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CRYOPRESERVATION

Cryopreservation of fruit germplasm

Monika Höfer[1] · **Magda-Viola Hanke**[1]

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Abstract Most temperate fruit species are genetically heterozygous and vegetatively propagated. Active collections of fruit genetic resources in Germany are generally maintained in the field, e.g., as potted plants for *Fragaria* and as trees for pome and stone fruit species. The plant material in active collections should be kept in duplicate to ensure security in case of disease or environmental disaster. The aim of this study was to develop an efficient complementary conservation strategy for fruit genetic resources. Although costly, fruit tree cultivars can be duplicated as field collections at a second site within the framework of the German Fruit Genebank, which is a decentralized fruit-specific network. Wild species accessions, particularly those of the genera *Malus* spp. (apple) and *Fragaria spp.* (strawberry) as well as strawberry cultivars, can also be duplicated by means of cryopreservation. In the current study, long-term cryopreservation was initiated for 194 *Fragaria* genotypes. A protocol combining vitrification with cold acclimation was effective and highly reproducible, with an average regrowth rate of 86%. In *Malus*, a general cryopreservation protocol based on dormant winter buds was adopted. Based on the results provided in this study, a combination of traditional *ex situ* conservation and cryopreservation can greatly improve the stability and security of fruit germplasm conservation.

Keywords Cryopreservation · Dormant bud · *Fragaria* · *Malus* · Vitrification

✉ Monika Höfer
monika.hoefer@julius-kuehn.de

[1] Julius Kühn Institute, Federal Research Centre for Cultivated Plants, Institute for Breeding Research on Fruit Crops, Pillnitzer Platz 3a, 01326 Dresden, Germany

Introduction

Fruit genetic resources and *ex situ* conservation The conservation and accessibility of broadly diverse fruit germplasm, including cultivars and wild relatives, represent a valuable genetic resource. Genetic resources, as donors of traits needed for plant breeding, are an essential starting point for plant improvement (Berthaud 1997). The world's gene pool of fruit crops is not expanding; instead, it is threatened with loss of diversity because of mankind's activity. In addition to the *in situ* conservation of wild fruit species at their centers of origin, it is also important to preserve landraces and local cultivars that have been used for many centuries and are adapted to different ecosystems (Stushnoff and Seufferheld 1995). Most of the temperate fruit species are genetically heterozygous and vegetatively propagated. Unique heterozygotic individuals which have been identified and selected for their special combination of genetic attributes cannot be regenerated by seed. Such genetic resources are maintained in the field as active collections, where the accessions are available for comprehensive characterization, evaluation, and distribution. An active collection provides breeders immediate access to flowering plants, and their phenotypic response to environmental fluctuations can be observed and monitored regularly. However, there are several disadvantages that limit the efficiency and threaten the security of active collections. Active collections are exposed to pests, diseases, and natural abiotic hazards and require considerable inputs, in form of land, labor, management, and materials that limit their capacity to ensure the maintenance of the diversity present in a species (Engelmann and Engels 2002). Despite the labor and operating costs, which include large areas of land, planting and pruning operations, and weed and pest management, conservation programs for woody fruit species rely on clonal orchards (Panis and Lambardi 2005). Periodic and careful

monitoring of the preserved trees is essential, particularly for diseases such as phytoplasmas, viruses, and bacteria. Many fruit diseases are difficult to eradicate, and the majority are listed as quarantine pests in the A2 list of the European Plant Protection Organization (EPPO 2016; https://www.eppo.int/QUARANTINE/listA2.htm).

Fragaria sp. field plantings have specific challenges, such as the need to regularly monitor runners spreading between different accessions and the threat of naturally occurring viruses, which cause periodic replanting (Maas 1998). Ideally, *Fragaria* sp. germplasm should be stored as potted plants under insect-proof screens with an active integrated pest management program to reduce the risk of virus contamination (Hummer 1991). Flowers and fruits must be removed from plants grown under a screen, and active field collections must be established from virus-free plant material for characterization and evaluation for breeding purposes. Reserve plant material, separate from the active collections, are needed to provide security in case of a disease or environmental disaster. A safety backup collection comprises accessions of an active collection at a different location (Engels and Visser 2003), such as being maintained at a second field site or in greenhouses, or held as *in vitro* cultures in the laboratory. Presently, biotechnology offers a broad range of techniques for such collections.

In vitro gene banks currently provide alternative short- and medium-term storage for a number of crops (Reed 2002), including temperate fruit trees and horticultural species (Hao and Deng 2003; Lambardi *et al.* 2006; Kovalchuk *et al.* 2009; Höfer 2011). However, these *in vitro* gene banks are not ideal for long-term storage of a collection, as the plantlets require repropagation, at intervals that depend on the method used, and may be lost due to contamination or technical difficulties. Cryopreservation is the preferred option for long-term storage of clonal germplasm, because this method requires minimum space, labor, culture medium, and maintenance (Engelmann 2000), and is now applied to a diverse range of plants (Reed 2008).

Benelli *et al.* (2013) reviewed the advances made over the last decade in cryopreservation of economically important, vegetatively propagated fruit trees. Cryopreservation protocols have been established using both dormant buds sampled from field-grown plants and shoot tips sampled from *in vitro* plantlets. Cryopreservation of dormant winter buds is of increasing interest for the conservation of genetic resources of woody plants, particularly fruit trees (Stushnoff and Seufferheld 1995; Forsline *et al.* 1998a, b; Towill *et al.* 2004; Volk *et al.* 2008). The technique is an alternative to other *in vitro* procedures and does not require *in vitro* laboratory support. Additionally, plants can be regenerated to flowering in a relatively short interval. Dormancy, the quiescent status of tissues and organs, is useful for cryopreservation, since physiologically it often involves development of cold-hardiness. Dormant winter buds survive the exposure to liquid nitrogen by first being slowly cooled down to −30°C, allowing the freezable water to move from inside the cells to the extracellular spaces, thus minimizing the damage caused by ice crystallization (Vertucci and Stushnoff 1992). Recent studies on *Fragaria* sp. cryopreservation were reviewed by Reed (2008) and Höfer (2016). Successful cryopreservation protocols for strawberry cultivars have used variations of plant vitrification solution 2 (PVS2) and encapsulation dehydration or encapsulation-vitrification.

Cryopreservation could serve as a long-term storage solution for reserve germplasm collections of fruit species; however, it will not replace the traditional *in situ* and *ex situ* approaches. A combination of *ex situ* conservation and cryopreservation has the potential to improve fruit tree germplasm conservation (Benelli *et al.* 2013) and to foster conservation of additional valuable germplasm.

Fruit genetic resources in Germany For many centuries, fruit crops were mainly cultivated in the abbeys, house gardens, and small farms of Central Europe (Morgan and Richards 1993), but today, fruit production is mostly in highly specialized farms, which has led to a dramatic loss of diversity in the forms of traditional and locally adapted cultivars (Way *et al.* 1990). The global development of agricultural production has resulted in a worldwide loss of biodiversity, a problem that was first addressed by the UN when the Convention on Biological Diversity (1992; https://www.cbd.int/convention/) was passed in 1992. In 1996, the "Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture" was adopted (1996; ftp://ftp.fao.org/docrep/fao/meeting/015/aj631e.pdf). Based on this, the German "National Program for Genetic Resources of Agricultural and Horticultural Plants" was published in 2002 and updated in 2012, providing the foundation for long-term conservation, evaluation, utilization, research, and development of agricultural and horticultural species in Germany (2012; http://www.bmel.de/SharedDocs/Downloads/EN/Publications/FachprogrammPflanzenRessourcen.pdf?__blob=publicationFile).

The conservation of fruit cultivars has a century-long tradition in Germany. Since the early twentieth century, numerous fruit crop cultivars have been maintained in public and private germplasm collections (Flachowsky and Höfer 2010). The current gene bank at the Julius Kühn Institute, Institute for Breeding Research on Fruit Crops, in Dresden-Pillnitz, Germany, focuses on fruit species native to Central Europe and on species which are important for fruit production in Germany in the present as well as in the past (Hanke *et al.* 2014). This gene bank contains approximately 4500 accessions and is the largest collection for fruit genetic resources in Germany. There are numerous other collections in Germany that belong to universities, other governmental institutions,

districts and communes, non-governmental organizations, and private individuals (Flachowsky and Höfer 2010). However, the risk of losing individual cultivars cannot be fully mitigated using a decentralized, uncoordinated strategy for conservation. Some cultivars are present in several collections whereas others are present in only one or a few collections. Therefore, at the national level the German Fruit Genebank (GFG) has been established to minimize the risk of losing fruit genetic resources (Flachowsky and Hanke 2011). The GFG is a decentralized gene bank that aims to coordinate the germplasm collections in Germany by utilizing existing structures, capacities, personnel, and financial resources as efficiently as possible and is organized into species-specific networks (e.g., apple network, strawberry network; etc. Fig. 1) by a coordination center at the Julius Kühn Institute. The coordination center is also responsible for supervising the national database of fruit genetic resources (http://www.deutsche-genbank-obst.de/). The GFG is part of the "National Program for Genetic Resources of Agricultural and Horticultural Crops" of Germany and contributes to an internationally coordinated strategy for conservation of fruit genetic resources in Europe.

The conservation strategy of the GFG requires that each selected cultivar be duplicated within the established fruit-specific network. The selected cultivars include German cultivars; cultivars with a cultural, local, or historical relationship to Germany; and cultivars with pomological traits that may be of interest for breeding purposes. Currently, six fruit species-specific networks exist: the apple network, with nine partner collection sites; the cherry and plum (*Prunus* spp.) network, each with seven collections; the *Rubus* network, with three collections; and the strawberry network, with only two

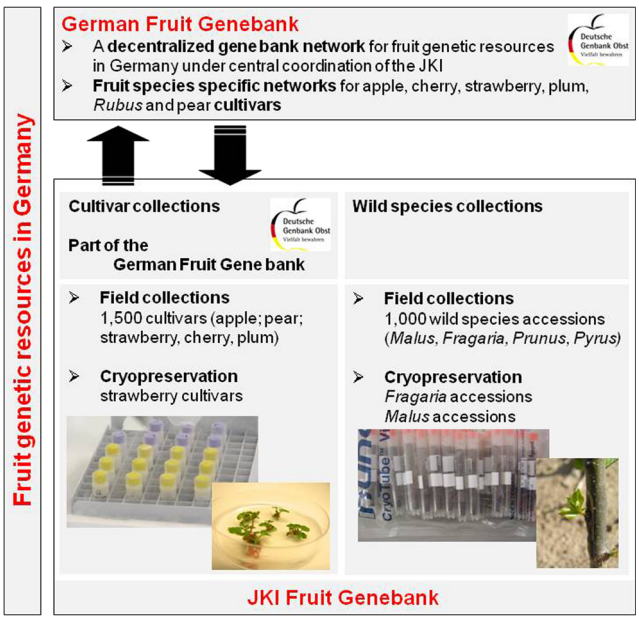

Figure 1. Schematic representation of the conservation strategy that includes cryopreservation for fruit genetic resources in Germany.

collections. The pear (*Pyrus* spp.) network was just started in 2017. Based on assessments performed by several institutions in Germany, currently 742 apple, 284 cherry, 209 plum, 202 strawberry, and 42 *Rubus* cultivars were selected for the GFG.

In addition to the cultivar collections, there are five collections of wild species from a range of genera held in the fruit gene bank in Dresden-Pillnitz, Germany. Special emphasis is on the genera *Malus* and *Fragaria*, the largest collections of these genera in Europe. The clonal *Malus* wild species collection comprises 518 accessions representing 46 species. Each accession was planted as two replicates on seedling rootstocks in the orchard in Dresden-Pillnitz. Most plants were collected decades ago from exchanges between arboreta, with 215 accessions obtained from the historical collection of the former Biological Central Center at Naumburg, Germany. The *Malus* material was expanded substantially through expeditions into the centers of origin (Hanke *et al.* 2012; Höfer *et al.* 2013). Although many of the *Malus* species are restricted to only one or a few accessions, nearly all botanical species of the genus are represented (Höfer *et al.* 2014). The *Fragaria* collection consists of 266 accessions representing 22 species and hybrids, maintained as potted plants in open fields and a partially replication in screen houses for virus-free plants. The material, including selections of cultivars, old landraces, and subspecies, is important not only from the point of view of taxonomy and phylogeny but also with regard to storing the breeding potential necessary for commercial exploitation of strawberries.

According to the conservation strategy of the GFG, each cultivar is to be conserved at no fewer than two field collections in the frame of the established fruit-specific network. Budwood exchange is organized after trueness-to-type evaluation, based on pomological and molecular characters. There are only two strawberry field collections in the network of the German strawberry gene bank. High operating costs and budget limitations for field collections do not allow further duplication of the *Fragaria* field collections. The effort required to establish and maintain an entire duplicate *Fragaria* collection under *in vitro* cold storage conditions has been calculated to be too labor intensive (Höfer 2011).

Wild species accessions of the various fruit species are not included in the GFG. Single accessions of wild *Malus*, *Pyrus*, *Prunus*, or *Sorbus* species can be found in the inventories of botanical gardens in Germany. However, duplication of the whole collection, especially for *Malus*, at a second field site is not a realistic solution. While accessions of *Malus* species can be duplicated in seed collections, a seed collection will not represent the true genotypes of clonal, heterozygous accessions. Additionally, growing out genotypes from seed takes too long, as field gene banks are used intensively in breeding and molecular biology and need to be available as living trees.

The aim of this study was to develop an efficient complementary conservation method for fruit germplasm, not only for the cultivar collections held at the GFG but also for wild

species accessions, with special emphasis on *Malus* spp. and *Fragaria* spp. within the Fruit Genebank of the Julius Kühn Institute (Fig. 1). The International Treaty on Plant Genetic Resources recognized the importance of apple and strawberry, denoting both as Annex 1 crops (2009; http://www.fao.org/3/a-i0510e.pdf). The present work describes the use of cryopreservation as part of the conservation strategy for fruit germplasm, specifically *Malus* spp. and *Fragaria* spp. Cryopreservation, as a complementary technique for conservation, provides a real guarantee against accidental loss of fruit genetic resources. A wider application of plant cryopreservation depends on the availability of efficient and reproducible protocols applicable to many different plant species (Panis and Lambardi 2005).

Material and Methods

Plant material All material was collected at the Fruit Genebank at the Institute for Breeding Research on Fruit Crops, Dresden-Pillnitz. The *Fragaria* collection consists of 266 wild species accessions and 193 cultivars and is maintained as potted plants with at least three plants under open field conditions and part of the collection in the screen house as virus-free plants (72 cultivars with two plants).

Shoot tips of 194 strawberry genotypes were cryopreserved for 8 y. The original donor material for the experiments was well-established *in vitro* cultures (Höfer and Reed 2010). Murashige and Skoog medium (MS; Duchefa, Haarlem, Netherlands; Murashige and Skoog 1962) supplemented with 0.44 μM 6-benzylaminopurine (BAP; SERVA, Heidelberg, Germany), 0.049 μM indole-3-butyric acid (IBA; SERVA), 30 g L^{-1} sucrose (Raffinade Zucker, EU-Quality, Mannheim, Germany), and 0.75% (*w/v*) agar (Difco™ Agar granulated, BD, Franklin Lake, NJ) was used for stock plant multiplication in 200-mL glass jars (40 mL per jar; WeckGläser, Wehr, Germany). Growth room conditions were 23°C with a 16-h photoperiod under 60–65 μmol m^{-2} s^{-1} photon flux (Osram L 36W/840 Cool White Lumilux, Hecker's Sohn, Dresden, Germany). Apical shoot tips (1–2 mm in length) were excised from 3- to 4-wk-old *in vitro* plants for further cryopreservation.

For *Malus* wild species, budwood (app. 50 cm) of the current season's growth was cut. Altogether, 63 accessions were cryopreserved for 4 y. Budwood was collected when the outdoor temperature was between 0 and −5°C for, at least, 72 h. The budwood was stored in plastic bags at −5 ± 1°C for a minimum of 5 d.

Cryopreservation Based on preliminary experiments in cryopreservation of strawberry (Höfer and Reed 2010) and apple (Höfer 2007), the focus of this research was to establish a technology that can be used for a large assortment of genotypes.

For *Fragaria*, the PVS2 vitrification method with cold acclimation was used again (Höfer 2016). After the last *in vitro* subculture, 2-wk-old shoots were cold acclimated for 14 d (16 h of darkness at −1°C and 8 h of light at 22°C; the same light intensity mentioned above). Shoot tips were then dissected and cultured on MS medium (Duchefa) supplemented with 5% (*v/v*) dimethyl sulfoxide (DMSO; neoLab, Heidelberg, Germany) and 1 g of additional agar (0.85% [*w/v*]; Difco™ Agar granulated, BD) for 2 d under cold acclimation conditions. Subsequently, the shoot tips were incubated in loading solution (2 M glycerol [neoLab] and 0.5 M sucrose [Grade II, Sigma-Aldrich®, München, Germany] in MS medium) for 15 min at room temperature (23°C) and finally transferred to 1.8-mL cryovials (Nunc™, Thermo Fisher Scientific®, Roskilde, Denmark). The explants were incubated on ice for 2.5 h with 0.75 mL PVS2 solution (30% [*w/v*] glycerol (neoLab), 15% [*w/v*] ethylene glycol (neoLab), 15% [*w/v*] DMSO (neoLab), 0.4 M sucrose [Sucrose Grade II, Sigma-Aldrich®]) in MS medium (Sakai *et al.* 1990). Finally, the cryovials were plunged directly into liquid nitrogen (LN) in a cryotank (BOSAFE, Messer Cryotherm, Kirchen, Germany). In order to test the recovery of the plant material stored in LN, the vials were rewarmed by plunging into sterile water (40°C) for 2 min after a minimum of 1 d of storage. After rewarming, the PVS2 was partly drained and replaced twice by 1.2 M sucrose (Sucrose Grade II, Sigma-Aldrich®) at 25°C. Subsequently, the shoot tips were transferred to Petri dishes (Ø 55 cm, Carl Roth, Karlsruhe, Germany) containing proliferation medium. For the first passage, a modified MS medium was used (Reed 2004) that contained MS salts and vitamins, 0.44 μM BAP (SERVA), 0.029 μM gibberellic acid (GA3; SERVA), 5.71 μM indole-3-acetic acid (IAA; SERVA), 170 mg L^{-1} NaH_2PO_4, (Carl Roth), 80 mg L^{-1} adenine sulfate (SERVA), 30 g L^{-1} sucrose (Sucrose Grade II, Sigma-Aldrich®), 3 g L^{-1} agar (Difco™ Agar granulated, BD), and 1.45 g L^{-1} Gelrite® (Carl Roth). Shoot tips in Petri dishes on proliferation medium were moved to the growth room for 1 wk in darkness, and then moved into the light conditions described above. Assessments of the recovery of the shoot tips were done 9 wk after rewarming.

For *Malus* sp., a protocol for cryopreservation of dormant apple buds was modified (Höfer 2015) from one developed at Fort Collins, Colorado (Forsline *et al.* 1998a, b). Stem sections 35 mm long, with only one bud, in the middle, were cut from dormant scions. These sections were dehydrated to 30% moisture on large-mesh, metallic trays in a −5°C cold chamber. The percent moisture was determined by gravimetric measurement. Two or three sections were then placed in 4.5-mL cryovials (Nunc™, Thermo Fisher Scientific®) in a controlled-rate temperature reduction freezer (Kryo 360-3.3, Messer Cryotherm) using the freezing protocol of 1°C h^{-1} to −30°C. After holding at −30°C for 24 h, the cryovials were transferred into cryoboxes and stored for 2 mo in the vapor

phase over LN. For recovery, vials were rewarmed to +4°C in a refrigerator for 24 h. For rehydration, the sections from each vial were placed separately in trays of wet, autoclaved sand at 4°C for 15 d. The chip budding technique (double-budded) was used to graft the sections onto 1-y-old M9 apple rootstocks planted in the orchard. Recovery data was taken 5 mo after grafting.

Statistical analysis The mean recovery rate and its standard deviation were calculated across the accessions of each species (Microsoft Office Excel 2007). In addition, the cumulative number of long-term cryopreserved *Fragaria* accessions were calcualted per year.

For each of the 194 *Fragaria* genotypes, 40 shoot tips underwent cryopreservation, 20 for long-term storage and 20 for recovery testing. Each vial was filled with five shoot tips; therefore, the long-term samples for each genotype were contained in four vials.

For the 63 *Malus* genotypes, 50 single-node sections were regularly used as a representative sample per genotype; of these, 30 sections were held for long-term storage and 20 sections were processed for recovery tests. Each vial was filled with two or three dormant buds, depending on the diameter of the genotype; therefore, the long-term samples of a genotype were contained by 10–15 vials.

Results and Discussion

***Fragaria* germplasm** To comprehensively screen the response of a large number of genotypes to cryopreservation, a method was selected based on the results of previous experiments which had compared four distinct cryopreservation protocols (two protocols using PVS2, encapsulation dehydration, and controlled rate cooling) (Höfer and Reed 2010). The chosen method was a PVS2 vitrification protocol that included DMSO pretreatment and a 14-d-long cold acclimation based on alternating day/night temperatures of 16 h at 1°C and 8 h at 22°C. This method was used to establish duplicates of accessions from the *Fragaria* collection (Höfer 2016). A minimum recovery of 40% was determined as the cutoff for successful storage of a given accession (Reed 2001). This optimized protocol was applied to additional genotypes, thus increasing the number of accessions in the long-term cryopreservation collection to 147 (Fig. 2). The cryobank is a third part of the National German Strawberry Genebank in addition to the two field collections located at different sites. Altogether, 79% of the 202 selected strawberry accessions are now conserved in both the traditional *ex situ* and the cryopreserved collection. The state-of-the-art (cultivars in cryocollection in JKI) can be reviewed at the website http://www.deutsche-genbank-obst.de/sammlung/index. The National German Strawberry Genebank holds some species selections, such as *Fragaria moschata* 'Capron' or 'Profumata di Tortona' and *F. vesca* 'Gartenfreude' or 'Ronja'. In addition, 47 accessions of wild *Fragaria* species are currently in long-term cryopreservation (Fig. 2), representing 18% of the *Fragaria* wild species collection, which consists of 22 species and their hybrids. Since the goal was to develop a system of methods that would work for the huge range of genotypes present in the gene bank, the data on recovery after cryopreservation were summarized as mean of the species (Table 1). A high recovery rate was achieved for all cultivars of *F.* × *ananassa* and all the wild *Fragaria* species (Table 1). The desired minimum recovery rate of 40% (Reed 2001) was achieved for all accessions, with most at >80% recovery. After recovery, plantlets with normal shoot formation were regenerated from shoot apices and were successfully transferred to soil in pots with no abnormalities observed. Some accessions have already been transferred back to the active collection.

There are several key factors for enhancing regrowth following cryopreservation. High survival of *in vitro*-grown material is determined not only by the cryogenic protocol itself but also by the physiological conditions of the material to be cryopreserved, such as growth stage, tissue size, and preculture conditions. The final protocol chosen included optimizations of a number of these factors (Höfer 2016). The optimal strawberry meristem, as described by Niino *et al.* (2003), has an apical dome fully covered by one to two leaf primordia, and the leaf bases are expanded. The leaf structure around the meristem acts to protect it from direct damage by phytotoxic substances and handling. Conditioning of donor plants through cold acclimation, sugar treatment, or a preculture of the dissected shoot tips on medium with sugar or DMSO improves dehydration tolerance (Reed 2008; Keller *et al.* 2013). Strawberry cryopreservation protocols using *in vitro* plants have used cold acclimation in different ways (Navatel and Capron 1997; Hirai *et al.* 1998; Clavero-Ramirez *et al.* 2005), i.e., as a preculture of the dissected shoot tips (Zhao *et al.* 2006; Pinker *et al.* 2009) or as a combination of preculture of both the donor plants and the dissected shoot tips (Niino *et al.* 2003). Reed and Hummer (1995) described alternating-temperature cold acclimation, with 22°C days and −1°C nights, combined with preculture of the shoot tips with 5% (*v/v*) DMSO. The present results also showed improved recovery following alternating-temperature cold acclimation combined with preculture of both donor plant and dissected shoot tips.

An effective osmoprotective treatment appears to be essential for a high recovery rate after cryopreservation. For the present data, the loading procedure was 2 M glycerol and 0.5 M sucrose for 15 min at room temperature (23°C) followed by 2.5 h of treatment with PVS2 on ice. The duration of the PVS2 treatment was extended because a lower temperature was used compared to the literature (Niino *et al.* 2003; Pinker *et al.* 2009; Yamamoto *et al.* 2012). The extended

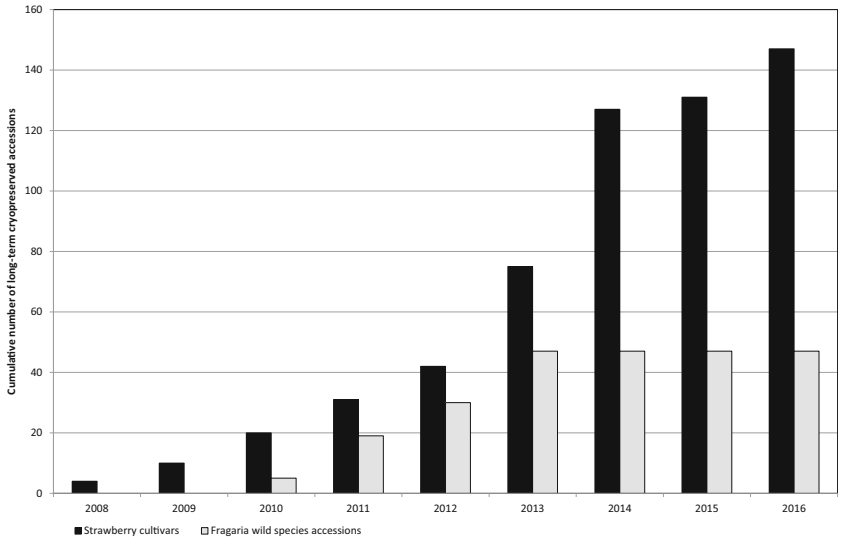

Figure 2. Cumulative number of long-term cryopreserved *Fragaria* accessions calculated per year.

PVS2 vitrification on ice and the alternating-temperature cold acclimation of the *in vitro* plants and of the isolated shoot tips were likely key factors in the successful recovery (Table 1). The post-thaw recovery medium is also important in a cryopreservation protocol. Although no comparisons were made, the modified MS medium may also have improved regrowth.

Table 1. Recovery after cryopreservation using PVS2 vitrification with cold acclimation, calculated across the *Fragaria* accessions tested for each species or hybrid

Species	No. accessions	Recovery (%)	SD
Fragaria bucharica	3	80.00	5.00
Fragaria chiloensis	5	82.89	13.90
Fragaria corymbosa	2	86.88	9.72
Fragaria gracilis	2	80.00	0.00
Fragaria iinumae	2	90.00	7.07
Fragaria mandshurica	2	95.00	7.07
Fragaria mosch. × *Fragaria viridis*	1	100.00	0.00
Fragaria moschata	6	85.00	12.65
Fragaria moupinensis	1	100.00	0.00
Fragaria nilgerrensis	2	75.84	12.96
Fragaria nipponica	2	92.50	10.61
Fragaria nubicola	2	80.60	6.23
Fragaria orientalis	2	100.00	0.00
Fragaria pentaphylla	2	82.50	10.61
Fragaria species	1	84.21	0.00
Fragaria tibetica	2	95.00	7.07
Fragaria vesca	18	80.78	17.97
Fragaria virginiana	4	88.75	10.31
Fragaria viridis	1	80.00	0.00
Fragaria × *ananassa*	128	87.31	14.10
Fragaria × *ananassa spp. cun.*	2	77.50	24.75
Fragaria × *bifera*	2	75.00	21.21
Fragaria yezoensis	2	80.00	0.00
Mean		86.27	13.92

Twenty shoot tips were screened for recovery for each accession ($n = 20$) after 1 d of storage in liquid nitrogen

There are only a few papers dealing with cryopreservation of a large number of strawberry accessions. Reed and Hummer (1995) reported cryopreservation of 56 strawberry accessions using cold acclimation and controlled rate cooling (0–100% recovery), while Yamamoto *et al.* (2012) established a vitrification method using aluminum-covered cryoplates for 15 strawberry cultivars, achieving 70–97% recovery. Okuno *et al.* (2005) mentioned the cryopreservation of about 60 strawberry accessions. The successive development of the cryobank of strawberry in the Institute for Breeding Research on Fruit Crops in Dresden was described in former articles (Höfer and Reed 2010; Höfer 2016). In the current study, long-term cryopreservation was finally initiated for 194 *Fragaria* genotypes, and the recovery tests revealed an average regrowth rate of 86% (Table 1). The accessions successfully cryopreserved represent four ploidy levels. The developed protocol is highly reproducible, efficient for a diverse set of accessions, and suitable for further genotypes.

A long-term study with selected genotypes was also initiated to investigate the recovery of plantlets after a longer duration of shoot tip cryopreservation. The literature indicates that it may be feasible to cryopreserve strawberry shoot tips for 28 y with no decrease in the viability of the meristems (Caswell and Kartha 2009).

***Malus* germplasm** According to the conservation strategy of the GFG, each cultivar will be maintained in duplicate field collection sites. Cultivars selected for duplication have to be evaluated for trueness to type based on pomological and molecular characters. Budwood of all true-to-type apple cultivars in the GFG are to be exchanged between the nine partner

collections; wild accessions generally are not included and are to be duplicated by means of cryopreservation. Previous experiments (Höfer 2007) optimized the original protocol of Forsline *et al.* (1998a, b) under the mild-winter conditions of Central Europe using a wide range of *Malus* sp. genetic material for a 4-y project and established a method usable for a wide range of *Malus* genotypes (Höfer 2015). The recovery after cryopreservation was summarized (Table 2). For *Malus* spp., the average recovery rates of long-term cryopreserved dormant buds, as a mean of each species, was 48.3%, just higher than the minimum allowable recovery rate of 40% (Reed 2001). Of the accessions cryopreserved in these experiments, only 36 accessions (56%) met this minimum standard, yet these 36 accessions had a mean recovery of 72%. In the previous studies, a relationship of viability after cryopreservation to taxonomic groups was discussed, but a large amount of variability in the *Malus* sections was also observed (Höfer 2015). Even when only a few accessions were tested for each species, high variability, correlated with a standard deviation of >35%, was seen within the species *M. transitoria*, *M. hupehensis*, and *M. floribunda* (Table 2).

Table 2. Recovery rate across a range of *Malus* genotypes after cryopreservation of dormant bud explants, calculated across the accessions tested for each species

Species	No. accessions	Recovery (%)	SD
Malus yunnanensis	1	10.00	0.00
Malus transitoria	2	30.00	35.36
Malus toringoides	3	22.60	15.33
Malus sylvestris	4	42.50	33.04
Malus spectabilis	2	5.00	0.00
Malus sikkimensis	1	22.20	0.00
Malus sieversii	3	56.67	27.54
Malus sieboldii	3	47.97	29.41
Malus sargenti	3	16.50	19.40
Malus prunifolia	3	81.67	23.63
Malus pratii	1	50.00	0.00
Malus orientalis	4	44.60	30.04
Malus komarovii	3	30.00	30.14
Malus ioensis	2	50.00	14.14
Malus hybr.	1	45.00	0.00
Malus hupehensis	2	38.60	47.52
Malus fusca	1	22.20	0.00
Malus floribunda	3	47.00	35.59
Malus florentina	2	14.05	11.53
Malus domestica	1	100.00	0.00
Malus coronaria	2	52.50	17.68
Malus baccata	3	96.70	5.77
Malus × *zumi*	4	46.25	30.38
Malus × *sublobata*	3	73.33	33.29
Malus × *soulardii*	1	95.00	0.00
Malus × *robusta*	3	65.47	26.99
Malus × *moerlandsii*	2	36.10	3.96
Malus × *dawsoniana*	1	88.90	0.00
Mean		48.35	31.28

Rootstock for chip budding M9. Twenty buds were used for recovery for each accession (n = 20)

Five steps are critical for successful cryopreservation of dormant apple buds (Stushnoff and Seufferheld 1995; Forsline *et al.* 1998a, b), namely, the collection of dormant buds in deep winter, suitable desiccation, cryopreservation technique, proper rewarming and rehydration, and successful grafting. Scion wood should be collected when the field temperature has been between 0 and −5°C for, at least, 72 h. While this is optimal for natural cold hardening, the climate in Central Europe does not provide these conditions. Therefore, in the current study, the branches were stored in plastic bags at −5 ± 1°C for a minimum of 5 d to improve their hardiness. This is consistent with the classic studies of Sakai (1966), who found that a temperature range of −3 to −5°C was the most effective temperature range for maximizing the frost hardiness of twigs. In the current studies, the three-step desiccation process—desiccation to 30% moisture content in a −5°C cold chamber, prefreezing using a controlled-rate freezer at 1°C h^{-1} to −30°C, and holding at −30°C for 24 h—provided an effective cold hardening. Vogiatzi *et al.* (2011) demonstrated that the −4°C desiccation step was obligatory and that a 24-h holding period at −30°C also improved recovery. In some *Malus* spp., desiccation prior to freezing was not necessary (Towill and Bonnart 2005.) Differences in the responses of dormant buds in these studies were likely due to the climatic differences, for the USA (New York) and Denmark, between continents and yearly fluctuations in temperatures at the field sites.

To test recovery rates, the cryopreserved tissues were warmed by transferring the vials to +4°C in a cold room for approximately 24 h before removing the sections from the vials and placing them in wet, autoclaved sand at 4°C for rehydration for 15 d. Slow rehydration and thawing of buds resulted in significantly better recovery than when buds were rapidly thawed (>5°C min^{-1}) (Grout *et al.* 2011). The length of the rehydration period was genotype-dependent (Vogiatzi *et al.* 2012), so a range of rehydration periods should be tested if diverse genotypes are being stored. At Dresden-Pillnitz, chip budding was performed in the orchard, as it was more practical, but was another modification compared to the literature mentioned above. Grafting can assess bud and cambium viability, both of which are needed to provide the graft union.

The genotype and its response to environmental conditions are also important factors that may influence the recovery of the dormant bud after cryopreservation. The percentage of the 1915 *Malus* accessions that met the baseline of 40% recovery in the USDA-ARS National Center for Genetic Resources

Preservation, Fort Collins, CO (Towill *et al.* 2004), was much higher than the results of genotype screening in the current study (Table 2), possibly due to the colder climate in New York (gene bank where the material was collected) producing better cold hardening. Stushnoff (1987) determined that genetically hardy cultivars had a "wider window" for sampling date and water content for successful cryopreservation, while less hardy cultivars produced inconsistent results. Using this information, the dormant fruit crops were classified as tolerant, moderately tolerant, or sensitive in relation to their cold hardiness, dehydration tolerance, and ability to be cryopreserved (Stushnoff 1991). An attempt was made to relate cold-hardiness with cryopreservability of dormant *Malus* buds (tolerant, moderate tolerant, sensitive; Towill *et al.* 2004). Growing season and winter conditions prior to scion cutting did affect the amount and distribution of water within the bud tissue and impacted the recovery rate of dormant buds following cryopreservation (Vogiatzi *et al.* 2010). Bilavcik *et al.* (2015) demonstrated that cryosurvival is correlated with cold hardening without direct regard to the particular phase of dormancy. Successful recovery after cryopreservation may be related to higher survival rates of secondary buds, despite lethal injury to the primary meristems (Höfer 2015). This secondary bud effect was highly dependent on the year, but the other conditions influencing the positive reaction of the secondary buds are not known.

Despite these unresolved questions, an adapted protocol was established for comprehensive cryopreservation of dormant *Malus* buds under the climate condition of Central Europe. This protocol was used to duplicate wild *Malus* species accessions held exclusively at the Fruit Genebank Dresden-Pillnitz. Sixty-three wild *Malus* species accessions were long-term cryopreserved (Table 2) which represents 12% of the *Malus* wild species collection. Further comprehensive application of the technique, especially using recalcitrant genotypes, could lead to a better understanding of the mechanisms involved in the induction of tolerance to dehydration and cryopreservation in the frozen buds.

Accurate records are vital to a long-term cryogenic storage plan (Reed 2008). Storage records link the cryopreserved samples to all information related to the original plant (passport information) and to the detailed protocol used to generate the preserved material. Furthermore, the organization of the Dewar vacuum flasks where the collections is stored is maintained in a logbook.

Conclusion

Currently, the fruit genetic resources of Germany are primarily maintained in the field, as active collections of potted plants for *Fragaria* and of trees for pome and stone fruit species. The aim of this study was to improve cryopreservation protocols for strawberry and apple genetic resources, as backups for plants in active collections in order to provide security in case of a disease or environmental disaster.

The cultivars selected for the GFG are to be duplicated at a second site as field collections within the framework of established, decentralized fruit-specific networks. Wild species accessions of the various fruit species, with special emphasis on *Malus* and *Fragaria*, are held exclusively at the Fruit Genebank of the Institute for Breeding Research on Fruit Crops Dresden-Pillnitz. These wild accessions will be duplicated by means of cryopreservation. The strawberry cultivars are not duplicated at multiple sites because only two field collections exist in the German strawberry network. Cryopreservation is a third way to fulfill the conservation strategy.

In the current study, long-term cryopreservation was initiated for 194 *Fragaria* genotypes, belonging to 22 species and hybrids, with an average regrowth rate of 86%. The PVS2 vitrification method with a 14-d cold acclimation period was highly reproducible, and worked for a diverse set of accessions. This makes it perfect for duplicating both the selected cultivars from the GFG and the *Fragaria* wild species accessions.

For *Malus* spp., the general protocol was adapted to Central European weather and laboratory conditions. Yearly weather variations are common, requiring, at least, 2 y of testing for the selected *Malus* accessions. For accessions with recovery rates below 40%, other possibilities should be considered for future storage. When low recovery cannot be overcome, more buds of each accession can be stored so that enough will be viable after thawing to re-establish a genotype (Dussert *et al.* 2003). There are also several other cryopreservation techniques that might be employed for use with recalcitrant genotypes (Suzuki *et al.* 1997; Seufferheld and Stushnoff 1999; Kovalchuk *et al.* 2014). In the future, the dormant bud method should be used for establishing a long-term, duplicate *Malus* collection at the Institute for Breeding Research on Fruit Crops, as it is a space- and cost-efficient method compared to a second field collection.

With the information that is provided in this study, cryopreservation can now be realistically used for reserve collections of important and irreplaceable genetic resources. A combination of traditional *ex situ* conservation and cryopreservation has great potential to improve conservation of fruit tree germplasm.

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