival when slow-cooled in the desiccated state compared to the survival when slow-cooled without prior desiccation. In contrast, MAL 0728 had significantly higher bud survival when it was slow-cooled without desiccation (Table 3). Overall, the bud survival after grafting 1999 for the 13 accessions was higher than the bud survival after grafting in 2006. This may be attributed to several factors: rootstock type, rootstock age, time in cryostorage, and the personnel performing the grafting at the PGRC in 1999 and the NCGRP in 2006.

Table 3. Percent of LNV-stored *Malus* buds that exhibited shoot elongation after budding in either 1999 or 2006. In 2006, budded accessions were either undried (45% moisture content), dried 1 week (37% moisture content) or dried (32% moisture content) prior to cooling. Means separation tests ($\alpha < 0.05$) were performed across the treatments within each accession. n.a.: not assessed.

Canadian Accession No.	Cultivar	1999[1]		2006[2]	
		Dried	Undried	Dried 1 week	Dried
MAL 0298	Snow Red Jones	90	100 ± 0 [a]	94 ± 6 [a]	100 ± 0 [aa]
MAL 0326	Minnesota no. 447	90	100 ± 0 [a]	n.a.	100 ± 0 [aa]
MAL 0348	Scarlet Pippen	70	56 ± 0 [a]	n.a.	100 ± 0 [bb]
MAL 0563	Geneva Crab	80	76 ± 13 [a]	n.a.	84 ± 6 [aa]
MAL 0604	Roy-A1	80	89 ± 11 [a]	n.a.	100 ± 0 [aa]
MAL 0698	Crab1	90	50 ± 6 [a]	n.a.	73 ± 17 [aa]
MAL 0699	Crab3	90	60 ± 10 [a]	84 ± 17 [a]	69 ± 2 [aa]
MAL 0728	Donald Wyman	70	95 ± 6 [a]	n.a.	39 ± 6 [bb]
MAL 0751	Sumac	80	55 ± 35 [a]	n.a.	73 ± 6 [aa]
MAL 0763	September Ruby	80	0 ± 0[3] [a]	13 ± 13 [ab]	50 ± 6 [bb]
MAL 0770	Ottowa 5410	80	34 ± 23 [a]	n.a.	28 ± 28 [aa]
MAL 0789	SJM 50 92-1016	90	n.a.	50 ± 17 [a]	60 ± 16 [aa]
MAL 0822	SRM no.18	80	53 ± 28 [a]	n.a.	78 ± 0 [aa]
Average		82 ± 2 [a]	64 ± 9 [a]	60 ± 18 [a]	68 ± 7 [a]

[1] One tube of 10 sections was budded; [2] To tubes of nine sections were budded; [3] Outlier datapoint was not included in column average.

We demonstrated that *Malus* dormant budwood can be successfully cryopreserved for at least 10 years. Buds in storage did not significantly decline in viability; however, there were differences in survival between buds grafted in 1999 and 2006. Accessions that did not survive the current methods employed at the NCGRP, which involve desiccation prior to slow-cooling, may have had higher success rates if slow-cooled without prior desiccation. Cold-tender *Malus* germplasm not included in this study may be less amenable to dormant bud cryopreservation methods than cold hardy materials (11). These accessions may have to be cryopreserved using shoot tip culture methods with either dormant buds or *in vitro* cultures as explants (6, 15, 16).

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