Prioritization of *Malus* accessions for collection cryopreservation at the USDA-ARS National Center for Genetic Resources Preservation

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Abstract

The USDA-ARS National Plant Germplasm System maintains a grafted collection of apple accessions representing 48 taxa in Geneva, NY. Dormant buds of many of these accessions have been routinely cryopreserved at the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO. In the standard procedure, dormant buds are sent to NCGRP in mid-winter. Scions are cut into 35 mm bud sections and desiccated at -5°C to a moisture content of 25 to 30% (fresh weight basis). Desiccated single-bud sections are then sealed into polyolefin tubes containing 10 to 12 sections each, slow cooled at -1°C h^{-1} to -30°C, held at -30°C for 24 h, and then placed into the vapor phase of liquid nitrogen (LNV) for long term storage. For viability testing, the buds from one polyolefin tube are rehydrated at 2°C in moist, sterile peat moss and grafted onto rootstocks. For most accessions, this left 3 polyolefin tubes of *Malus* buds in long-term storage for each accession. For this analysis, successfully cryopreserved accessions were defined as those that have a total of 19 or more predicted viable buds remaining in LNV at NCGRP. Of the 2291 accessions currently cryopreserved at NCGRP, 2052 accessions meet this requirement. Criteria were established to prioritize the apple accessions that are either inadequately backed up at NCGRP or have not yet been processed. These criteria include the likelihood of success in cryopreserving the *Malus* taxon, the vulnerability of the field trees, the number of viable buds currently stored in LNV, and previous failures in response to the standard procedure.

Keywords: apple, dormant buds

INTRODUCTION

Field collections of plant genetic resources are expensive to maintain and are vulnerable to climatic, abiotic, and biotic threats. Cryopreservation has emerged as a technology by which clonally propagated field collections can be conserved in a safe, secure, secondary location. Apple cultivars can be cryopreserved in the form of dormant buds (Forsline et al., 1998; Höfer, 2015; Towill et al., 2004; Vogiatzi et al., 2011) or in the form of excised shoot tips (Condello et al., 2011; Feng et al., 2013; Halmagyi et al., 2010; Kushnarenko et al., 2009; Li et al., 2014; Liu et al., 2004; Niino et al., 1992; Paul et al., 2000), with dormant buds often being a more economical choice (Volk et al., 2010). The success of the dormant bud cryopreservation method is dependent upon having genetic backgrounds that are amenable to the procedure, having field collections that achieve adequate levels of winter dormancy, and having robust, reliable processing and recovery techniques available. Over the past 25 years, dormant bud cryopreservation methods have been successfully used to conserve most of the cultivars in the USDA National Plant Germplasm System apple collection, maintained at the Plant Genetic Resources Unit (PGRU) in Geneva, NY (Towill et al., 2004). These dormant buds are expected to remain viable in liquid nitrogen vapor (LNV) for extended lengths of time (Volk et al., 2008).

The USDA-ARS National Plant Germplasm System maintains a grafted collection of apple accessions representing 48 taxa managed by the PGRU. Apple accessions were placed into LNV as part of a research program at the USDA-ARS National Center for Genetic

Resources Preservation (NCGRP) in Fort Collins, CO between 1988-1992 (3-19 accessions processed year^{-1}), and for routine back-up between 1993-2014 (22 to 350 accessions processed year^{-1}) (Table 1).

Table 1. Number of *Malus* accessions processed per year for cryopreservation at NCGRP.

Year	Accessions (no.)
1988	3
1989	6
1990	10
1991	19
1992	15
1993	94
1994	138
1995	236
1996	219
1997	350
1998	307
1999	220
2000	222
2003	94
2004	169
2005	46
2007	91
2013	22
2014	30

The goals of this research were to 1) determine the *Malus* species-level response to the standard dormant bud cryopreservation protocol that has been implemented at NCGRP, and 2) establish criteria to determine the adequacy of accession back-up and priority status for apple collection materials.

MATERIALS AND METHODS

Cryopreservation procedure

Dormant budwood from the previous season's growth were collected from trees in the NPGS apple field collection in January or February and sent to NCGRP for processing. Scions were cut into sixty 35 mm bud sections and desiccated at -5°C to 25-30% moisture content. Desiccated single-bud sections were sealed in polyolefin tubes, slow cooled (-1°C h^{-1}) to -30°C, held at -30°C for 24 h, and then placed into the vapor phase of LN for long term storage with 10 to 12 buds tube^{-1}. For viability testing, one polyolefin tube for each accession was shipped to PGRU for grafting. Buds were rehydrated for 12 days at 2°C in moist, sterile peat moss and then grafted onto *Malus* seedling rootstocks, with no more than two grafts per seedling, to assess viability (Forsline et al., 1998; Volk et al., 2008).

Data compilation

Species and field inventory data were recorded for each of the *Malus* accessions that have been processed for cryopreservation at NCGRP between 1988 and 2014. Data collected for cryopreservation results for each accession included the processing date, number of buds processed, number of predicted viable buds in LN, and percent viability (number of viable buds as determined by grafting/number of buds grafted*100).

The percent viability for each accession was calculated and summarized by species (Tables 2 and 3). The species with more than 25% of the accessions tested that did not have viability levels of at least 40% were determined to be "difficult" species (Table 3). These

difficult species were not particularly amenable to cryopreservation and were marked as such for prioritization assessments.

Table 2. *Malus* species with high levels of viability of dormant buds after LNV exposure.

Taxon	No. accn. processed	No. accn <40% viability	% accn. <40% viability	Average % viability
Malus baccata	49	6	12	72
Malus brevipes	2	0	0	65
Malus coronaria	49	8	16	56
Malus domestica	1344	42	3	77
Malus florentina	3	0	0	47
Malus floribunda	11	2	18	54
Malus hybr.	326	13	4	77
Malus ioensis	37	5	14	61
Malus kansuensis	5	0	0	88
Malus mandshurica	3	0	0	73
Malus orientalis	16	3	19	56
Malus orthocarpa	1	0	0	70
Malus prunifolia	35	2	6	75
Malus sieversii	88	16	18	58
Malus sieversii var. *kirghisorum*	8	0	0	76
Malus sieversii var. *turkmenorum*	3	0	0	77
Malus sikkimensis	12	0	0	75
Malus spp.	26	3	12	67
Malus sylvestris	20	0	0	78
Malus toringo	22	4	18	68
Malus toringoides	7	0	0	89
Malus transitoria	4	0	0	75
Malus × *adstringens*	2	0	0	95
Malus × *arnoldiana*	2	0	0	70
Malus × *asiatica*	16	0	0	72
Malus × *dawsoniana*	2	0	0	80
Malus × *hartwigii*	5	0	0	76
Malus × *magdeburgensis*	2	0	0	70
Malus × *micromalus*	15	1	7	75
Malus × *moerlandsii*	2	0	0	50
Malus × *platycarpa*	7	1	14	59
Malus × *purpurea*	5	1	20	72
Malus × *robusta*	13	1	8	76
Malus × *scheideckeri*	2	0	0	70
Malus ×*soulardii*	3	0	0	98
Malus zhaojiaoensis	2	0	0	50
Malus zumi	3	0	0	80

To identify prioritization levels for future cryopreservation efforts, field inventories were assembled. The cryopreservation status of each accession was classified as 1) never processed previously, 2) successfully backed-up, or 3) cryopreserved but depleted. Availability of field materials for future cryopreservation efforts was documented.

Table 3. *Malus* species classified as "difficult to cryopreserve" because 25% or more of the accessions had viability levels of less than 40% after cryoexposure of dormant buds.

Taxon	No. accn. processed	No. accn <40% viability	% accn. <40% viability	Average % viability
Malus angustifolia	17	10	59	34
Malus fusca	40	19	48	34
Malus halliana	14	6	43	39
Malus honanensis	3	1	33	37
Malus hupehensis	16	5	31	54
Malus prattii	3	1	33	47
Malus sargentii	16	4	25	64
Malus spectabilis	8	3	38	45
Malus tschonoskii	3	3	100	0
Malus ×astracanica	1	1	100	10
Malus × atrosanguinea	2	2	100	10
Malus × sublobata	4	1	25	60
Malus yunnanensis	12	3	25	68

RESULTS AND DISCUSSION

Malus dormant buds have been cryopreserved at NCGRP for decades. For routine back-up at NCGRP in the 1990s, it was standard to process 60 dormant buds apple^{-1} accession and use 12 of those buds to assess viability during the same season. This left a maximum of 48 buds in LNV per accession. This protocol resulted in fewer propagules than are currently recommended for long-term conservation at NCGRP (60 predicted viable propagules with at least 40% viability accession^{-1}), and thus alternative viability standards were established for previously processed materials. Accessions were considered to be adequately backed-up at NCGRP if they have 19 or more buds predicted to be viable in LNV. Of the 2291 accessions currently cryopreserved at NCGRP, 2052 accessions meet this requirement. There are currently 239 *Malus* accessions that do not have 19 predicted viable buds in LNV that will be reprocessed as resources are available.

Viability results at the species level are presented in Tables 2 and 3. Thirteen *Malus* species and hybrid species had at least 25% of the accessions with viability levels lower than 40%. These included *M. angustifolia*, *M. fusca*, *M. halliana*, *M. honanensis*, *M. hupehensis*, *M. prattii*, *M. sargentii*, *M. spectabilis*, *M. tschonoskii*, *M.* × *astracanica*, *M.* × *atrosanguinea*, *M.* × *sublobata*, and *M. yunnanensis*. Accessions belonging to these species were classified as "difficult to cryopreserve" for prioritization classification. A previous report attempted to correlate species cryopreservability and taxonomic relationships in *Malus*. It was found that some North American *Malus* species, such as *M. fusca* and *M. angustifolia* are from more mild regions and have lower cryosurvival levels than species that are from more winter hardy locations (Towill et al., 2004).

Accessions with fewer than 19 buds predicted to be viable in LNV (including those that were completely depleted) were targeted for reprocessing. In addition, accessions with permanent inventory status in Geneva, NY (with PI number assigned), but not yet cryoprocessed, were considered to be candidates for cryopreservation.

A comparison of field records and cryopreservation records resulted in a total of 568 *Malus* accessions in current need of cryopreservation at NCGRP. These accessions were classified into three priority levels. Accessions have been classified as priority 1 if they belong to a "non-difficult to cryopreserve" species, only one tree is present in the PGRU field collection, the tree is not in the nursery (large enough to provide budwood), and there are no viable buds in LNV at NCGRP. In addition, priority 1 accessions do not have a history of failure in previous cryopreservation efforts. At this time, 64 accessions are classified as

priority 1. Priority 2 materials were described as follows: “non-difficult to cryopreserve” species, less than 19 viable buds in LNV at NCGRP, one or more trees available in the PGRU field collection, and may have had <40% viability in a previous cryoprocessing effort. Currently, 387 accessions are classified as priority 2. Priority 3 materials are those that are considered to be “difficult to cryopreserve” species, had low viabilities (<40%) after two attempts at cryopreservation, and have fewer than 19 viable buds in LNV at NCGRP. There are currently 117 priority 3 *Malus* accessions.

NCGRP will determine the number of slots available for cryoprocessing on an annual basis, and accessions classified as priority 1 will be processed first. A minimum of 170 dormant bud segments for each accession will be sent to NCGRP in January for cryoprocessing. After processing, 140 buds will be placed into cryo-storage. Of those, 1 tube (10 dormant buds) of each accession will be returned to the curator for viability testing in the spring. Accessions will be considered successfully cryopreserved when viability levels are >40% and 60 or more dormant buds are predicted to be viable.

Materials will be processed according to their priority level. The cryopreservation priority list will be updated as new materials are added to the permanent apple collection and as additional viability data become available. As time and resources permit, research will be performed to identify improved methods for the cryopreservation of “difficult to cryopreserve” species and accessions.

Our assessment revealed six accessions that are only available at NCGRP in LNV and have fewer than 19 predicted viable buds (PI 589378-*M. baccata*, PI 589985-*M. coronaria*, PI 589743-*M. floribunda*, PI 589266-*M. fusca*, PI590082-*M.* hybrid, and PI 590077-*M.* hybrid). These accessions have been targeted for repropagation in the PRGU field collection.

Cryopreservation success levels for diverse *Malus* accessions may be dependent upon collection field conditions in a given year (Jenderek et al., 2011; Vogiatzi et al., 2011). If low levels of viability are obtained, accessions will be reprocessed at a later date. Since there are a limited number of buds cryopreserved for most of the accessions in the *Malus* collection, routine viability assays are not performed for all of the accessions. However, there were no significant changes in viability in the Canadian *Malus* dormant bud collection after 10 years of LNV at NCGRP (Volk et al., 2008). Some *Malus* accessions have been cryopreserved as “controls” on a routine basis, and long-term viability assessments of these materials will be published in the future.

Establishment of a prioritization of accessions for *Malus* cryopreservation efforts at NCGRP provides a roadmap for future preservation efforts. When 32 priority 1 accessions are successfully cryopreserved in each of the winters of 2016 and 2017, these materials will be secure. This work has identified *Malus* species for which additional research is needed to improve cryopreservation procedures and also determined which species are particularly amenable to the dormant bud cryopreservation method that has been implemented at NCGRP.

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