DISSERTATION

CRYOPRESERVATION AND RECOVERY OF TEMPERATE FRUIT GERMPLASM USING DORMANT BUD TECHNOLOGY

Submitted by

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ABSTRACT

CRYOPRESERVATION AND RECOVERY OF TEMPERATE FRUIT GERMPLASM USING DORMANT BUD TECHNOLOGY

Cryopreservation of dormant budwood allows for the efficient processing and long-term storage of some temperate deciduous genetic resources and holds promise for the secure storage of countless others. The method is of particular interest because it is more cost effective than preservation using *in vitro* methods. By investigating the process of dormant bud cryopreservation reported in literature in conjunction with novel studies of pretreatment effects on regrowth after liquid nitrogen exposure and bud freeze resistance, critical factors affecting survival have been identified. These factors can be broadly classified into two categories; factors related to the condition of budwood before storage and factors impacting recovery after storage. Preconditioning treatments such as air drying to ~30% moisture content and slow cooling of dormant budwood before liquid nitrogen storage are among the most critical pre-storage factors for survival. Preconditioning treatments can also have a significant effect on increasing freeze resistance. Differential thermal analysis was used to investigate the pretreatment effects and identify the best preconditioning method for the cryostorage of peach dormant buds. Apple, apricot, cherry, peach and pear were used for cryorecovery studies but not tested by differential thermal analysis. Treatments tested include air desiccation of budwood to 30% moisture content, exposure to sucrose solution using concentration and exposure treatment levels selected for greatest increase in freeze resistance in pretrial experiments, and a combination treatment of sucrose solution exposure before air desiccation. An additional group of twig segments was not treated but

processed in the fresh state to compare treatment effects. Of the four pretreatment groups, desiccation alone had the greatest impact at increasing freeze resistance and cryosurvival in most species tested. A follow up experiment was conducted to investigate moisture content effect cryosurvival and freeze resistance in peach dormant buds. For all treatment levels tested, survival and shoot development were low. Best pre-storage moisture level in peach was higher than expected at 35% (fresh weight basis) suggesting desiccation sensitivity may be contributing to low cryosurvival. A linear relationship between dormant bud moisture content and LT50 was also observed in freeze resistance trials.

Posttreatment factors affecting growth and establishment include warming rate and recovery method. Recovery of cryostored dormant budwood can be accomplished by either grafting, *in vitro* culture, or direct rooting. In order to increase efficiently in storage and recovery, a new approach to recovery was tested; antimicrobial forced bud development followed by *in vitro* culture initiation using shoot tips of sprouted buds. This approach aims to combine the efficiency of forced bud development with secure establishment of *in vitro* shoot cultures after cryopreservation. Successful establishment of shoot cultures from dormant buds recovered using antimicrobial forced bud development before culture initiation was correlated with the development of shoots with leaves.

The earlier developmental stages were not significant for culture establishment. This method has successfully been applied to several fruit and nut species namely apple, apricot, cherry, peach, pear and little walnut and has the potential for use with many other species of deciduous species in need of clonal preservation. As a two-step process, antimicrobial forced bud development can be used as a standalone viability test, or when needed, used as a platform for shoot culture induction for the reestablishment of cryopreserved dormant bud germplasm.

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DEDICATION

This work is dedicated to Hannah Romo. Thank you for all you have done to encourage and support me through the years. I couldn't have done it without you.

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OVERVIEW

Cryopreservation of clonal plant germplasm is a reliable way to preserve important agronomic traits and protect against loss of crop genetic diversity of many horticultural species. Dormant bud cryopreservation techniques present an efficient alternative to the labor-intensive shoot tip cryopreservation process and may allow a single technician to preserve large quantities of germplasm in a season. This method of cryopreservation takes advantage of the natural dormancy in cold hardy crops, making it a viable technique only for deciduous trees and shrubs. Many factors must be considered when attempting to perform dormant bud research methods to applied-level germplasm preservation efforts. This process is necessarily a seasonal endeavor, which puts strain on labor and facilities particularly in winter. Integration of methods and procedures using different crop species or new equipment provides additional challenges that must be tested in advance. By identifying variables of dormant bud processing in cryopreservation literature, options emerge that allow for the modification of reported methods to work within the confines of institutional resources. Infrastructure, pre-processing, and recovery stages are discussed in terms of necessity and available alternatives to allow informed decision making in establishing an applied dormant budwood genebank.

INTRODUCTION

Germplasm conservation is essential to preserving the genetic diversity of cultivated and wild species. Clonal plant collections can successfully maintain specific genetic combinations of highly heterozygous crops, like fruit trees, that rely on asexual propagation. While clonal collections may be maintained in field plots, greenhouses, screenhouses, and *in vitro* culture labs, long-term preservation in cryogenic conditions provides an option that is less susceptible to environmental damage, more secure, and stable with very little maintenance (Engelmann 2004).

Dormant bud (DB) cryopreservation was developed as a technique for storing clonal germplasm of temperate, deciduous trees and shrubs beginning in the early 1960's (Sakai 1960). This approach relies on the naturally occurring state of dormancy. During dormancy, deciduous tree shoots enter arrested development, allowing them to withstand the unfavorable growing conditions of winter. Capitalizing on this natural cold hardy state, often when paired with certain pretreatment techniques, allows material to withstand the extreme cold of cryogenic storage (Stushnoff 1991). The ability of this approach to preserve genetic integrity makes it a promising option for preserving temperate clonal tree fruit cultivars (Choudhary et al. 2013; Yi et al. 2015).

The DB cryopreservation method utilizes material collected directly from the field and does not rely on tissue culture introduction and multiplication to provide the propagules that are cryopreserved. Furthermore, the cost of DB cryopreservation is ten times less than the cryopreservation of excised shoot tips from *in vitro* cultures (Jenderek et al. 2019). Additionally a study comparing cryopreservation of apple germplasm using DB and *in vitro* shoot tips found that

the DB takes about 40% of the time and 50% of the labor needed for in vitro shoot tips cryopreservation (Lambardi et al. 2011). As a result of the efficiency of DB cryopreservation, it has been studied in a variety of deciduous woody species, which have been reported to be amenable to the technique with varying degrees of success: almond (Choudhary et al. 2014), apple (Stushnoff 1987; Forsline et al. 1996b, c, 1998a; Wu et al. 2001; Towill and Bonnart 2005; Lambardi et al. 2008; Yi et al. 2015; Höfer 2015; Pathirana et al. 2018; Tanner et al. 2020), apricot (Tanner et al. 2020), ash (Volk et al. 2009), aspen (Aronen and Ryynänen 2014), birch (Ryynänen 1996), black walnut (Morrissey and Gustafson 1990; Jenderek et al. 2011b), blueberry (Jenderek et al. 2017), cottonwood and willow (Towill and Widrlechner 2004; Bonnart et al. 2014), currant (Rantala et al. 2019), elm (Harvengt et al. 2004), grape (Esensee et al. 1990; Forsline et al. 1996a), little walnut *Juglans microcarpa* (Tanner et al. 2020), mulberry (Niino 1995; Atmakuri et al. 2009; Fukui et al. 2011; Choudhary et al. 2013), pear (Oka et al. 1991; Reed et al. 1998; Zhumagulova et al. 2014; Tanner et al. 2020), peach (Tanner et al. 2020), peony (Seo et al. 2007), persimmon (Matsumoto et al. 2004, 2015; Ai and Luo 2005; Benelli et al. 2008) and sour cherry (Towill and Forsline 1999; Kovalchuk et al. 2014). Although the list of species reported to be cryopreserved as budwood is long, many species are reported to have low to no survival, especially with cold and drought sensitive individuals. Special attention should be given to reported recovery rates and number of reported accessions attempted for a given species to understand the general ease or difficulty of cryopreservation.

In addition, several informative and helpful reviews of cryopreservation methods have been published which include valuable information on the state of DB technologies (Stushnoff 1987; Reed 2001, 2002, 2004, 2008; Towill and Ellis 2008). Genebank standards for plant genetic resources provide an overview of available preservation methods on everything from germplasm

collection, characterization, and storage for seed pollen and clonal resources (Food and Agriculture Organization 2016). This review aims to provide information about the DB cryopreservation method with a focus on its many variables and permutations reported in literature to allow genebanks and germplasm managers to make informed decisions regarding the designation of critical resources.

INFRASTRUCTURE AND HUMAN RESOURCES

FACILITIES AND EQUIPMENT

Appropriate infrastructure is crucial to any endeavor to preserve critical germplasm. Since these collections are intended to be maintained indefinitely, a reliable source of funding is essential. Resources for processing DB minimally include tools to uniformly cut segments (bandsaw, pruners), balances to accurately weigh individual segments or batches of segments, as well as vessels and labels for storing material during processing such as trays or screens. Because maintaining dormancy is crucial, reduced-temperature storage facilities must be used to store and precondition material prior to cryopreservation. Cold storage allows for the processing of many accessions collected during peak mid-winter dormancy (Forsline et al. 1996c). Temperatures for housing DB before liquid nitrogen vapor (LNV) storage range from -5 ºC to 5 ºC in the literature (Forsline et al. 1998a; Fukui et al. 2011). Air flow and humidity control are essential features for cold rooms during desiccation steps, as humidity greatly influences budwood drying rate (Chrusciel et al. 1999). Liquid nitrogen (LN) compatible packaging and labels are also required for long-term storage. A programable freezer capable of reaching ≤-30 °C is usually needed

because a slow cooling step is used in most protocols (Tyler and Stushnoff 1988a; Vogiatzi et al. 2012). A less costly alternative to programmable freezers is the use of a non-programmable low temperature freezer to cool budwood step-wise by reducing the freezer set point by 5 ºC manually every 24 hours (Towill and Bonnart 2005). Additionally, it has been demonstrated that programmable freezers can be constructed by modifying domestic freezers for slow cooling budwood but requires some proficiency with electronics and programming (Pathirana et al. 2018).

Collections in long-term cryogenic storage require a permanent, dedicated space. Liquid nitrogen storage tanks must possess the capacity to support the needs of the individual program and may require periodic maintenance and/or replacement. The availability and efficient use of the storage space is an important consideration because samples intended for long-term storage will be occupying this space indefinitely. Storage of accessions as DB requires more space than excised meristem shoots because DB progagules are much larger. The needs of any cryopreservation storage operation balance preservation security with resource efficiency.

Cryopreserved DB are typically stored in LNV. Even though LNV storage requires less LN than collections stored in the liquid phase, a reliable supply of LN is needed. Many LN supply options are commercially available, ranging from delivery services to on-site generators that allow for in-house production.

Infrastructure for DB recovery depends upon the selected recovery method, from the ones that are discussed below in detail (as discussed in detail in the recovery section). A plant tissue culture laboratory is required for methods that make use of a tissue culture recovery system. A tissue culture laboratory will require facilities for media preparation, culture vessels, laminar flow hoods, scalpels and forceps, instrument sterilization equipment, an autoclave, and a growth chamber with lighting and temperature control. Healthy seedling or clonal rootstocks are necessary for recovery

methods utilizing grafting. A greenhouse, growth chamber, or field plot is needed to house rootstocks and recovering DB. Growth chambers and greenhouses are preferable, as the environment in the field plots are not controlled and may only be suitable for use during a small seasonal period when weather is conducive to regrowth.

Environmental monitoring systems are critical parts of infrastructure that protects sensitive processes, material, and the facility from quality-compromising situations by monitoring temperature, smoke, oxygen and other environmental sensors and alarming if abnormal conditions are detected (Biringer et al. 2013). This allows for unexpected environmental changes to be addressed before they can cause irreversible damage. Temperature monitoring is helpful to identify equipment failure and potential quality compromising situations in storing, desiccating, and slow cooling DB. Temperature monitoring systems are most useful if operating thresholds are predefined and set to alarm if conditions diverge in any step of the process. Fire, smoke, and water leak detection capabilities help to protect against catastrophic loss. Oxygen sensors inside the LN tank room are critical to protecting workers from asphyxiation by ensuring levels do not fall below 19.5% (Yanisko and Croll 2012). Duplication of critical infrastructure helps to ensure continued facility operation by avoiding service interruption. Duplicate electrical generators, LN supply tanks, cold rooms, and growth chambers are recommended for this reason.

Data management systems are essential for keeping track of germplasm information such as material identity, source location, collection and storage dates, cryoprocessing method, recovery techniques, estimated viability, quantity of units available for distribution, and storage location of material for each cryopreservation event. As the material will be stored for many years into the future, database information needs to be organized and complete to aid in recovery when needed. To ensure the integrity of all information associated with a germplasm collection, data must be

accurate, up-to-date, and backed up regularly across multiple servers. Additionally, digital color images of the budwood with ruler to indicate size of budwood is a simple but useful addition to database records.

HUMAN RESOURCES

Large-scale implementation of DB cryopreservation requires a skilled workforce. Technicians ideally need to have an understanding of plant physiology, horticulture, and botany. Expertise in plant physiology allows technicians to identify critical developmental challenges and to modify existing protocols to match specific crop requirements as necessary. For *in vitro* recovery methods, technicians must be proficient in media preparation and have experience with sterile culture techniques including surface sterilization, culture initiation/maintenance, and micropropagation. For graft recovery methods, technicians must have experience in the relevant grafting techniques and have shown reliable success maintaining rootstocks and grafting fresh untreated material.

Time management skills are required, as dormant budwood cryopreservation is a seasonal effort with several stages of processing. During the initial stages of large-scale DB processing, a greater commitment of labor is required to collect material from the field, cut, dry, assess the moisture content and package several accessions simultaneously without comprising bud dormancy.

SOURCE MATERIAL

SOURCE SELECTION

When selecting a source of material, considerations include preservation goals, the access to healthy plant material grown under climate and weather conditions that foster dormancy, and the availability of staff for collecting and preparing budwood. Location of source trees may have an effect on cold hardiness and cryo-recovery (Stushnoff and Junttila 1986; Toldam-Andersen et al. 2007; Jenderek et al. 2011a). It has been demonstrated in *Malus* DB cryopreservation that species with greater cold tolerance regrow in higher numbers compared to species with lower cold tolerance (Towill et al. 2004).

COLLECTION

Budwood used for cryopreservation is collected during mid-winter dormancy for optimal regrowth (Stushnoff 1987; Forsline et al. 1996c). During mid-winter dormancy, material has greater desiccation tolerance compared to material harvested at the onset of cold acclimation during the fall season (Tyler and Stushnoff 1988a; Vertucci and Stushnoff 1992). When buds deacclimate following chilling requirement completion due to warming temperatures in spring, bud growth resumes and buds become cold tender and susceptible to freeze damage (Sakai 1966; Arora and Taulavuori 2016). Twigs collected outside of mid-winter dormancy can be expected to have very low survival using the DB cryopreservation method. Such actively growing material may have better cryo-recovery using vitrification-based protocols (Seufferheld et al. 1992).

As weather plays a role in preconditioning material for DB cryopreservation, recovery results are variable from year to year (Jenderek et al. 2011a). To ensure maximum cold hardiness, twigs are collected during mid-winter dormancy after at least 72 hours (h) of exposure to an average air temperature of 0 °C or colder when possible (Forsline et al. 1998a). Cold hardening may not always possible, or be needed for apple cultivars from warm winter locations such as Mediterranean climates (Contaldo et al. 2018). In some cases, dormant twigs can be artificially acclimated by exposure to gradually cooler temperature under controlled laboratory conditions after twig harvest (Sakai 1966). If cultivar specific chilling requirements during dormancy are not satisfied, budwood may have difficulty developing shoots during recovery (Toldam-Andersen et al. 2007).

To collect budwood, twigs should be cut with a clean, sharp set of pruning shears (Figure 1.1a). To eliminate mechanical vectoring of viruses within a field collection, shears can be cleaned with a solution of sodium hypochlorite at a concentration of 0.5-1% by immersion of the blades for a few seconds (Ling 2017).

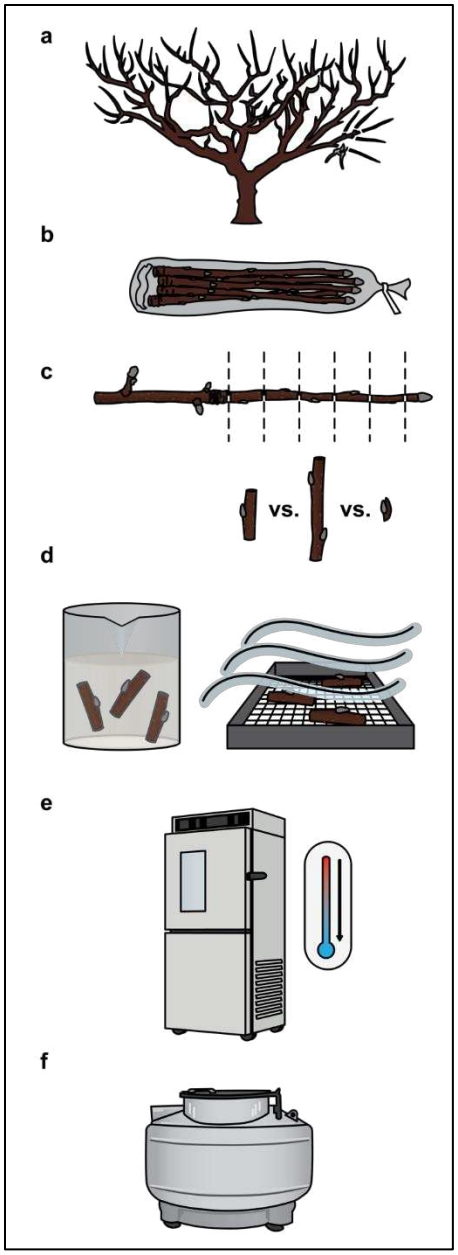

FIGURE 1.1. Dormant budwood cryopreservation processing steps. a. Budwood is collected from source tree during mid-winter dormancy, b. Twigs are sealed in plastic with identifying label ready for processing or shipment to processing location, c. Twigs are divided into individual segments or isolated bud chips; only previous season's growth is used, d. Segments of DB are preconditioned for storage by exposure to high osmotic solutions and/or reducing moisture content by dry air exposure inside a cold chamber, e. Segments of DB are slowly cooled at a controlled rate to a set prefreezing temperature and held for 24 hours, f. Segments of DB are transferred to liquid nitrogen tank for long-term storage.

Twigs are collected in enough quantity to securely preserve the accession (Volk et al. 2017). The United States Department of Agriculture - National Plant Germplasm System routinely stores 150 to 160 propagules per accession (Jenderek and Reed 2017). More material must be stored when post cryopreservation viability is expected to be low to protect against loss. Ideally, each dormant budwood container should have at least one viable twig segment and enough containers in storage to allow for several recovery events (Reed 2001). Based on the viability assessment of a subset of cryopreserved twig segments, the number of viable samples in storage can be estimated (Volk et al. 2017). Storing more samples of a single accession increases confidence that the accession is securely preserved, but also increases the amount of storage space needed. For species where expected regrowth is low after LN exposure, more samples may be required for storage (Reed 2001; Volk et al. 2017). Having an initial viability score of 35% or greater will ensure that at least one sample will be viable in every group of 10 samples stored, with a confidence level of 95% (Volk et al. 2017). For example, if 180 DB segments are placed into long-term storage and are estimated to have 35% viability or greater, at least 53 segments are estimated to be alive in storage.

SELECTION OF PLANT MATERIAL

Twigs selected for DB cryopreservation should ideally be healthy and come from source trees that are free from diseases (Reed 2008); avoid collecting material showing signs of insect damage or other biotic stress. Twigs selected from source trees are ideally uniform in size and diameter to allow for even desiccation between segments; this is also helpful for graft recovery, which requires using rootstocks of similar stem diameter to match scion budwood.

For DB cryopreservation to be successful, surviving vegetative buds must be able to develop into shoots after LN storage. Several fruit crops have compound or multiple buds per node. A compound bud contains multiple growing points inside a single bud structure. Compound buds of

mixed bud types will have floral and vegetative structures within the same node or bud (Figure 1.2). For example, mulberry and apple nodes include compound mixed buds, whereas peach nodes are composed of single vegetative and floral buds. Nodes of multiple or compound buds are acceptable for use in DB cryopreservation, as the vegetative structure is preserved. In species which have compound bud structures, growth from secondary buds may increase survival of twig segments when larger primary buds do not survive (Vogiatzi et al. 2010).

Knowledge of temperate fruit crop bearing habits and reproductive biology is necessary for proper protocol development and DB cryopreservation implementation. Floral and vegetative buds produced in separate structures are referred to as single bud types. These may be found clustered together at a node site (Figure 1.2b) as in peach shoots or spread apart at separate node sites as with blueberry shoots (Figure 1.2c, 1.2d). In species having single bud types like blueberry, it is necessary to avoid twig segments containing only floral buds; these usually occur on the terminal ends of twigs (Figure 1.2c). Single floral buds are visually distinct from single vegetative buds as they are much larger. Floral buds also develop on lateral spurs of two-year old twigs of apple (Figure 1.2d) and are not used for DB cryopreservation. Twigs from previous season's growth are best for cryopreservation.

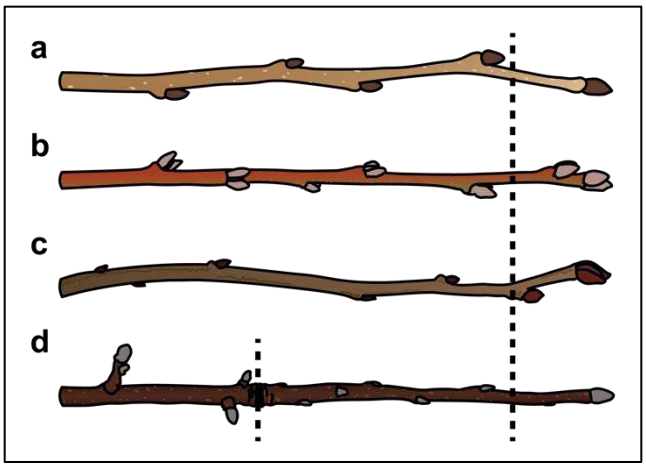

FIGURE 1.2. Dormant shoots of various species. Dotted lines represent separation between usable material, shoot tips, and two-year-old wood to be discarded. a. Mulberry shoot with compound buds, b. Peach shoot with a single vegetative bud located in between much larger single floral buds, c. Blueberry shoot with several small vegetative buds and a large single floral bud on the terminal position, d. Apple shoot showing spur buds on two-year-old wood to the left of the dotted line.

PACKAGING TWIGS FOR SHIPMENT OR HOLDING UNTIL CRYOSTORAGE

Once collected, twigs are organized into bundles, labeled, and packed into plastic bags (Figure 1.1b). These are kept in refrigerated conditions until processed or shipped to the processing location. Typical storage temperatures range from -5 to 3 ºC (Forsline et al. 1998b; Fukui et al. 2011). If shipping is needed, twigs can be carefully packed into cardboard a cardboard box, then sent using next day shipping to ensure quick delivery; this is the standard shipping method reported in literature(Tyler and Stushnoff 1988b; Forsline et al. 1998a; Volk et al. 2009; Bonnart et al. 2014). A battery-powered data logger can be included in the shipping box to track the temperature exposure of the material during transit. Additionally, shipping with gel-based cold packs inside insulated Styrofoam shipping boxes will keep budwood cool if high temperature is a concern during transit. Shipping arrangements need to consider holidays, weekends, and weather conditions to avoid unnecessary delays.

PRECONDITIONING

MATERIAL STORAGE AND PREPARATION

Once received, the twigs are unpacked and inventoried before being placed back into cold storage. Twigs are kept in sealed plastic bags in cold storage until processing. For apple buds, preprocessing storage temperature is reported at -3 to -5 °C (Forsline et al. 1998b), however temperatures as warm as 3 to 5 ºC have been used for mulberry(Fukui et al. 2011). Taking inventory of the twigs allows the material to be inspected a final time to ensure the absences of dead, damaged, or diseased twigs, all of which must be discarded. Proper twig storage and processing as soon as possible ensures that the quality of the budwood does not deteriorate; however, one study shows that apple DB can be stored up to six months without reduction in post-cryopreservation viability (Forsline et al. 1998b).

To begin processing, the material is typically cut (Figure 1.1c) and the apical shoot tip and base of each twig are discarded. Dormant twigs are then cut into segments of uniform length. Single-node twig segments of 3.5 cm are commonly used for species recovered by grafting, while longer segments containing multiple buds may be used for species recovered by direct rooting (Bonnart et al. 2014). Well-centered buds and uniform diameter are particularly important for even moisture loss during the desiccation step.

An alternative processing method is employed in the preservation of mulberry, which utilizes an isolated bud containing only a small cross section of connecting stem (Figure 1.1c) (Niino 1995; Atmakuri et al. 2009; Fukui et al. 2011). These bud chips are cut from twigs with a sharp knife or

scalpel and allowed to dry for a few hours before cryo-exposure. This method allows for quick processing and has an additional benefit of requiring less storage space compared to twig segments. A drawback of this method is that it requires the use of an *in vitro* system for recovery and it is reliant on surface disinfection and on recovery media that may not be optimized to the needs of all genotypes of a given species. *In vitro* micrografting is a promising approach to recovery which may address the issues of growing diverse genotypes on a single media. The *in vitro* micrograft recovery approach has been successfully implemented in citrus cryopreservation (Volk et al. 2015).

PRETREATMENT BY DESICCATION

DB-cryo regrowth is often increased when the moisture content (MC) of twig segments is reduced before exposure to LNV (Tyler and Stushnoff 1988a, b). Desiccation (Figure 1.1d) is typically performed under cold and dry conditions to maintain dormancy. This preconditioning step reduces the amount of water, thereby reducing potential cellular damage caused by freezing in subsequent steps. In most reports of successful DB cryopreservation, an initial MC of about 45-55% is reduced to 25-30% by monitoring weight loss of material (Forsline et al. 1996c; Towill and Forsline 1996; Forsline et al. 1998b; Volk et al. 2009; Vogiatzi et al. 2011c; Benelli et al. 2013; Bonnart et al. 2014). Tolerance to desiccation is highest during midwinter and may be optimized for a specific accession or species by identifying the threshold moisture level below which cellular damage occurs(Vertucci and Stushnoff 1992). Storage of budwood at MC below critical levels results in reduced regrowth post LN. Cryo-storage of DB with excess moisture reduces viability as cells are damaged by ice crystal formation that causes rupture of cell membranes. Damage caused by freezing may also result in the formation of cracks in bark and that may provide entry for invading pathogenic microorganisms during regrowth (Helton 1962). MC is established on a fresh weight

basis using the formula, [(FW-DW)/FW]*100, where FW is the fresh weight of a twig segment before drying, and DW is the weight of the same twig segment after oven drying (Towill and Ellis 2008). Oven drying is accomplished by placing individually weighed twig segments into an oven set to temperatures between 85 to 100 ºC to drive off moisture for three to four days. Oven drying time is complete when the weight of individual segments does not change with additional drying and may take longer for large diameter segments or cooler drying temperatures.

The time required to properly desiccate twig segments can range greatly from hours to several months depending on protocol and a number of variables: species, temperature, humidity, air movement, segment diameter and length, presence of residues, treatment coatings, and storage vessels presenting a physical barrier to moisture loss. Desiccation temperatures reported in the literature range from room temperature conditions for small bud chips for mulberry (Niino 1995) to below freezing temperatures for longer twig segments, such as -3 to -5 °C for apple (Forsline et al. 1998a). As relative humidity has an inverse relationship to temperature, humidity increases at colder temperatures compared to warmer conditions for the same amount of water vapor. This inverse relationship causes desiccation at lower temperatures to slow compared to warmer conditions, except where humidity inside the chamber is actively reduced by dehumidification systems. Humidity conditions during desiccation are rarely mentioned in literature but can directly affect desiccation rate (Chrusciel et al. 1999). Increased air movement inside the chamber will also aid in moisture loss. Longer, wider twig segments desiccate slower than short, thin segments (Tyler and Stushnoff 1988b).

The desiccation step may not be necessary for cryopreserving extremely cold-hardy genera and species such as Populus trichocarpa, Salix sp. and some cultivars of apple, provided that the material is slowly cooled (see section below) to -30 ºC prior to LN exposure (Towill and Bonnart

2005; Bonnart et al. 2014). Extremely cold-hardy genotypes can tolerate the stress of desiccation while less hardy cultivars may not survive cryogenic temperatures at all without it; therefore, most established protocols uniformly employ desiccation to achieve regrowth of many different genotypes within a species.

PRETREATMENT BY SOLUTION EXPOSURE

While most DB cryopreservation methods have shown success with desiccation prior LN exposure, there is also evidence to suggest that over-desiccation can cause damage that negatively impacts the post-cryo regrowth of DB (Seufferheld et al. 1999). One report showed that a sucrose-alginate treatment before the moisture reduction step could reduce desiccation damage and increase post-cryo regrowth in cold-tender genotypes of apple with traditionally low viability rates (Seufferheld et al. 1999). This treatment was accomplished by immersing twig segments in 5% medium viscosity alginic acid and 0.5 M sucrose before transferring segments individually to a solution of 100 mM CaCl2 and 0.5M sucrose at 0 ºC for 24 hours. Sucrose alginate encapsulated twig segments where then transferred to 0.7 M sucrose solution for 24 hours before exposure to 1.0 M sucrose and 0.2 M raffinose solution for 24 hours. Further research is needed to determine the usefulness of alginate-sucrose pretreatments in DB before applying this method to large-scale preservation efforts. A study using differential thermal analysis to quantify freeze resistance of sucrose pretreatments in peach found that while exposure to high osmotic concentrations increased freeze resistance compared to untreated controls, this effect was insignificant when compared to the much greater freeze resistance and post-cryo recovery imparted by desiccation alone (Chapter 2).

Treatment of DB by exposure to high osmotic solutions has been reported to be beneficial to cryo-regrowth when applied with and without prior artificial desiccation (Kovalchuk et al. 2014). This

approach aims to limit cellular damage by increasing cells osmotic concentrations using compatible osmolytes, such as sugars, to promote cellular vitrification over ice formation (Figure 1.1d). A study of pear DB cryopreservation found that treatment in PVS3 before exposure to LN was better than PVS2, PVS4, honey, 35% glycerol + 10% DMSO + 10% PEG-8000 + 0.4 M sucrose or 50% glycerol, 50% glucose in liquid MS medium with 0.4 M sucrose, pH 5.7(Zhumagulova et al. 2014). Solutions which promotion of vitrification have been highly successful in increasing cryosurvival but usually require buds be surface sterilized and precutltured using in vitro techniques before cryopreservation and recovery in tissue culture.

PACKAGING FOR CRYOSTORAGE

Dormant twig segments are quickly sealed inside a container to prevent further desiccation after any preconditioning steps and before LN exposure. Either cryovials with screw cap lids or plastic polyolefin tubing sealed with an impulse sealer have been used. The number of twig segments inside each container should be consistent. When loading containers, it is beneficial to divide the segments into groups of representative diameters to homogenize each subset and minimize recovery variability between tubes. Each tube must be labeled to uniquely identify the material inside. Labels need to be resistant to degradation when exposed to cryogenic temperatures. Labels may include barcodes, QR codes, or other scannable formats but should always include some identifying information on the label in addition to the code.

After labeling, tubes are organized and loaded into vessels, such as aluminum boxes with vent holes, that reduce potential temperature insulation during slow cooling and the subsequent transfer to LNV. It is crucial that these vessels are resistant to degradation in LN and do not significantly impede cooling and warming rates. If space allows, multiple accessions can be stored together

inside a box; however, this may increase the handling of stored tubes as well as the risk of rewarming material during inventory or material retrieval processes.

CRYOPRESERVATION

SLOW COOLING

Slow cooling tubes containing dormant twig segments (Figure 1.1e) to temperatures of -30 to -35 ºC, prior to LN exposure, greatly increases their post-cryo regrowth when compared to direct exposure without prior precooling (Tyler et al. 1988). The speed at which dormant twig segments are cooled affects the amount of freeze-induced desiccation that occurs within cells. The initial ice formation in vascular conduits and extracellular spaces causes a negative pressure gradient that pulls water out of cells. Fast cooling rates do not allow enough time for water to sufficiently migrate out of cells before freezing occurs, resulting in intracellular damage by ice crystal formation (Towill and Bonnart 2005).

Slow cooling protocols depend on the species, material moisture, and the capability of the facility's programmable freezer. While cooling rates reported in literature vary, they are generally around 1 to 2 ºC per h (Stushnoff 1987; Tyler and Stushnoff 1988b; Forsline et al. 1998b, a) or 5 ºC per day (Towill and Bonnart 2005; Volk et al. 2009; Bonnart et al. 2014). After slow cooling to -30 or -35 ºC, DB are typically held at this temperature for 24 h before transferred to LNV for long term storage. The most cold-hardy species may not require a holding period after slow cooling, but are not adversely affected by a holding period; for more cold sensitive species, this holding period significantly increases regrowth (Tyler and Stushnoff 1988b). There are many companies that

produce temperature controlled environmental test chambers for industrial uses such as testing critical equipment or predicting product shelf life (Waterman and Adami 2005; Jung 2012) that can be used for slow-cooling dormant budwood for cryopreservation. Options exist for chambers that are cooled using LN or advanced electric powered multistage refrigeration systems (Jung 2012).

STORAGE

After slow cooling and/or holding for 24 h at -30 to -35°C, material must be immediately transferred to LN temperatures without rewarming, either by way of a transfer tank or direct loading into long-term cryo-tank storage (Figure 1.1f). While the first reports of DB cryopreservation utilized direct immersion in LN (Sakai 1960; Sakai and Yoshida 1967; Sakai and Nishiyama 1978), subsequent protocols place material into LNV for storage (Tyler and Stushnoff 1988b; Niino 1995; Forsline et al. 1996c; Matsumoto et al. 2004; Towill and Bonnart 2005; Towill and Ellis 2008; Volk et al. 2009; Fukui et al. 2011; Jenderek et al. 2011b; Bonnart et al. 2014; Höfer 2015; Pathirana et al. 2018; Tanner et al. 2020). There may be no practical difference in storing DB in LN or its vapor phase as no comparison study of DB survival has been reported.

As cryopreserved accessions will be stored long term, it is critical to organize tanks to allow for the efficient retrieval of accessions when they are needed. Standardized labeling and organizational systems ensure order is maintained as cryogenic collections grow. If possible, it is beneficial to split the storage of an accession into two or more independent storage tanks to minimize loss due to tank mechanical failure. Duplicate collections housed in geographically separated locations provides even greater protection from catastrophic loss to a single storage location (Food and Agriculture Organization 2016). During storage, minimal sample handling will prevent rewarming.

LONGEVITY

Longevity of budwood is an important factor that affects curation decisions. No reduction of viability was observed after 4 to 11.5 years of apple and mulberry dormant budwood cryostorage (Forsline et al. 1998b; Volk et al. 2008; Fukui et al. 2011). More long-term studies across diverse taxa are needed to better understand how long DB remain alive in LN storage but, theoretically, material may be viable indefinitely (Engelmann 2004). A study of moisture content of apple DB during cryoprocessing showed that no moisture loss occurs when material was sealed in cryovials and slow cooled to -30 ºC or warmed after LNV exposure (Vogiatzi et al. 2012). However, desiccation could occur if there are cracks or incomplete seals in the DB cryostorage packaging. This could affect viability and longevity of the cryo-stored DB material.

REGENERATION OF CRYOPRESERVED MATERIAL

WARMING

After samples are retrieved from LN storage tanks, they must continue with the warming process. Rapid warming at temperatures of 38 °C may not be suitable for cryopreserved DB as it was observed to cause bark shattering in apple DB segments and reduced viability compared to gradual warming (Tyler and Stushnoff 1988b). Gradual warming is accomplished by transferring DB to a cold chamber at temperatures ranging from 2 to 5 °C for 16 to 24 h before recovery (Tyler et al. 1988; Tyler and Stushnoff 1988b; Volk et al. 2009; Bonnart et al. 2014). In mulberry, regrowth was observed to be higher for samples warmed at 25 °C for 24 h compared to samples at -1 °C for

24 h or 40 °C for 15 minutes (Matsumoto et al. 2004). In some cases, warmed DB samples may be rapidly shipped to a secondary location for regrowth assessments.

RECOVERY

Successful recovery of DB is marked by the formation of a complete plant having either a shoot and root system, or the ability to further multiply through micropropagation(Food and Agriculture Organization 2016). Grafting (Forsline et al. 1995, 1998b; Towill and Bonnart 2005; Grout et al. 2009; Atmakuri et al. 2009; Volk et al. 2009; Lambardi et al. 2011; Bonnart et al. 2014), direct rooting (Bonnart et al. 2014), *in vitro* recovery (Yakuwa and Oka 1988; Wu et al. 2001; Matsumoto et al. 2004, 2015; Lambardi et al. 2011; Choudhary et al. 2014; Rantala et al. 2019), and tissue culture of shoots from antimicrobial forced bud development (Tanner et al. 2020) are examples of established DB recovery systems. Selection of the appropriate recovery system is dependent upon the species, the availability of resources, and the desired use of the material.

GRAFTING

For graft recovery (Figure 1.3a), rootstocks are planted three to four weeks before scions will be grafted to allow stock plants to break dormancy and resume growth (Volk et al. 2009). After warming, twig segments can be rehydrated in moist peat moss for as long as 14–15 days at 4 °C(Forsline et al. 1998b; Volk et al. 2009). Chip-budding, similar to patch budding, is the grafting technique most often used for DB cryo-recovery (Forsline et al. 1998b). The success of this recovery method is dependent upon a number of factors that include rootstock compatibility with scion, rootstock health, environmental conditions, the skill of the grafter, and aftercare of trees(Jenderek and Reed 2017). The benefit of this recovery system is its ability to produce a tree

that can be grown to flowering phase in a relatively short period of time due to the lack of juvenility.

DIRECT ROOTING

Recovery by direct rooting (Figure 1.3b) is performed by dipping the basal end of the segment in commercially available formulations of indole-3-butyric acid and/or 1-naphthaleneacetic acid before planting in a soilless medium (Bonnart et al. 2014). Direct rooting of DB can also be accomplished without the application of rooting hormone, but root formation may be variable (Towill and Widrlechner 2004). This method is the simplest recovery system and is performed in either growth chamber or greenhouse conditions. While it is not suitable for most species, this technique holds promise for species that readily form adventitious roots and are extremely cold-hardy. After cryo-exposure in less cold-hardy species, the cambium tissue is generally damaged to a degree that it is no longer functional and direct rooting would not be possible.

SHOOT CULTURE

Surface disinfection and media must be optimized in advance for direct induction into tissue culture (Figure 1.3c). The growing medium should be tailored to the general needs of the species and work for a variety of different genotypes, both cultivated and wild, to allow for large scale implementation (Kim et al. 2006). As DB are sourced from field trees, they may have high rates of contamination and will require intense surface sterilization treatments to establish sterile cultures. While this recovery method may be more labor and resource intensive than others, it may be a desirable option when grafting is not possible. In addition, cryopreserved DB segments can be warmed and propagated throughout the year. Resulting plants can be easily multiplied through standard subculturing techniques.

FORCED BUD DEVELOPMENT -SHOOT CULTURE

Forced bud development (Figure 1.3d) may also be used as a recovery system, if followed by tissue culture induction in a two-step process. For this recovery method, warmed budwood sections are planted and maintained in high humidity growth conditions. Because forced shoots are not sterile, high contamination levels by microorganisms may preclude them from establishing sterile cultures, unless forcing solutions containing antimicrobial agents such as 8-hydroxyquinoline citrate are used in conjunction with this technique (Tanner et al. 2020). In this study shoots produced using antimicrobial forcing solution after cryopreservation were successfully brought into tissue culture using the aforementioned techniques. Successful shoot development has been accomplished in cryopreserved apple, pear, sweet cherry, , apricot, peach and little walnut using this method (Tanner et al. 2020).

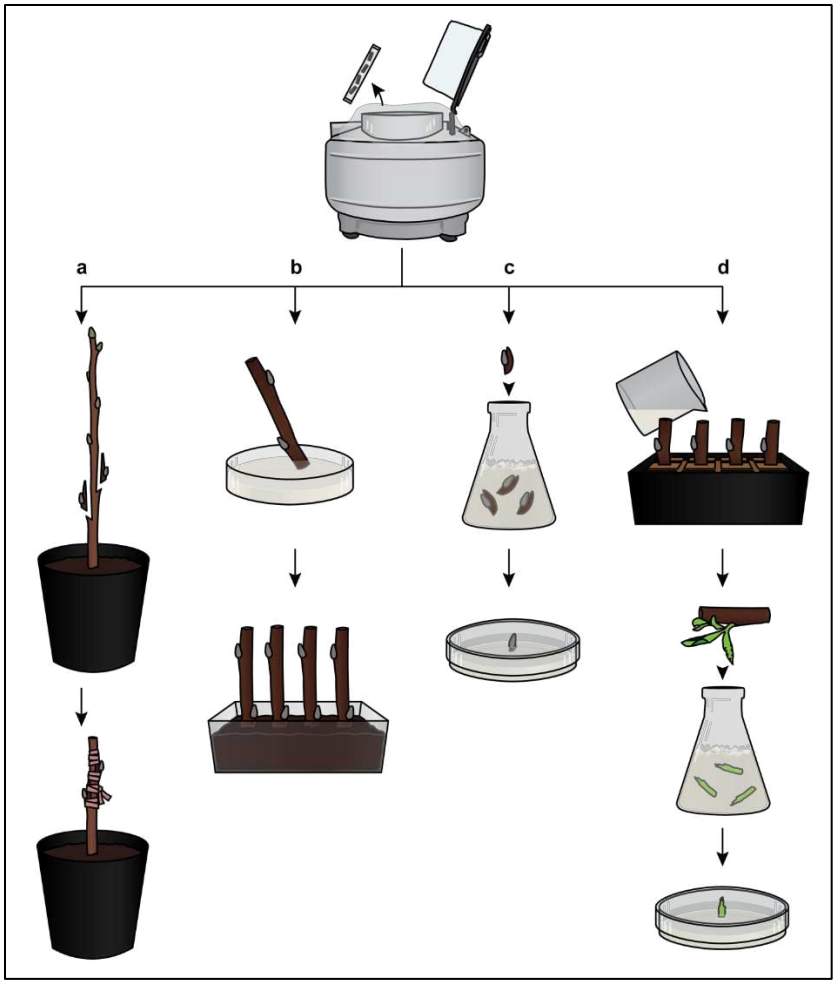

FIGURE 1.3. Examples of recovery systems for dormant buds after storage in liquid nitrogen. a. Recovery by grafting using chip-budding technique onto rootstock (Forsline et al. 1998b), b. Direct rooting of twig segments using rooting hormones before planting in growing environment (Bonnart et al. 2014), c. Bud chip recovered using *in vitro* system (Yakuwa and Oka 1988), d. Recovery using antimicrobial forced bud development to produce new growth, then induced into shoot cultures (Tanner et al. 2020).

VIABILITY ASSESSMENT

Viability assessments may sometimes be used to determine if an accession meets the criteria for being successfully preserved. In some cases, DB viability assessments such as staining or bud sprouting may provide insights about the regrowth capacity of cryopreserved accessions. Forced bud development is accomplished by placing DB into a high relative humidity growing

environment allowing for surviving buds to sprout; viability is determined based on the visual identification of survival and growth (Tanner et al. 2020). These conditions may allow some species to grow roots; however, the majority of sprouted DB are incapable of forming a complete plant system without the use of additional propagation techniques.

Tissue browning assessment relies on the development of reactive oxygen species that oxidize phenols after freeze damage occurs at the site of cellular injury (Calkins and Swanson 1990). When these phenolic cellular compounds are exposed to air, tissues around them oxidize and become visibly darker. This method requires an incubation period at temperatures above freezing for tissues to oxidize and turn brown. For tissues incubated at room temperature and 100% relative humidity, browning can be visually observed after 5 days (Stushnoff and Seufferheld 1995).

For tissue staining methods, buds are soaked in a dye solution, cross sectioned, and evaluated based on the presence/absence of stain in tissues of interest (Calkins and Swanson 1990). A major disadvantage of tissue staining as a viability test is the lack of clear delineation between damaged tissues that will recover and tissues that are irreparably damaged. Triphenyltetrazolium chloride (TTC) has been used extensively as a viability test in seed evaluation but much less in DB cryosurvival studies. TTC works by reacting with cellular respiration in living tissues changing from colorless to red dying living tissues. DB are stained with a 1% solution of TTC for 24 hours at 25 ºC before cutting into the bud to observe the localization and intensity of stain in the meristem (Zhumagulova et al. 2014; Kovalchuk et al. 2014).

CONCLUSION

Dormant budwood cryopreservation is a useful tool in managing temperate clonal tree and shrub genetic resources. Compared to shoot tip preservation, this approach reduces the front-end labor of establishing and maintaining *in vitro* cultures and requires less technical labor in order to achieve preservation. For established cryopreservation facilities considering incorporating DB germplasm into their repertoires, the seasonal commitment and initial investment in appropriate infrastructure must be considered. Furthermore, any laboratory adopting published DB cryopreservation protocols must conduct small-scale testing prior to any attempts at large-scale implementation, as source material and processing conditions of different facilities can lead to variable results. While dormant budwood cryopreservation can be an efficient means of preserving large quantities of critical germplasm, careful planning is required to balance the use of limited resources for successful implementation of this technology on a year-to-year basis.

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CHAPTER TWO: DETERMINING THE EFFECT OF PRETREATMENTS ON FREEZE RESISTANCE AND SURVIVAL OF CRYOPRESERVED TEMPERATE FRUIT TREE DORMANT BUDS

OVERVIEW

Freeze resistance is critical to successful dormant bud cryopreservation, and is affected by genotype, environmental conditions, dormancy phase and processing techniques. Pretreatment induced freeze resistance may contribute to more successful and efficient protocols for cryopreserving dormant buds. Differential thermal analysis (DTA) was used to quantify the effects of cryopreservation pretreatments on freeze resistance of dormant budwood. Low temperature exotherm profiles created by DTA could rapidly identify pretreatments that are contributing to increased freeze resistance in tree fruit species. In this study, DTA was used to help elucidate the effects of varying pretreatments (sucrose, desiccation and their combination) on peach, a model crop in tree fruit physiology that has shown little cryosurvival using the dormant bud method in the past. Post cryopreservation recovery trials using an antimicrobial forced bud development protocol evaluated the ability of selected pretreatments, that improved freeze resistance based on

DTA, to improve recovery rates of dormant budwood of various deciduous tree fruit and nut species. Pre-cryogenic exposure to sucrose solution (5.0 M, 96 hours), desiccation to 30% moisture content (MC) and their combination tested for their efficacy on improving post cryogenic viability in peach, apricot, sweet cherry, little walnut, black walnut, English walnut, apple, and pear. Among the different pretreatments tested, desiccation to 30% MC had the greatest impact on increasing freeze resistance and cryosurvival across most fruit species tested and little walnut. Gradual reduction of MC (40 to 25%) levels increased freeze resistance in peach (R^2=0.95) and increased some recovery outcomes (leaf, shoot and bud swell), however, this was not correlated with equal cryorecovery outcomes as severe bud cracking was observed. Overall, our approach linking freeze resistance and preconditioning treatments could help establish efficient species-specific protocols to preserve a number of important temperate woody crops.

INTRODUCTION

The importance of preserving genetic resources to support the changing needs of future agricultural endeavors cannot be overstated as crop genetic diversity provides the basis for the development of new cultivars. Cryopreservation has proven to be a viable method of securing important biological collections against loss. Cryopreservation of dormant buds (DB) has the potential to be a more efficient and cost-effective germplasm backup method compared to field collections or tissue culture derived meristem shoot cryopreservation. Successful protocols using the DB cryopreservation method have been developed for some deciduous clonal tree fruit crops. However, more work must be done to develop reliable methodology for preserving other tree fruit

species that do not survive liquid nitrogen vapor (LNV) exposure using the standard processing protocols.

Successful cryopreservation of DB relies upon the material's ability to survive preconditioning to low moisture and exposure to the extreme cold of LNV. DB cryopreservation uses last growing season's shoots that are collected following midwinter dormancy. Acclimation begins in late summer/early fall when daylength is reduced and low daily temperatures begin to drop. The gradual daily decrease of air temperature in temperate climates is associated with increasing resistance to freezing in perennial plants, thus acclimating to maximum cold hardiness around midwinter. Timing, degree, and duration of chilling events are variable from year to year and influence the rate of acclimation and cold hardiness; this may contribute to the lack of replicability of recovery outcomes on a season by season basis. One study on *Vaccinium* dormant budwood cryopreservation found a positive correlation between cryosurvival and the 10 day average of the maximum daily air temperature preceding the twig harvest [9]. During the peak of their dormant physiological state, buds have reached maximum cold hardiness and are most prepared for surviving cryogenic storage conditions [20].

Additionally, supplementing the budwood's natural cold hardiness with laboratory applied pretreatments that increase freeze resistance by supporting naturally occurring processes of freeze induced desiccation of critical cells in shoot primordia may increase the chances for survival at unnaturally low temperatures. Internal moisture content of budwood has been identified as an important factor influencing cryosurvival of apple buds [24]. In dormant apple shoots, initial moisture content of freshly collected DB varies from year to year but is usually between 45-55% when collected from source trees in field collections. Placing shoot segments into refrigerated chambers and exposing them to air movement will slowly desiccate the material. When the desired

moisture content is reached, twig segments can be sealed, and then placed in a programmable freezer to be slowly cooled to -30°C before LNV storage. Gradually reducing the temperature allows for intercellular water to migrate into extracellular spaces, where it can be frozen without causing damage to cell membranes [17]. At about -38°C all water spontaneously freezes; this is known as the homogeneous ice nucleation point. Using adequate slow cooling rates to freeze DB during cryopreservation allows sufficient time for water to migrate from intercellular spaces to extracellular locations in order to limit the severity of freeze damage to a survivable level [23]. Most apple genotypes have shown greater post cryogenic regrowth when reduced to 20-30% moisture content before LNV exposure.

Previous studies on cold hardiness in plants have found a positive relationship between carbohydrate concentration and cold tolerance [4,5,8,10,11,14,16]. In response to freezing conditions in winter, these temperate plants supercool their buds by freezing extracellular water, thereby increasing sugars, sugar alcohols, and reducing water content in cells. Naturally occurring sugars present in DB have been found to have cryoprotective effects related to stabilization of cellular components during dehydration [21]. Oligosaccharides, such as sucrose and raffinose, are involved in cold tolerance and have been shown to increase in deciduous twigs in the fall during the development of hardiness [6,18]. Additionally, increasing sucrose concentration in dormant twigs has been linked to repair of freeze induced embolism in dormant twigs of walnut [1]. Sucrose is a common ingredient used as a pretreatment in the cryopreservation of *in vitro* shoot tips of some species [15]. Exposure of in vitro shoot tips of sour cherry to high concentrations of sucrose or glycerol has been effective for preparing shoots for LN exposure and eliminating the need for artificial cold hardening in climate controlled growth chambers [2]. In dormant bud

cryopreservation, the application of sucrose and raffinose imbedded in a alginate matrix has been shown to increase cryotolerance of cold tender apple cultivars [19].

The goal of this research is to optimize dormant bud cryopreservation techniques to support the efforts of gene banking facilities in conserving clonal germplasm of deciduous fruit trees with greater efficiency, and to increase survival after LNV storage. This study investigates the relationship between various preconditioning treatments and their effect on freeze resistance and regrowth after LNV exposure. By identifying the most successful combination of pretreatments that overcome each crop's barriers to survival and regeneration, we hope to influence protocols for preservation of pear (*Pyrus communis* L. cv. 'Bartlett'), peach (*Prunus persica* (L.) Batsch cv. 'Cresthaven'), sweet cherry (*Prunus avium* L. cv. 'Bing'), apricot (*Prunus armeniaca* L. cv. 'Tilton'), apple (*Malus x domestica* Borkh. cv. 'Gala'), English walnut (*Juglans regia* L. accession 'DJUG 568.1'), black walnut (*Juglans nigra* L. cv. 'Sparrow') and little walnut (*Juglans microcarpa* Berl. accession 'DJUG29.4').

MATERIALS AND METHODS

Several agriculturally important species of hardy fruit were used to investigate the impact of pretreatments on cryo-survival of dormant winter buds. Walnut, black walnut, little walnut, cherry, peach, apricot and pear were selected for this research, as these crops have historically shown minimal success using standard DB cryopreservation techniques. We additionally selected one accession of apple for this study, as it has historically had good post-cryopreservation viability.

Dormant peach budwood [*Prunus persica* (L.) Batsch cv. 'Cresthaven'] of the previous season's growth were collected on November 6, 2017 from the experimental orchard of Colorado State University at Western Colorado Research Center-Orchard Mesa (WCRC-OM) in Grand Junction, Colorado, and shipped overnight to the US Department of Agriculture's National Laboratory for Genetic Resources Preservation (NLGRP) in Fort Collins, Colorado. A preliminary study utilized these twigs to investigate the effects of sucrose at various concentrations and durations of exposure on internal moisture content and cold hardiness to determine optimum treatment rates and duration to be further tested in a variety of species.

The cryo-survival tests were conducted on material harvested in January 2018. Dormant twigs of peach, apple (*Malus x domestica* Borkh. cv. 'Gala'), sweet cherry *(Prunus avium* L. cv. 'Bing'), apricot (*Prunus armeniaca* L. cv. 'Tilton'), and pear (*Pyrus communis* L. cv. 'Bartlett') were collected from WCRC-OM on January 4, 2018; this collection date is referred to as Sampling Time 1 (ST1). An additional sampling (Sampling Time 2 or ST2) of apple and peach was conducted on January 15, 2018. A third sampling (Sampling Time 3 or ST3) of peach was performed on January 29, 2018. Dormant twigs of little walnut (*Juglans microcarpa* Berl. accession 'DJUG29.4'), English walnut (*Juglans regia* L. accession 'DJUG 568.1'), and black walnut (*Juglans nigra* L. cv. 'Sparrow') were collected from the US Department of Agriculture's National Clonal Germplasm Repository (NCGR) in Davis, California on January 15, 2018.

Air temperature data for WCRC-OM were accessed through CoAgMet (coagmet.colostate.edu), and air temperature data for NCGR were accessed through UC Davis Atmospheric Science website (http://atm.ucdavis.edu/weather/uc-davis-weather-climate-station/); these are shown in Table 2.1.

TABLE 2.1. Air temperature data of field sites contributing twig samples. Maximum and minimum temperatures (°C) averaged over 10 days preceding twig sampling. NCGR contributed English walnut, black walnut and little walnut twigs, while all other material came from WCRC-OM.

Location	Collection Date	10d Ave. Tmax	10d Ave. Tmin
WCRC-OM	11/6/17	15.62	2.88
WCRC-OM	1/4/18	7.91	-6.84
WCRC-OM	1/15/18	7.06	-3.21
WCRC-OM	1/29/18	5.63	-7.87
NCGR	1/15/18	14.56	6.67

At NLGRP, budwood was cut into 3.5 cm segments, each containing a single node with a vegetative bud located close to the segment midpoint. After cutting, twig segments of each cultivar were mixed before being divided into treatment groups of 30 segments; the exception was little walnut, which was limited to 20 segments due to material availability.

EXPERIMENT 1: EFFECT OF SUCROSE ON PEACH DORMANT BUD FREEZE RESISTANCE

The first line of investigation explored the effect of sucrose on freeze resistance. A preliminary study on peach sampled in November 2017 showed that treatment in weak sucrose solutions significantly increased the moisture content (gravimetrically determined on a fresh weight basis) of the dormant twig segments (Figure 2.1). Using material from the same sampling time, a follow-up experiment was conducted using the following factors: 0.0 M, 1.66 M, 3.33 M, and 5.0 M sucrose solutions, for 24, 48, 72, and 96 hours of exposure on a rotary shaker at 4°C. After sucrose exposure, a differential thermal analysis (DTA) freeze test was conducted on this material to

evaluate the effect these treatments had on budwood freeze resistance. Details on DTA test described in Freeze Resistance Analysis section below.

EXPERIMENT 2: EFFECT OF SUCROSE AND DESICCATION PRETREATMENTS ON CRYO-RECOVERY OF DIFFERENT TREE FRUIT AND NUT SPECIES

Material from all crops harvested in January 2018 (ST1 and ST2) was used for the next line of inquiry, which examined the differences between additive and reductive pretreatment methods. The pretreatments in this study included: exposure to a 5.0 M sucrose solution, desiccation to 30% moisture content (MC), exposure to 5.0 M sucrose plus desiccation to 30% moisture content, and an untreated control group.

Segments treated in 5.0 M sucrose solutions were exposed at 4°C for 96 hours, as this was determined to be the optimal concentration and exposure time during preliminary experiments described in Experiment 1. Moisture content was determined gravimetrically on a fresh weight basis after drying sacrificial material at 100°C for 4 days in a laboratory oven. Material was desiccated at -4 ± 1°C and weighed regularly until the target moisture content was reached. A MC of 30% (fresh weight basis) was selected as the desiccation target as it is the standard MC level reported in apple DB literature[7]. This MC has also been successfully used for the cryopreservation of ash, cottonwood, willow, pear, apricot, and peach, [3,22,27].

LNV exposure and FBD cryo-recovery tests were conducted as described below on 30 segments per pretreatment (except little walnut) for each fruit species (ST1): apple, peach, sweet cherry, apricot, pear, little walnut, English walnut, and black walnut. Additional samples were collected for apple and peach (ST2) to replicate the first sampling time.

EXPERIMENT 3: EFFECT OF MC ON FREEZE RESISTANCE AND CRYO-RECOVERY

A further line of inquiry tested the hypothesis that moisture content was having the greatest impact on freeze resistance in peach twigs collected during midwinter (ST3). This experiment evaluated the effect of pretreatment on freeze resistance and cryo regrowth potential of twig segments treated with one of four different cryo pretreatments. These pretreatments were the same as described in Experiment 2. Additional treatment groups were desiccated to various MCs to investigate the relationship of freeze resistance to tissue MC to compare with previous treatment results of Experiment 2. These additional MC groups were desiccated to six MC targets; 40%, 37.5%, 35%, 32.5%, 27.5%, and 25%. Each treatment group consisted of 60 twig segments: 30 used to quantify freeze resistance by DTA and 30 segments to evaluate post cryo-regrowth potential using the methods and conditions mentioned below.

FREEZE RESISTANCE ANALYSIS

DTA was used to quantify freeze resistance of peach following a methodology [12] which uses thermo electric modules (TEM) (Laird Technologies, Chesterfield, MO) to detect temperature gradients generated by the exotherms. This method was modified and upgraded for increased efficiency and sensitivity [13]. This updated DTA equipment was constructed by mounting TEMs inside rectangular candy tin cans with folding lids sized just large enough to house the TEM. Eleven tin cans each containing a TEM were fixed onto aluminum plate which were connected to a multimeter/data acquisition system (Keithley 2700, Tektronix Company, Beaverton, OR) to constitute the sensor array. This DTA setting provides an improved signal/noise ration allowing for precise exotherm detection [13].

For each treatment group, 30 dormant bud segments of peach were used to assess freeze resistance. Peach was selected for its capacity to show distinctive peaks for low temperature exotherms and its lack of survival after cryostorage following standard processing procedures of the dormant bud cryopreservation method. The twig segments were trimmed just above and just below each node that contained two floral and one vegetative bud, and were cut in half, longitudinally, to reduce stem biomass and folded into aluminum foil to reduce moisture loss during DTA run. Five prepared segments were placed inside each aluminum box where the buds could contact the TEM.

Dormant peach buds were frozen at a rate of 4°C per hour from 0°C to -40°C using a programmable controlled rate freezer (model ZP-8, Cincinnati Sub-Zero, Cincinnati, Ohio). Temperature and voltage data were recorded by a datalogger located outside of the test freezer with wires routed through a port which connected to TEMs using Kiethley KickStart Software (Tektronix, Beaverton, Oregon). After a controlled freeze event, data were exported from Kiethley KickStart Software to Microsoft Excel (Microsoft Corp., Redmond, Washington) analyze for exotherms. Low temperature exotherms (LTE) represent intracellular freezing events inside the sample tissue and are considered lethal events to individual buds. High temperature exotherms (HTE) mark the freezing of extracellular water as tissues begin to freeze and are nonlethal events. Exothermic data were collected from the datalogger and analyzed using Microsoft Excel by subtracting voltage values of a reference TEM from sample TEM readings. Analyzed data were plotted using GraphPad Prism version 8.3.0 to create DTA profiles used for the identification of exotherm peak temperatures.

CRYO-RECOVERY

After applicable pretreatments were applied, material designated for cryo-recovery was frozen at a rate of 1°C per hour from -5°C to -30°C and held at -30°C for 24 hours using a programmable controlled-rate freezer (model ZP-8, Cincinnati Sub-Zero, Cincinnati, Ohio). Twig segments were then transferred to an LNV storage tank (MVE/Chart model XLC1830) for at least two weeks before rewarming and viability testing.

Twig segments were rewarmed by removing samples from LNV and warming at -5°C for 24 hours, then submersing twig segments in a 1.5 mM ascorbic acid solution for 15 minutes at 5°C before planting in antimicrobial forced bud development (AFBD) regrowth environment [20]. AFBD after cryo was accomplished by planting twig segments directly into Oasis Horticube media (product no. 5240, Oasis Grower Solutions, Kent, OH), each tray containing 3 L of a forcing solution consisting of 200 mg L^{-1} of 8-hydroxyquinoline citrate (Alpha Chemistry, Holtzville, NY) and 20 g L^{-1} of D-Sucrose (Phytotechnology Laboratories, Shawnee Mission, KS) as previously described in Tanner et al. [20]. AFBD was conducted inside plastic nursery trays covered by clear plastic humidity domes placed inside a Percival growth chamber (model number 166LL) set to 21°C with a diurnal photoperiod of 18 hours light/6 hours dark. Twig segments were allowed to develop in this regrowth environment for 6 weeks before recovery assessment.

A progressive bud development rating scale designed to evaluate the regrowth potential of DB after cryopreservation previously reported by Tanner et al. [20] was used to quantify development of DB after cryopreservation (Figure 3.1). Twig segment diameters were recorded for each segment using a digital caliper (World Precision Instruments LLC., Sarasota, FL) to investigate the relationship between survival of LNV-exposed twigs and segment width. The diameter at the segment midpoint was measured for all material designated for cryo-recovery regrowth trials.

STATISTICAL ANALYSIS

Analysis of the FBD viability tests is based on percent viable, where viable is defined as having the tip greening or leaf/shoot development outcomes. The freeze resistance assessment is based on exotherms of peach buds, captured by DTA. Both bud viability and freeze resistance were analyzed with the Kruskal Wallis test. Dunn's Multiple Comparison test is used to compare the results from each treatment. All data were analyzed on GraphPad Prism version 8.3.0.

RESULTS AND DISCUSSION

EFFECT OF SUCROSE AND DESICCATION PRETREATMENTS ON FREEZE RESISTANCE AND CRYO-RECOVERY

Cryoprotectants have been used for cryopreservation of meristematic tissue and have therefore been explored in the realm of dormant bud cryopreservation. Sucrose has been used because it is a naturally occurring carbohydrate found in overwintering DB of hardy plants and is nontoxic and affordable cryoprotectant. In this study, we employ DTA to quantify the affect that simple sucrose pretreatments have on freeze resistance. Experiment 1 which focused on understanding the effect of sucrose solution exposure; concentration and duration, on DB freeze resistance to identify the best preforming sucrose treatment. The results of this experiment showed that sucrose treatment did alter freeze resistance of twig segments compared to untreated material. Sucrose concentrations below 3.33M (0M and 1.66M) reduced freeze resistance compared to untreated controls. In the case of water treatment (0.0 M sucrose), after 24 hours of treatment exposure freeze resistance was significantly less than that of untreated buds. Buds exposed to water only lost all ability to resist

freezing after 72 hours of treatment exposure. We also found that very high concentrations (3.33-5.0 M) of sucrose solution applied for long exposure times (96 h) do increase freeze resistance (Figure 2.1). The temperature of freezing initiation may relate to solute concentration present in the extracellular matrix of bud tissues as suggested by Vogiatzi et al. [26] and is supported by our data. The amount of extracellular freezing appears to be reduced as sucrose concentration and exposure levels increase. This idea is supported by the reduction of the initial freezing event, the HTE. This reduction in the HTE was observed in both duration and degree of magnitude in the DTA profile suggesting osmotic desiccation is occurring during sucrose treatment. Based on the findings of this preliminary study, Experiment 1, the effective pretreatment level with sucrose solution of 5.0 M for 96 h (4°C) was selected to be used in subsequent cryo-survival trials, Experiment 2 and 3, as it was expected to potentially increase survival after LNV exposure compared to untreated material.

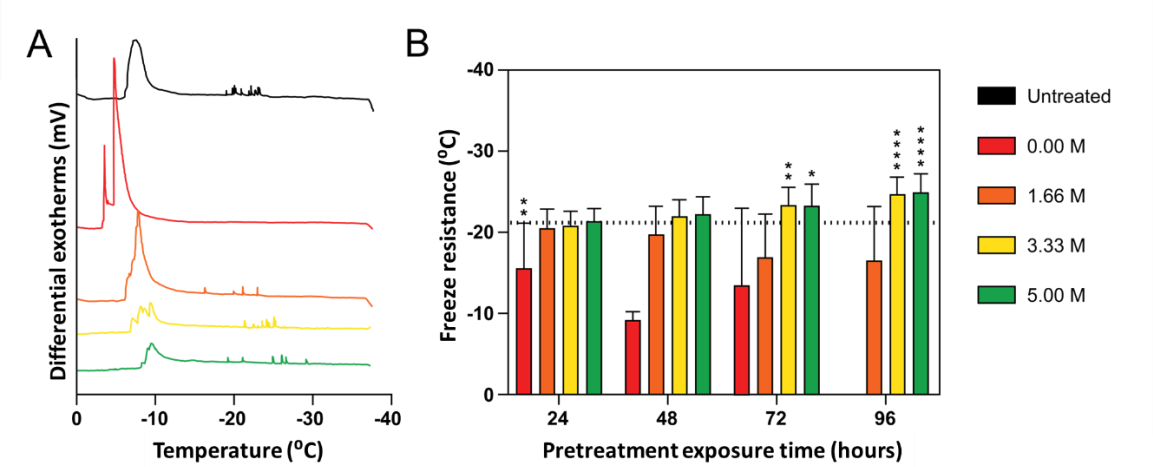

FIGURE 2.1. Sucrose concentration and exposure effect on freeze resistance in peach dormant twig segments collected in November 2017. (A) DTA exotherm profiles showing high temperature exotherms on the left and distinctive low temperature peaks on the right. DTA profiles shown are after 96 hours of sucrose exposure. (B) Exotherm temperatures for each treatment group showing significant effect of sucrose treatment on freeze resistance. Baseline freeze resistance from untreated buds shown as a horizontal black dotted line. Statistical significance level is indicated with the symbol *. * $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. n=30

DTA was also used to compare the effects of pretreating by solution exposure and conventional dormant budwood pretreatment by air desiccation on freeze resistance in peach. The sucrose pretreatment that had the greatest impact on freeze resistance was exposure to 5.0 M solution for 96 hours in our preliminary studies (Experiment 1, Figure 2.1), though it was not statistically different from the control in our follow up experiment (Experiment 2, Figure 2.2). On the other hand, it was shown that the conventional pretreatment (desiccation) significantly increased freeze resistance, as did the combined method (desiccation plus a sucrose cryoprotectant), but to a lesser degree. DTA peak height observed to be variable but was influenced by moisture content with larger peaks observed from buds with higher moisture levels (Figure 2.2A)

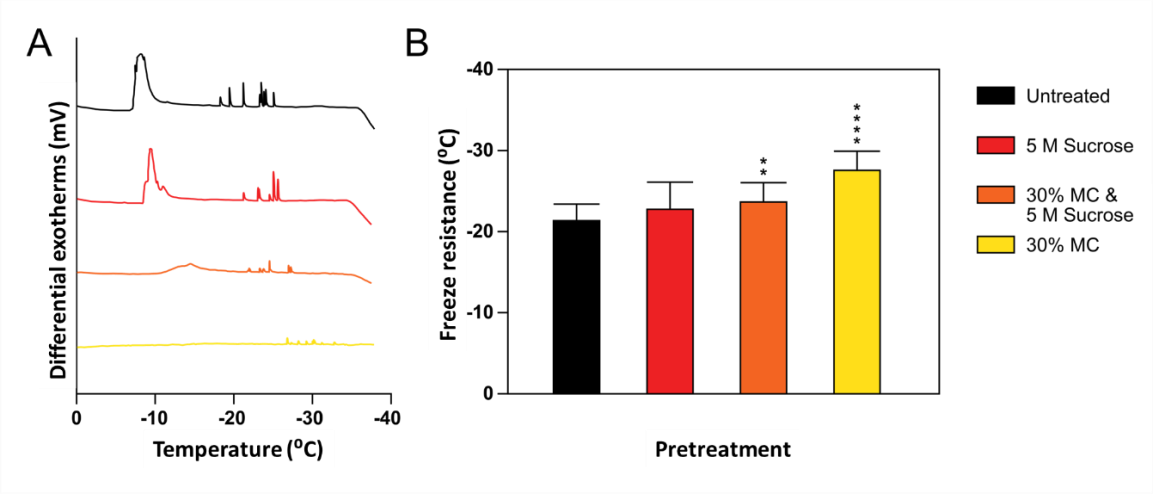

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FIGURE 2.2. Effects of various preconditioning treatments on freeze resistance of peach dormant twig segments collected in January 2018. (A) DTA profiles corresponding to each pretreatment. (B) Exotherm temperature values showing significant effect of pretreatment on increasing freeze resistance compared to an untreated control. Statistical significance level is indicated with the symbol *. * $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. n=30

Although our preliminary studies of Experiment 1 showed high molarity sucrose exposure to increase freeze resistance of peach twig segments compared to an untreated control, these pretreatments were not effective at increasing survival after LNV exposure (Figure 2.3). LNV temperature ranges from -140° to -180°C and is thus significantly colder than the few degrees of increased cold tolerance produced by sucrose pretreatment detected by DTA. Reduction of internal

MC to 30% by air drying at -4°C had a greater effect on survival in LNV than sucrose solution treatment alone or in combination with desiccation to 30% MC in peach.

Recovery outcomes for twig segments after LNV exposure for all taxa tested are reported in Figure 2.3, except for English walnut and black walnut, which showed no recovery across all treatments (data not shown). Twig segments that were frozen in the fresh state (*i.e.* without artificial desiccation) and twigs treated with 5.0 M sucrose alone did not recover (tip greening/ leaf and shoot) after LNV exposure in any species tested. Of all the groups tested, apple twigs desiccated to 30% MC had the highest recovery rate, 63.33%. In pear and sweet cherry, sucrose treatment in combination with desiccation to 30% MC was the most beneficial pretreatment tested. In pear, twig segments treated with 5.0 M sucrose for 4 days before desiccation to 30% MC at -5°C had the same shoot recovery rate as those desiccated to 30% MC without sucrose exposure, though the former had more bud swelling. In sweet cherry treated with 5.0 M sucrose and desiccated to 30% MC, 13.33% of twig segments developed shoots compared to only 3.33% for desiccation to 30% MC alone. For pear and sweet cherry, both factors could be optimized in the future.

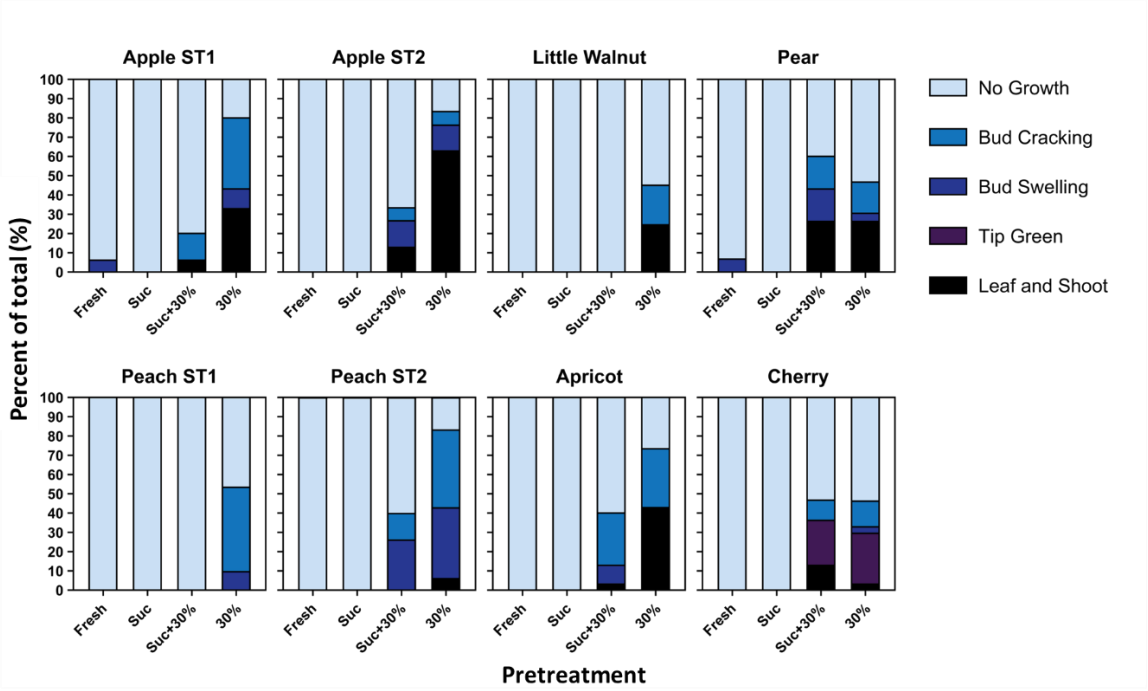

FIGURE 2.3. Recovery outcomes of various hardy fruit tree species after LNV exposure. English walnut and black walnut had no growth and are not shown. n=30 for all species except little walnut (n=20). Some crops were tested from two harvests, sampling time 1 (ST1) occurred on January 4,2018 and sampling time 2 (ST2) on January 15, 2018.

For most, desiccation was the pretreatment that showed the greatest increase in viability. This corresponds with the peach DTA results, which suggested that air drying twig segments to 30% MC at -4°C is more beneficial for increasing freeze resistance compared to all other pretreatments tested. Although 30% MC has shown to be a beneficial pretreatment in this study, this particular MC is not necessarily optimized for peach or other hardy tree fruit crops. The original desiccation recommendations for DB were developed for apple, which is somewhat desiccation tolerant and cold hardy; desiccation to 23.4% increased survival for apple twig segments [27]. In subsequent work, the MC range of desiccated apple twig segments was narrowed to between 28-32% with 30% ideally targeted [7]. Optimum desiccation levels for crops and cultivars may differ from those reported for cold hardy apple cultivars and needs further investigation to increase survival and regrowth following LNV exposure [25]. Since cold tolerance is crucial to material survival, the significant improvement ($p<0.0001$) of freeze resistance seen in some treatments further suggests

that DTA can be a time and resource saving approach to optimizing cryoprotectant application in future studies.

Two sample times were used in apple and peach. The first twig collection was conducted on January 4 (ST1) and the second on January 15, 2018 (ST2). For both crops, survival rate improved in the second date of January 15, 2018 compared to January 4, 2018. While the average maximum air temperature was similar for both and statistically not significant, the average minimum air temperature was significantly colder for ST1 ($p = 0.0333$). These results indicate additional factors might be influencing the improved survival and regrowth observed in sample time 2 such as increased cold hardening or the satisfaction of chilling requirements due to a cold weather event that occurred after ST1 but before ST2.

For a more robust analysis of preconditioning factors that affect cryo-survival of dormant winter buds, we compared the twig diameter of every segment to its cryo-survival ranking. In all species tested, the smallest diameter twig segments in each treatment did not survive LNV exposure (Figure 2.4). For apple, shoot growth was observed in twig segment of diameters of 3.85 and 5.88 mm. The smallest 3% of diameter segments of apple did not grow after LN exposure and were 3.48 to 3.72 mm in diameter. In peach, shoot development was seen in sizes between 3.79 and 4.52 mm; however only two segments were observed viable and the actual size range is expected to be larger than observed here. The smallest 36% of peach twig segments did not grow after cryo; having diameters of 2.9 to 3.7 mm. With apricot, shoot development was observed from 3.57 to 6.88 mm. The smallest 23% of segment diameters did not regrow after LN exposure and were 2.36 to 2.75 mm wide. For pear, 4.91 to 6.77 mm size ranges showed shoot growth after storage in LN. The smallest 6% of segment diameters did not grow after LN exposure and were between the sizes of 3.21 to 3.41 mm. In sweet cherry, viability was observed in segment diameters sized between

4.8 and 7.08-mm. Bud swelling occurred in sweet cherry in even the smallest diameters tested. In little walnut regrowth was observed between 4.1 to 5.81 mm segment diameters. The smallest 5% of diameters did not regrow after LN storage and ranged in size from 3.27 to 3.96 mm. Histograms of recovery outcomes and size classes are presented in Figure 2.4.

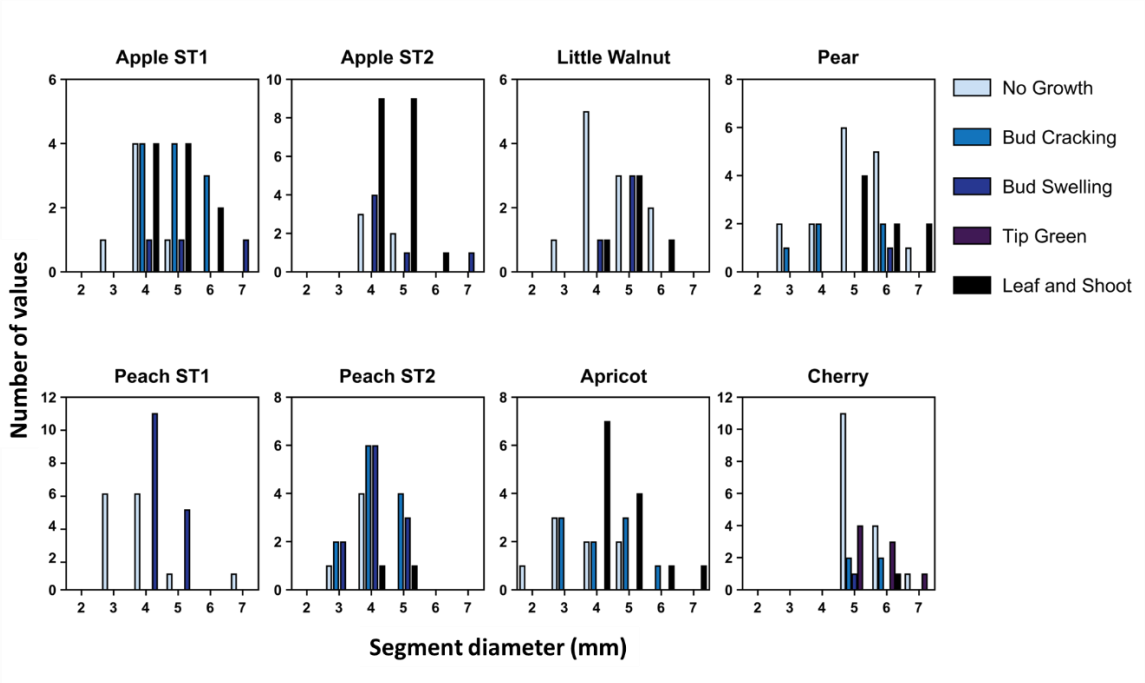

FIGURE 2.4. Twig segment diameters of various deciduous fruit tree species showing the influence of twig diameter on cryo-recovery. n=30 for all species except little walnut (n=20).

Twig segment with the smallest diameters were not observed to survive LNV exposure. This result suggests that the most apical portions of the twig should be discarded, leaving the most uniform middle and basal twig sections for processing and LNV storage; this practice would increase survival of the remaining sample group and divert storage resources from the least successful to the most successful material. In germplasm storage, anything that can be done to increase survival before storage is warranted. In discarding twig tips, a small amount of material will be lost but can be compensated by the addition of more twigs for storage, allowing for the stored material to have a greater overall survival.

EFFECT OF SHOOT INTERNAL MOISTURE CONTENT ON FREEZE RESISTANCE AND CRYO-RECOVERY

Since moisture content showed a greater impact on freeze resistance in peach and cryo-survival across almost all crops tested in Experiment 2, we use DTA to further explore the relationship between internal moisture content and freeze resistance and conducted regrowth trials to validate our findings in Experiment 3. The effect of moisture content on freeze resistance illustrated by DTA profile of buds at various moisture contents (Figure 2.5A). We found that lower internal moisture content increased freeze resistance, particularly in those groups desiccated to 25-35% MC (significantly different from the control, $p<0.0001$) (Figure 2.5B). When comparing moisture content to freeze resistance (LT_{50}), we found a linear relationship between these two factors (Figure 2.5C and Figure 2.7).

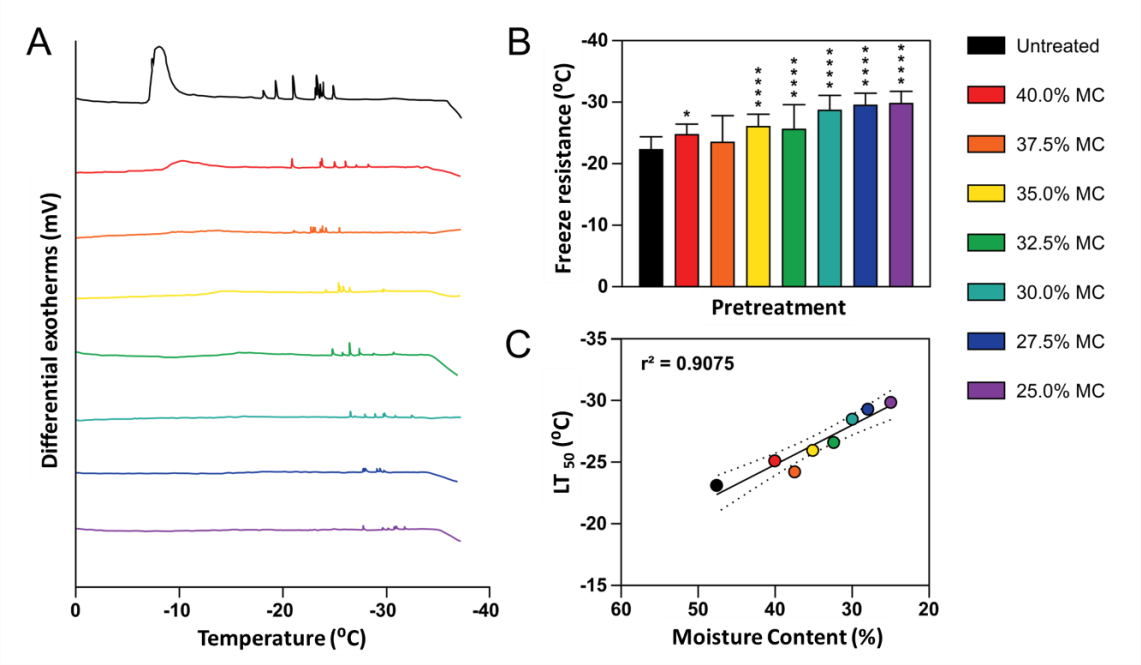

FIGURE 2.5. Dormant twig segments of peach (ST3) collected on January 29, 2018 desiccated by air drying at -4°C±1 to various moisture contents before controlled cooling at a rate of 4 °C/hour from 0 to -40 °C. Moisture content (fresh weight basis) treatment groups of Fresh (47% MC), 40%, 37.5%, 35%, 32.5%, 30%, 27.5% and 25% are shown. (A) DTA exotherm profiles display the effect of moisture content on freeze resistance. (B) Exotherm values of DTA freeze resistance trail showing significant influence of reduced moisture content on increased freeze resistance. (C) Relationship between moisture content and LT_{50} values of peach DB. Statistical significance level is indicated with the symbol *. * $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. n=30

Although freeze resistance was greatly improved in those samples desiccated below 35% MC (Figure 2.5), the cryo-survival assessment did not exactly match the patterns seen in the DTA (Figure 2.6). The limited regrowth seen in material desiccated below 35% MC (figure 2.6) suggests that desiccation damage may be limiting recovery after LNV exposure.

While ideal internal moisture content of DB should be optimized for peach and other hardy deciduous fruit crops that do not survive LNV storage, future studies must address desiccation damage in order to maximize sample regrowth. We speculate that different tissue types in budwood may lose moisture at different rates. Tissues such as the bud axis may become drier than

surrounding tissues as water is removed more efficiently within the vascular system during resulting in the formation of cracks as tissues rehydrate during recovery.

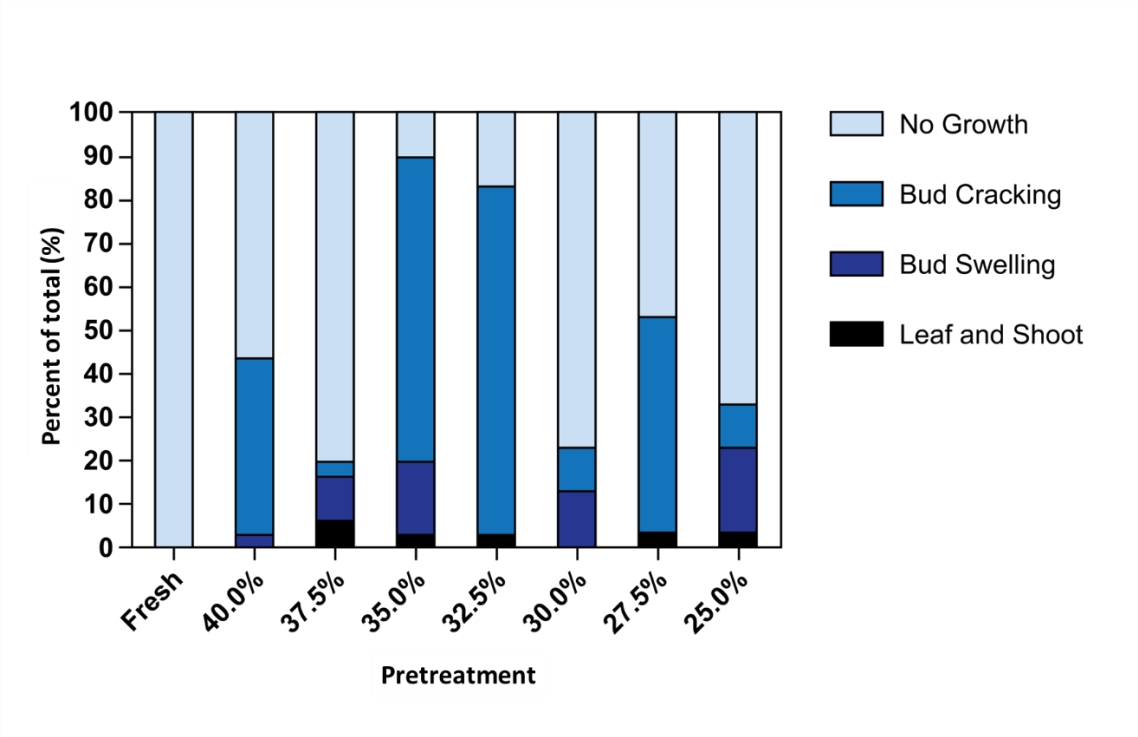

FIGURE 2.6. Recovery outcomes of dormant peach twig segments (ST3) collected on January 29, 2018 and pretreated by desiccating to various MC after LNV exposure. n=30

CONCLUSION

Cryopreservation of dormant winter twigs is a promising method for efficiently storing clonal hardy deciduous tree and shrub species. Cryo-survival is dependent on many factors, such as collecting budwood during periods of peak dormancy, reducing internal moisture before LNV exposure, and slowly cooling twig segments to allow for freeze-induced intracellular desiccation

before storage in LNV. Applying the DTA method has provided insight into the temperature at which buds are freezing and the effect that various pretreatments have on freeze resistance. DTA has allowed us to quickly identify pretreatment methods that have the greatest effect on increasing freeze resistance in vital crops such as peach. In general, pretreatments showing the greatest freeze resistance also had increased recovery after LNV exposure, although more work is needed to mitigate the effects of desiccation damage. Through our study, we show that a multi-tiered approach allows us to test our hypotheses about the effect these preconditions will have on DB before focusing our resources on optimizing the pretreatments that will most effectively preserve such limited and vital genetic material. Through this approach, establishing efficient species-specific protocols for preserving a number of important temperate woody crops can become a reality in the near future.

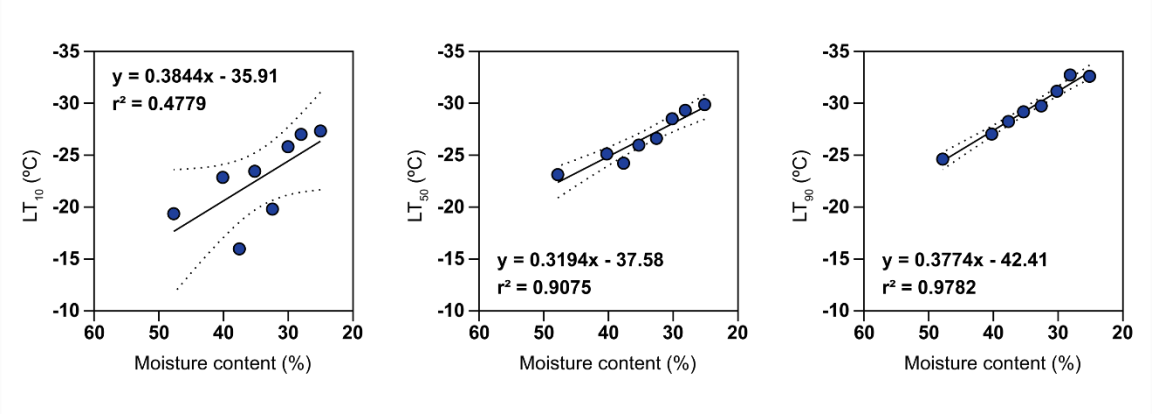

FIGURE 2.7. Relationship between lethal temperatures of freezing DB and the internal moisture content in peach. (A) Lethal temperatures that killed 10%, (B) 50% and (C) 90% of dormant buds are shown above. n=30.

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CHAPTER THREE: ANTIMICROBIAL FORCING SOLUTION IMPROVES RECOVERY OF CRYOPRESERVED TEMPERATE FRUIT TREE DORMANT BUDS

OVERVIEW

Dormant bud cryogenic preservation is a cost- and labor-efficient method of genetic resource backup compared to *in vitro* derived meristem shoots cryopreservation. While protocols have been developed for cryopreserving apple dormant buds, effective and reproducible protocols are yet to be developed for several temperate fruit and nut species. Dormant bud cryopreservation typically requires material to be grafted to evaluate viability and recover a plant. Forced bud development has been used on a very limited scale for cryostored dormant budwood recovery, however, it provides a labor-efficient alternative viability assessment. To increase the utility of this approach, regrowth must be optimized to allow complete plant recovery. We hypothesized that bacterial attacks are limiting regrowth, thus, an antimicrobial forcing solution can maximize regrowth potential. This study examined the effects of an antimicrobial forcing solution (8-hydroxyquinoline citrate and sucrose, 8-HQC) on the cryosurvival and recovery of dormant buds of fruit (*Malus x domestica*, *Prunus armeniaca*, *Prunus avium*, *Prunus persica*, *Pyrus communis*), and nut species (*Juglans regia, Juglans nigra, Juglans microcarpa*). Recovery and shoot development were significantly improved for all the fruit and one nut species (*J. microcarpa*) treated with the 8-HQC, compared to standard recovery under high humidity alone ($P<0.001$). Additionally, this post cryo recovery

approach led to successful *in vitro* shoot tip establishment across all surviving fruit species. 8-HQC embedded forced bud development method increased viability and efficiency for existing cryostored material and can be used as a benchmark to develop protocols for different crops that could potentially lead to complete plant recovery.

INTRODUCTION

Cryogenic storage contributes to food security and preserves genetic diversity for crop-breeding programs by allowing an efficient storage of important genetic resources at lower cost, labor, space, and risk associated with field collection maintenance. Cryopreservation of deciduous fruit and nut trees using dormant winter buds is an important tool for germplasm conservation [21].

Survival of dormant budwood exposed to liquid nitrogen was first reported nearly 60 years ago using mulberry and willow twigs [16]. Since then, technology and methodology have developed to encompass a variety of cold-hardy woody taxa and increase overall survival. Cryopreservation using dormant winter buds holds some advantages over tissue culture mediated preservation methods for temperate fruit trees as it is a simpler, more cost-effective process; however, it is only applicable to dormant woody plant species. Another advantage of dormant budwood cryopreservation is the speed at which accessions can be processed, which results in higher throughput of cryopreserved accessions compared to *in vitro* meristem shoots [7].

Dormant bud cryopreservation has limitations, including its short seasonal harvest window and restricted application to cold-hardy woody taxa; however, the biggest limitation to its widespread use is the lack of effective protocols for many crops. At the National Laboratory for Genetic

Resource Preservation (NLGRP) in Fort Collins, CO, dormant bud cryopreservation is used to store genetic material of apple (*Malus spp.*) [3] and tart cherry (*Prunus cerasus* L.) [25]. While refinement of standard processing methods is currently underway for other cold-hardy fruit trees and shrubs at the NLGRP, a greater concentration of effort is still needed to make this process useful for a broader variety of fruit and nut crops [6].

Decreasing internal moisture content of dormant budwood has been shown to increase survival in liquid nitrogen exposed twigs segments and has become standard practice in dormant bud cryopreservation [2,18,26–31]. In dormant apple twigs, initial moisture content is usually between 50-55% when collected from source trees in field; however, most genotypes have shown greater regrowth when reduced to 25-30% moisture content by air-drying under refrigerated conditions before slow cooling and liquid nitrogen vapor (LNV) exposure [29]. This process removes extraneous moisture before dormant budwood is exposed to LNV. Slowly cooling the twig segments allows time for symplastic water to migrate into extracellular spaces, in order to limit the severity of freeze damage to a survivable level [23]. Further studies have investigated the use of cryoprotectants to improve the ability of dormant buds to survive cryostorage [18,22].

While research on treatments prior to cryoexposure has been invaluable to developing processing protocols, further research on optimizing recovery conditions for various temperate clonal tree fruit species is still needed. In cultivated apple, evaluating cryorecovery is usually accomplished by grafting a subset of twig segments onto seedling rootstocks using the chip budding or patch budding method [4,20,29]. The shoot development from the grafted bud indicates the viability of the stored samples, and further allows for complete recovery of the material into cultivation.

Since grafting is a highly skilled technique, regrowth results may vary dependent on the technique of the grafter. Except for the need for skilled grafting personnel, this technique is resource-limited

and labor-intensive as it requires rootstocks to be propagated or procured, and specific conditions to be controlled in order to conduct the twig segment rehydration and graft regrowth trials [4].

Sprouting of shoots from dormant buds after cryostorage has been established as an alternative viability test [23]. Compared to grafting, bud sprouting or forced bud development (FBD) is far less labor-intensive. Using the FBD technique, twig segments are planted into a growing environment with high relative humidity and are allowed to recover from dormancy and develop shoots. Viability is evaluated after several weeks depending on the tree fruit species. NLGRP is currently working towards developing crop specific cryostorage protocols using FBD as a promising and efficient alternative to grafting to test dormant bud post-cryopreservation viability.

Tissue necrosis during recovery in FBD may limit the regrowth potential of cryopreserved dormant bud segments. Due to desiccation and freezing damage to cell membranes and walls, cryoprocessed dormant buds are extremely vulnerable to microbial attack [10,17]. Forcing solutions containing the antimicrobial 8-hydroxyquinoline citrate (8-HQC) and sucrose have been useful at preventing plant pathogenic microorganisms (bacteria, fungi etc.) from building up and blocking xylem tissues, allowing buds to develop after flower stems have been harvested, thus, increasing the vase life of fresh cut flowers [9,12,13,19]. In woody plants, this forcing solution has been used to extend the softwood cutting season and increase success in sterile culture induction [33]. In the present study we used an 8-HQC and sucrose forcing solution as a microbial suppressant in cryoprocessed dormant buds undergoing regrowth in an FBD viability assay. By reducing biotic barriers to shoot regrowth, we hypothesize that the dormant bud cryosurvival of various fruit and nut species will be improved, and efficiency of future and past material stored through dormant bud cryopreservation protocols will be increased.

While FBD is a great alternative to grafting as a viability assessment, it is not yet useful for plant regeneration [23]. Cryopreserved dormant winter twig segments retain maturity when recovered by grafting; this allows for the rapid production of flowers within two years of recovery, which is helpful for plant breeders [21]. While using the ability of stored budwood to sprout a shoot as a determination of cryoviability is efficient compared to the labor of grafting, the method usually fails to establish material for regrowth after storage. The present study addresses the major deficiency in the FBD system using antimicrobial forcing solution. Following this approach, viable meristems from the segments used for FBD viability assays, could be potentially excised and induced into tissue culture, allowing for multiplication and re-cultivation after cryostorage.

MATERIALS AND METHODS

DORMANT BUDWOOD

Eight species of domesticated and wild fruit and nut crops were used to investigate survival and regrowth after prolonged exposure to LNV. Dormant twigs of the current season's growth were collected at peak dormancy, and shipped overnight to NLGRP in Fort Collins, Colorado.

Sweet cherry (*Prunus avium* L. cv. 'Bing'), apricot (*Prunus armeniaca* L. cv. 'Tilton'), peach [*Prunus persica* (L.) Batsch cv. 'Cresthaven'], and European pear (*Pyrus communis* L. cv. 'Bartlett') dormant twigs were sampled on January 4, 2018 from CSU's experimental orchard at the Western Colorado Research Center-Orchard Mesa (WCRC-OM) in Grand Junction, CO. These tree fruit crops were chosen because they represent a broad genetic variability and they can fit for preservation protocols using the dormant budwood method. Dormant twigs of cultivated apple

(*Malus x domestica* Borkh. Cv. 'Gala') were also sampled from WCRC-OM at the same time. Apple is a model fruit tree species that has been successfully tested in prior dormant budwood cryopreservation trials [3].

A second twig sampling of peach and apple was conducted at WCRC-OM on January 15, 2018. These served both as a replication of all treatments, as well as an intra-seasonal comparison of performance during cryostorage and post cryopreservation treatments. These groups of apple and peach are differentiated by sample time, where ST1 refers to those sampled on January 4, 2018, and ST2 refers to those sampled on January 15, 2018.

Three nut crops were also included in this study: little walnut (*Juglans microcarpa* Berl. accession 'DJUG29.4'), English walnut (*Juglans regia* L. accession 'DJUG 568.1'), and black walnut (*Juglans nigra* L. cv. 'Sparrow'). These were sampled on January 15, 2018 from the US Department of Agriculture-Agriculture Research Service, National Clonal Germplasm Repository, Davis, CO.

Air temperature data for WCRC-OM were accessed through CoAgMet (coagmet.colostate.edu), and air temperature data for NCGR were accessed through UC Davis Atmospheric Science website (http://atm.ucdavis.edu/weather/uc-davis-weather-climate-station/) and are shown in Table 3.1.

TABLE 3.1. Air temperature data of field sites contributing twig samples. Maximum and minimum temperatures (°C) averaged over 10 days preceding twig sampling. National Clonal Germplasm Repository in Davis, CA (NCGR) contributed English walnut, black walnut and little walnut twigs. All other material came from Western Colorado Research Center-Orchard Mesa, Grand Junction, CO (WCRC-OM).

Location	**Harvest Date**	**10 d Ave. T_{max}**	**10 d Ave. T_{min}**
WCRC-OM	1/4/2018	7.9	-6.8
WCRC-OM	1/15/2018	7.1	-3.2
NCGR	1/15/2018	14.6	6.7

CRYOPROCESSING AND PRE-TREATMENTS

Upon arrival, twigs were cut into 3.5 cm segments containing one node located close to the segment midpoint. After cutting, twig segments of each batch were mixed before being separated into treatment groups. All treatment groups consisted of 30 twig segments per species per harvest, except little walnut, which was limited to 20 segments per treatment due to material availability. Based on standard processing techniques developed for apple [3], all groups were desiccated to 30% moisture content through air drying at -3 °C. Moisture content was determined gravimetrically on a fresh weight basis after drying a subset of 20 twig segments at 100 °C for 4 days in a laboratory oven. Moisture content of little walnut was determined using a subset of 15 segments per treatment group.

After desiccation, each treatment group was frozen at a rate of -1 °C per hour from -5 °C to -30 °C, and then held at -30 °C for 24 hours using a programmable controlled rate freezer (model ZP-8, Cincinnati Sub-Zero, Cincinnati, Ohio). After controlled cooling, twig segments were transferred to and held in an LNV storage tank (MVE/Chart model XLC1830) for at least two

weeks between the temperatures of -140 °C and -180 °C before rewarming and viability tests were conducted.

POST CRYO RECOVERY TREATMENTS

Twig segments were rewarmed by removing samples from LNV, thawing at -5 °C for 24 hours and then submerging them in a 1.5 mM ascorbic acid solution for 15 minutes at 5 °C. Twig segments were then divided into two recovery test groups: (1) FBD standard protocol (namely STD), (2) FBD with 8-HQC antimicrobial forcing solution (namely HQC).

Segments undergoing the standard (STD) protocol developed for apple dormant buds [3] were rehydrated in sterile peat for 14 days at 3 ± 2 °C, then planted in Oasis Horticube media product no. 5240 (Oasis Grower Solutions, Kent, OH) containing approximately 3 L of water per flat. Segments undergoing FBD with an antimicrobial forcing solution (HQC) were planted directly into Oasis media, with each flat containing 3 L of a forcing solution. The forcing solution, originally used to increase the vase life of cut flowers [8,9,11], consists of 200 mg L^{-1} of 8-hydroxyquinoline citrate (Alpha Chemistry, Holtzville, NY) and 20 g L^{-1} of D-sucrose (Phytotechnology Laboratories, Shawnee Mission, KS).

All trays were covered with clear domes and kept in a Percival growth chamber (model number 166LL) set to 21 °C with a diurnal photoperiod of 18 hours light/6 hours dark. These incubated for 6 weeks before viability assessment was conducted.

VIABILITY EVALUATION

A progressive dormant bud development classification system was created to quantify development of cryopreserved twig segments in STD and HQC FBD and report recovery outcomes in this study 6 weeks post thaw. This classification system is illustrated visually in diagram form

in Figure 3.1. Cryopreserved twig segments were evaluated by classifying the development of dormant buds for each twig segment into any of the following five developmental outcomes: no growth, bud cracking, bud swelling, tip greening, or leaf and shoot development (Figure 3.1).

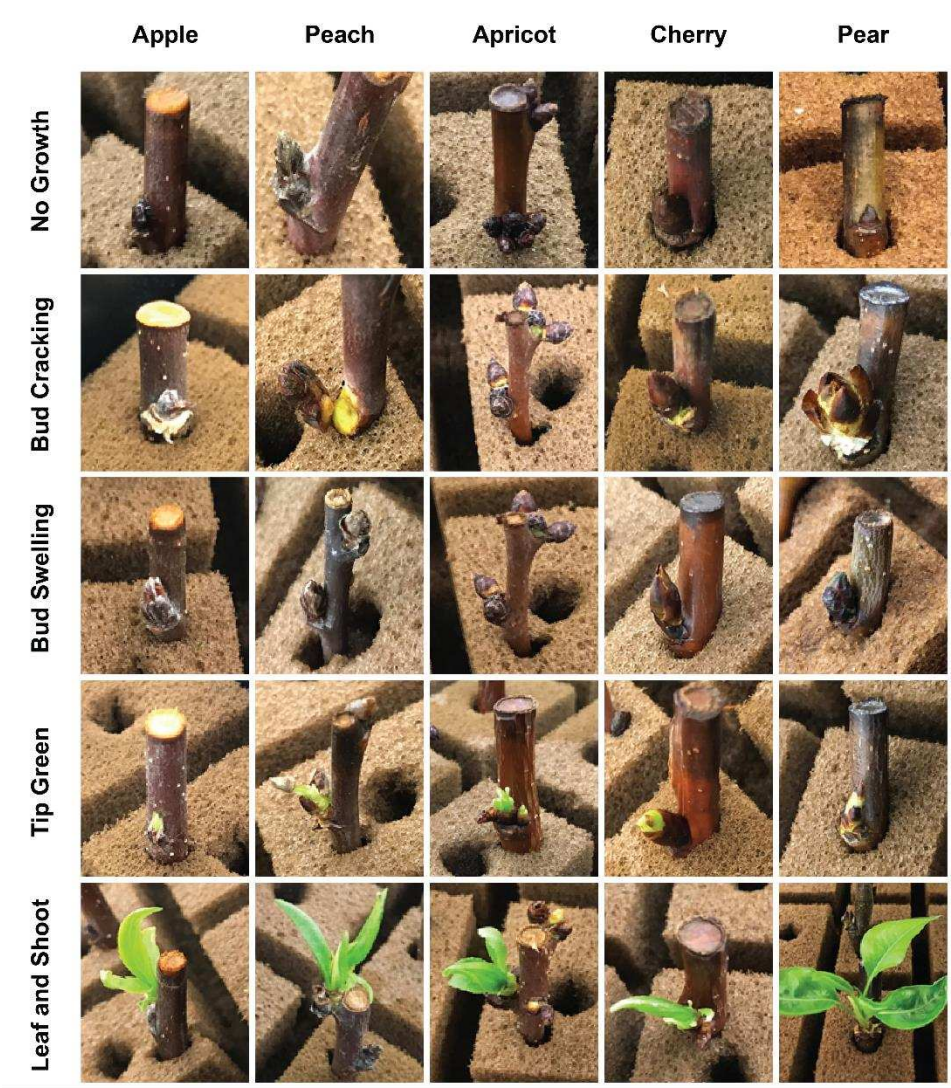

FIGURE 3.1. Visual diagram of developmental outcomes (Y axis) of dormant winter twig segments after liquid nitrogen exposure recovered under forced bud development conditions illustrating distinctions used for evaluating development potential after cryopreservation. Five developmental stages where recognized in this study; no growth, bud cracking, bud swelling, tip green, and leaf and shoot. Developmental stages are shown for apple (cv. 'Gala'), peach (cv. 'Cresthaven'), apricot (cv. 'Tilton'), Sweet cherry (cv. 'Bing'), and European pear (cv. 'Bartlett').

TISSUE CULTURE INDUCTION

Cultivated apple, peach, pear, apricot, and sweet cherry were targeted for this experiment. At least three shoot tips from each fruit species that showed growth in FBD were excised after no further growth was expected. Cultures were established after surface sterilizing shoot tips in 0.525% sodium hypochlorite for 10 minutes and rinsing 3 times in sterile deionized water. After surface sterilization, explants were plated on Linsmaier and Skoog pH Buffered Basal Salts Product: LSP03-100LT (Caisson Laboratories Inc, Smithfield, Utah). Explants were cultured under Philips Greenpower TLED lights at a temperature of 26 °C±1 and relative humidity of 32.5%±22. Tissue cultures were evaluated after 4 weeks and considered successful when shoot tips resumed growth.

STATISTICAL ANALYSIS

A classification system of five distinctive developmental outcomes was applied to each twig segment individually and used to convert observed recovery outcomes into numeric values (0-4) for statistical analysis. No growth of the dormant bud was scored as 0. Bud cracking was scored as 1. Bud swelling was scored as 2. Tip green developmental stage was scored as 3. Leaf and shoot development were scored as 4. The Mann-Whitney test was used to compare developmental outcome differences among post cryogenic methodologies across all species studied. Statistical analysis and graph artwork were conducted using GraphPad Prism v8.1.2 for Windows (Graph Pad Inc., San Diego, CA, USA). Observed recovery outcome values where converted to percentage using fraction of the total calculation for graphical representation.

RESULTS AND DISCUSSION

8-HQC ANTIMICROBIAL FORCING SOLUTION IMPROVES RECOVERY OF CRYOSTORED BUDWOOD

Exposure to LNV, as well as subsequent rewarming and rehydration, is very stressful to plant cells; expanding water inside tissues may deform and damage the functionality of membranes during recovery, causing cellular leakage and oxidation of phenolic compounds after thawing [15,29]. While desiccation and slow freezing may mitigate lethal intracellular freezing, some amount of damage is unavoidable. Stressed plant tissues are predisposed to infections and disease development [17]. Microbial attacks during recovery of LNV-exposed dormant budwood can limits regrowth potential and rot twig segments.

In FBD using high humidity alone, excessive amounts of fungal growth were observed on budwood segments. Cobweb mold and blue/green sporulating mold were observed to colonize the top cut surface of the twig segments and engulf the bud and lenticels, with the worst infections covering the entire surface of the bark. The presence of these decomposers indicated that the twig segments were in decay. All segments regrown under high humidity alone had some amount of mold (Figure 3.2A); in contrast, almost none of the twig segments treated with the antimicrobial forcing solution were observed to be colonized by molds after 6 weeks of recovery (Figure 3.2B).

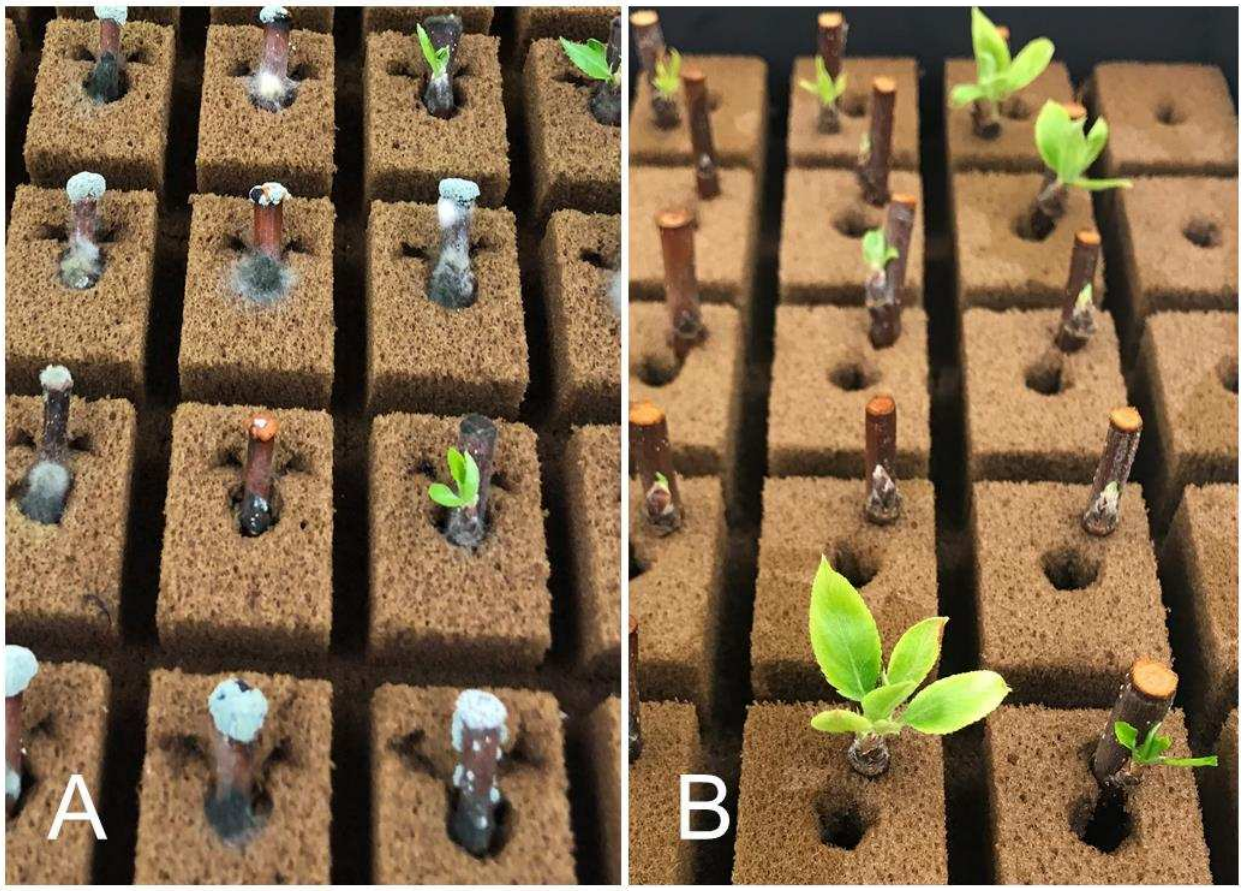

FIGURE 3.2. ‘Gala’ apple twig segments after LNV exposure under forced bud development: (A) grown under high humidity alone (STD), and (B) using an HQC antimicrobial forcing solution.

Antimicrobial treatment of buds during cryorecovery aids survival by discouraging colonization of microbes in the xylem of twig segments [1]. By precluding microbial decomposers from colonizing the plant material during this critical period, tissues have time to recover from the stress of desiccation and damage to cell walls, as well as the oxidation of phenolic compounds that leak out of damaged cells.

In six out of eight species tested, the signs of regeneration (combined bud cracking, bud swelling, tip greening, and leaf/shoot development) were consistently higher under the antimicrobial forcing solution, compared to the standard FBD method (Figure 3.3). Two species, English walnut and

black walnut, showed no signs of regrowth after LNV exposure under either post-cryopreservation recovery condition; for this reason, data from these two species are not presented herein.

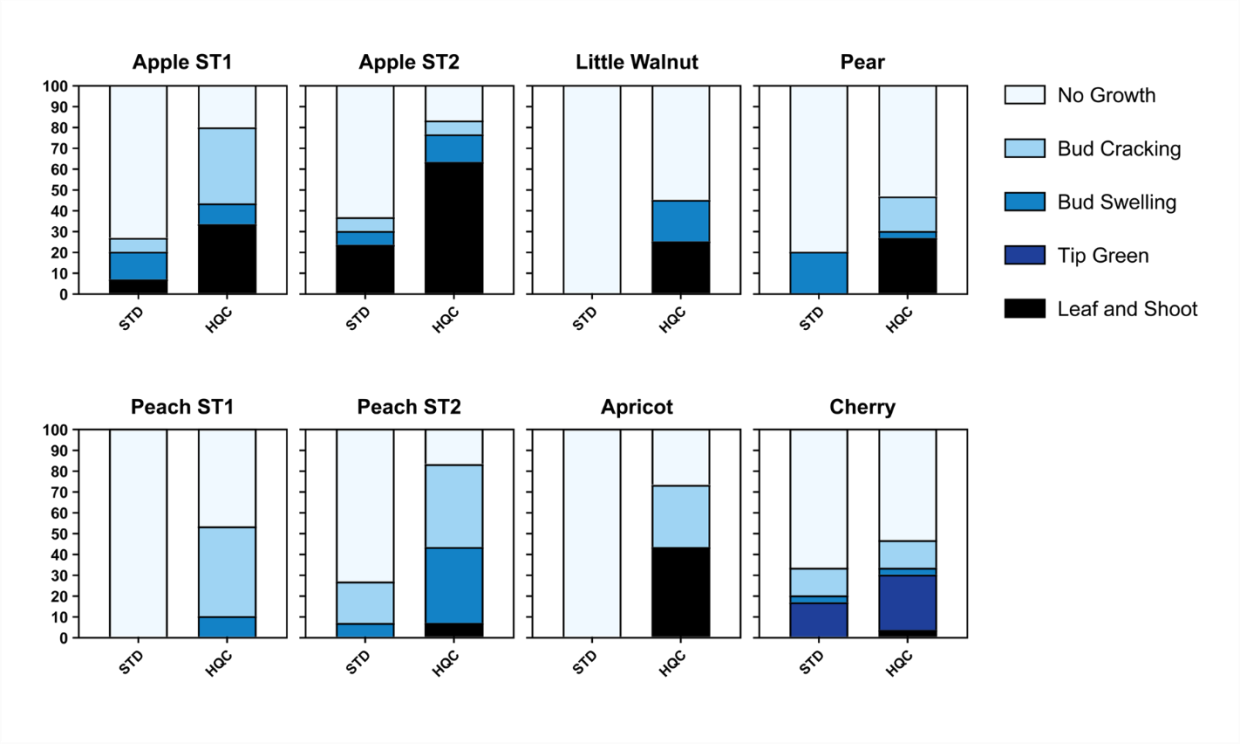

FIGURE 3.3. FBD recovery outcomes of cryoprocessed dormant winter twig segments under standard high humidity (STD), or in 8-Hydroxyquinoline citrate and sucrose forcing solution (HQC) post cryo recovery strategies. Some crops were tested from two harvests, distinguished by ST1(January 4,2018) or ST2 (January 15, 2018).

Percent viability (tip greening and leaf/shoot development) was compared between the material grown under the standard condition versus that grown with the HQC antimicrobial forcing solution. A Mann-Whitney test showed significantly different outcomes between the two conditions (P= 0.0005), with greater regeneration when the FDB solution was enriched with the antimicrobial 8-HQC and sucrose (Table 3.2).

TABLE 3.2. Mann-Whitney *U* test (two-tailed) of statistical differences between dormant buds recovered using 8-HQC forcing solution and standard (STD) recovery using high humidity alone.

	Mann-Whitney *U* test outcome
Median value of STD (%)	0 (n=10)
Median value of HQC (%)	28.3 (n=10)
Exact *P* value	0.0005 (***)
Actual difference	28.3
Hodges-Lehmann difference	26.7

***, significant difference at the level of 0.001.

For pear, little walnut, peach, and apricot, viability was only observed in the presence of the 8-HQC in the FDB solution (Figure 3.3). The results of this study suggest that material such as little walnut, apricot, sweet cherry and peach, which have previously been evaluated as non-viable through conventional FBD tests at NLGRP (unpublished data), may have low levels of survival if recovered with an antimicrobial forcing solution. This approach may therefore be used as a tool to improve further research into the cryopreservation of woody crops that have historically had little to no success under the methodology developed for apple.

Apple and sweet cherry were the only crops that had viable dormant budwood under standard FBD conditions following cryopreservation. In sweet cherry, the antimicrobial forcing solution increased viability from 16.7% to 36.7%. In apple, an increase in viability from 6.7% to 33.3% was observed for ST1, and 23.3% to 63.3% for ST2.

The results of the apple trials suggest that antimicrobial forcing solutions could be used to further optimize existing protocols. While grafting is still often used for cryoviability testing, the success shown in this study suggest that FBD could be used as an efficient and viable alternative. Further study is, however, needed to provide a direct recovery rate comparison between grafting and FBD.

The higher viability scores for segments recovered in an antimicrobial forcing solution compared to those recovered under standard FBD, suggests that some material stored at NLGRP may in fact have a higher viability than first reported. As the use of forcing solution in recovery is a post-recovery treatment, it can thus be applied to dormant twig segments already processed and stored in LNV from previous years of processing at the NLGRP.

While the 8-HQC forcing solution seemed to improve the overall recovery of LNV-exposed dormant buds, a major recovery issue was tissue cracking occurring on tissues supporting the bud. Bud cracking was most severe in peach where callus tissue production resulted in the complete separation of the bud from the stem. This was a major limiting factor in peach cryorecovery, with 43% of ST1 and 40% of ST2 impacted by bud cracking. All species that showed any signs of regrowth also had some segments affected by bud cracking.

We hypothesize that tissue cracking observed in cryorecovered dormant budwood relates to differential drying rates across tissue types during desiccation preconditioning. One study looking at the effect of desiccation on cultivated apple cryosurvival found that budwood damage increased when moisture content was brought below 40%; they subsequently applied a sucrose-alginate stabilization treatment before desiccation, which reduced desiccation damage and increased viability [18]. Stabilization treatments can secure uniform desiccation across tissue types and potentially lead to the preclusion of this type of damage, and further increase the viability of material grown using an 8-HQC forcing solution.

IN VITRO ESTABLISHMENT OF LNV-EXPOSED SHOOTS

As field derived buds are prone to contamination in tissue culture, grafting was seen as the best option for dormant bud cryorecovery [24]. Based on the research of Yang and Read [33] that suggested that new growth produced under 8-HQC and sucrose could be used for *in vitro* establishment, our work expanded this scope to include complete plant recovery of cryoprocessed dormant buds. In this study, LNV-exposed buds were recovered under FBD and successfully introduced into tissue culture for all five temperate fruit species tested: apple, peach, apricot, pear, and sweet cherry (Figure 3.4). Tissue culture establishment considered successful when shoot tips resumed growth with at least two to three leaves present after 4 weeks in cultures free of microbes.

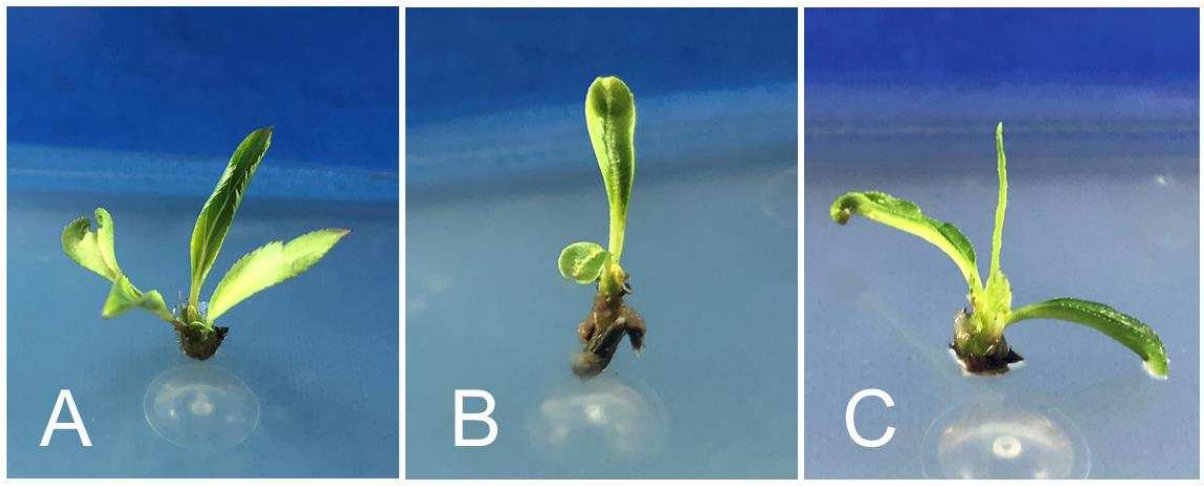

FIGURE 3.4. Shoot tips established *in vitro* from excised buds of LNV-exposed twig segments of (A) 'Gala' apple, (B) 'Bartlett' pear, and (C) 'Cresthaven' peach.

We found that the presence of the antimicrobial solution made surface sterilization possible and allowed us to establish tissue cultures from all shoot tips (three replicates) and fruit species used in this intermittent and preliminary study. The practical implications of this finding are that the FBD method using a forcing solution of 8-HQC and sucrose can be used, not only as a viability test, but additionally to induce a complete plant recovery system for cryopreserved dormant winter twig segments. It has been reported that forcing solution can also be combined with plant growth

regulators to further increase shoot development and proliferation of shoots for culture induction [5,14,32].

Additional studies focused on *in-vitro* establishment rates of LNV stored twig segments would be useful. Since, the first objective of this study was to observe the effect of FBD conditions on recovery of LNV-exposed budwood, *in vitro* establishment of rescued shoot tips occurred after the ideal timing of culture induction. As shoots began to stall and no further growth was expected, shoots were surface sterilized and plated onto sterile media to investigate the feasibility of *in vitro* establishment of dormant buds recovered using 8-HQC forcing solution. Future work focusing on *in vitro* cultivation would ideally utilize shoots in their optimal stage of growth for the purpose of quantifying tissue culture establishment.

Overall, we found that culture induction was possible when an 8-HQC forcing solution was applied. This technique holds promise for improving the efficiency of dormant budwood cryopreservation protocols. Surface sterilization of shoot tips produced in FBD on an as needed basis allows recovery work to be performed anytime of the year, as dormant winter twig segments are held in LNV until recovery can be accomplished according to workflow needs. The added utility of being able to quickly micro propagate and multiply material that was initially stored as dormant bud segments may further serve as a tool that germplasm curators can use to manage their collections and preserve stored propagules.

CONCLUSION

While dormant budwood cryopreservation is a promising method of preserving temperate clonal tree fruit species, more work is needed to tailor the method to new species. There are many important factors affecting survival and any improvement in survival outcomes is beneficial. As the process of cryostorage is stressful to plant cells, they are extremely vulnerable to attack during the recovery stage after LNV exposure. We have found that reducing the microbial pressure during this critical stage can significantly increase bud survival for many different temperate tree fruit species. Our improved FBD approach, can become an efficient alternative to grafting for viability assessment. Furthermore, when cryopreserved budwood is recovered with an antimicrobial forcing solution, culture induction becomes possible. By uniting the viability assessment tool with a germplasm recovery system, curators and preservation germplasm units may have better options tailored to managing their valuable and limited collections.

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CHAPTER FOUR: ANTIMICROBIAL FORCED BUD DEVELOPMENT CAN LEAD TO COMPLETE PLANT RECOVERY FROM CRYOPRESERVED POME AND STONE FRUIT DORMANT BUDS

OVERVIEW

Cryopreservation allows for the secure storage of agriculturally important genetic resources. Tree fruit cultivars are highly heterozygous and cannot be stored in seed form requiring the preservation of shoots from field or in vitro collections. Dormant bud cryopreservation is a time and labor efficient option of cryo-storage compared to *in vitro* derived shoot tips for temperate deciduous species. This method utilizes twigs from field collections harvested during winter dormancy in the volume needed for secure preservation to quickly protect vulnerable germplasm against loss compared to the lengthy process of establishment, multiplication, meristem excision and cryopreservation of *in vitro* storage methods. Recovery of dormant buds by grafting or tissue culture has been the primary methods of reestablishment after liquid nitrogen storage but have their own challenges and disadvantages. Grafting is laborious and results are variable, influenced by grafter consistency, rootstock suitability and the regrowth environment. Tissue culture recovery

allows for greater control of variables which may contribute to regrowth but is more complex. *In vitro* recovery is reliant on specialized media to support the shoot development from diverse genotypes of a given genera or species and may not be adequate for all. Viability tests aim to address the question of regrowth potential while reducing labor demands associated with full plant recovery. Viability tests such as tissue staining, tissue browning assessment, electrolyte leakage and bud sprouting have been used to estimate survival but may not relate to actual recovery rates for grafting or culture induction. After cryopreservation dormant budwood is in a state of stress caused by cellular desiccation and freeze induced damage to cell membranes increasing vulnerability to attach by microbes. By modifying bud sprouting with an antimicrobial agent, 8-hydroxyquinoline citrate, and sucrose, regrowth after cryopreservation has increased for several clonal fruit species and cultivars compared to sprouting in high humidity alone. When antimicrobial forced bud development is used as an intermediate step in recovery between liquid nitrogen storage and *in vitro* shoot culture, establishment has improved for most genotypes tested. This report investigates the utility of antimicrobial forced bud development in several genotypes of apple and sour cherry as well as an apricot, a peach and a pear cultivar to assess the usefulness of this approach to a wide array of important temperate fruit crops for viability assessment and culture establishment after cryopreservation.

INTRODUCTION

Cryopreservation allows for the secure storage of agriculturally important genetic resources and is especially useful for storage of clonally propagated crops that cannot be stored in seed form, such as tree fruit cultivars or other highly heterozygous crops [3]. Germplasm of clonally propagated crops must be stored as dormant buds or shoot tips, either because seed production will not result in reproduction of the elite clone or because seed production is not possible. Cryopreservation is complimentary to field collection efforts as it provides a duplicate backup to safeguard against loss [9].

For temperate deciduous trees, dormant bud cryopreservation is a time- and labor-efficient approach to germplasm storage. Dormant bud (DB) cryopreservation holds great promise of increased processing efficiency, benefitting large-scale preservation goals for temperate woody crops. Cryoprocessing dormant bud germplasm is ten times less expensive than *in vitro* derived shoot tip cryopreservation [9]. Budwood can be collected directly from trees during mid-winter endodormancy in the volume needed for effective germplasm storage and can be processed and stored relatively quickly. Cryopreserving tissue culture (TC) shoots requires a longer process including the establishment of sterile *in vitro* cultures using species-specific media, multiplication over several subculturing cycles, meristem excision from shoot cultures, and cryoprocessing.

To determine if a plant has been successfully cryopreserved, material must be recovered and regenerated into a plant. Complete plant recovery may be achieved by grafting the rewarmed bud onto suitable rootstock or by establishing an uncontaminated culture that is able to be

micropropagated [4]. Grafting is a common recovery method [2,6,15] but is a resource and labor-intensive process that requires suitable rootstocks, greenhouse space, tools, and a skilled grafter. One study successfully surface sterilized and initiated DB into culture before storage in liquid nitrogen (LN) [11]; this procedure does, however, mirror TC shoot preservation in many of the steps involved. In an effort to reduce the amount of processing prior to storage, some research has focused on initiating DB germplasm to TC after LN storage [1,7,8,11–13]. This approach has led to the successful recovery of many cryopreserved accessions of apple, pear, mulberry, gooseberry, and currant.

Viability tests aim to address the question of regrowth potential while reducing labor demands associated with full plant recovery. Viability tests such as tissue staining, tissue browning assessment, electrolyte leakage, and bud sprouting have been used to estimate survival but may not relate to actual recovery rates for grafting or culture induction. Antimicrobial forced bud development (AFBD) is a new method of sprouting cryopreserved buds that increases post-preservation regrowth by suppressing microbial growth; it utilizes an antimicrobial forcing solution comprised of 0.2g 8-hydroxyquinoline citrate (HQC) and 20g sucrose per liter to support healthy bud growth while suppressing microbial colonization after cryogenic storage [16]. Not only is AFBD an improved method for estimating DB regrowth potential, it holds further promise as an intermediary step to establishing clean TC with greater efficiency. As budwood is sourced from field trees, twigs are naturally covered with microbes which may invade and decompose cryostressed buds after storage and hinder culture induction efforts.

Preservation efforts are racing to prevent loss of vulnerable, rare, wild, and diverse species. Efficient and reliable methods of storing and recovering germplasm are essential to success. It is therefore crucial to research and utilize economical preservation and recovery techniques, such as

DB cryopreservation and AFBD, that may allow gene banks to successfully store greater quantities of material in less time. This research seeks to determine the utility of AFBD as a reliable indicator of cryopreserved DB regrowth potential, as well as its effect on establishing clean TC and therefore its ability to create a complete plant recovery system. This study first compares the results of AFBD viability assessments to that of TC recovery systems. To determine the effect AFBD has on the DB's ability to establish plantlets, two recovery systems are compared: A) TC induction of buds directly after rewarming, and B) TC induction of shoots produced through AFBD. Budwood in this study came from 17 genotypes spanning three genera of agriculturally important deciduous fruit trees: *Malus*, *Prunus*, and *Pyrus*.

MATERIALS AND METHODS

COLLECTION OF BUDWOOD

Dormant twigs of the previous season's growth were collected from two orchards during mid-winter dormancy. Dormant budwood from peach (*Prunus persica* L. Batsch) cv. 'Cresthaven', apricot (*Prunus armeniaca* L.) cv. 'Tilton', and pear (*Pyrus communis* L.) cv. 'Bartlett' was collected from Colorado State University at Western Colorado Research Center – Orchard Mesa (WCRC-OM) in Grand Junction, Colorado on January 6, 2020. Budwood was collected from several accession of apple (*Malus* Mill.) and sour cherry (*Prunus cerasus* L.) on the week of January 20, 2020 from US Department of Agriculture – Agriculture Research Service, Plant Genetic Resources Unit (PGRU), located in Geneva, NY. Six and seven unique accessions of apple and sour cherry, respectively, representing a diversity of genotypes was selected for this study. All

accessions tested are listed in Table 4.1. Budwood material was packaged and shipped overnight to the processing facility at US Department of Agriculture – Agriculture Research Service, National Laboratory for Genetic Resources Preservation (NLGRP) in Fort Collins, Colorado.

TABLE 4.1. Accession information, twig collection date, and source information for all 17 accessions included in this study.

ID code	Accession number	Genus	Species	Cultivar name	Collection date	Source
A1	PI 107196	*Malus*	*domestica*	Antonovka 1.5 pounds	1/20/2020	PGRU
A2	PI 588880	*Malus*	*domestica*	Granny Smith	1/20/2020	PGRU
A3	PI 588943	*Malus*	*domestica*	Liberty	1/20/2020	PGRU
A4	PI 590185	*Malus*	*domestica*	Jonathan	1/20/2020	PGRU
A5	PI 589976	*Malus*	*coronoria*	GMAL 2892	1/20/2020	PGRU
A6	PI 613813	*Malus*	*sargentii*	GMAL 397.1	1/20/2020	PGRU
P03	PI 657712	*Prunus*	sp.	Rosi de Bistrista	1/20/2020	PGRU
P06	PI 592860	*Prunus*	*cerasus*	Csengodi Csokros	1/20/2020	PGRU
P07	PI 592862	*Prunus*	*cerasus*	Maliga Emleke	1/20/2020	PGRU
P09	PI 592878	*Prunus*	*cerasus*	Balaton	1/20/2020	PGRU
P22	PI 657743	*Prunus*	*cerasus*	Tamaris	1/20/2020	PGRU
P08	PI 592872	*Prunus*	*cerasus*	Studencheskaya o.p. IV-6-15	1/20/2020	PGRU
P10	PI 657730	*Prunus*	sp.	Stevns	1/20/2020	PGRU
P12	PI 657733	*Prunus*	sp.	Early Ludwig	1/20/2020	PGRU
Apricot	PI 290818	*Prunus*	*armeniaca*	Tilton	1/6/2020	WCRC-OM
Peach	PI 673787	*Prunus*	*persica*	Cresthaven	1/6/2020	WCRC-OM
Pear	PI 267940	*Pyrus*	*communis*	Bartlett	1/6/2020	WCRC-OM

PROCESSING AND LN STORAGE

Upon arrival at NLGRP, twigs were inventoried and placed into a cold storage room set to -4 °C until they could be processed. Twigs were cut into 3.5 cm segments containing a bud positioned near the midpoint. Each accession was evaluated for initial moisture content (MC) gravimetrically on a fresh weight basis. Accession groups were air dried at -4 °C with ~75% relative humidity and weighed daily to monitor MC changes. Once segments reached an estimated 28–30% MC, they were packaged and sealed in polyolefin tubing, labeled for storage, then slow cooled at a rate of 1

°C per hour from -5 °C to -30 °C and held at -30 °C for 24 hours using a programmable controlled-rate freezer (model ZP-8, Cincinnati Sub-Zero, Cincinnati, Ohio). Due to equipment malfunction, three accessions of sour cherry, P08, P10, and P12, were heated to 34 °C just prior to the slow cool step. After 24 hours at -30 °C, DB segments were quickly transferred to an LN storage tank (MVE/Chart model XLC1830) where they were stored in the vapor phase of LN for at least two weeks.

REWARMING AND RECOVERY

Budwood segments were removed from LN storage and warmed at 4 °C for 24 hours. Material was split into three groups: A) AFBD for evaluation of shoot growth, B) direct TC recovery of the buds, and C) forced shoot development through AFBD before TC recovery (AFBD-TC). Each group contained 30 twig segments per accession.

For AFBD, DB segments were planted into Oasis Horticube media product no. 5240 (Oasis Grower Solutions, Kent, OH) containing 3 L of forcing solution. Forcing solution consisted of 200 mg L^{-1} of HQC and 20 g L^{-1} of sucrose [16]. Trays containing DB were covered with clear plastic humidity domes and placed into a growth chamber set for 21 °C and day length of 18 hours light/6 hours dark. Segments were monitored daily and forcing solution was refreshed as needed. For the AFBD viability assessment, DB segments were regularly evaluated and ranked according to the maximum level of growth achieved within six weeks. Growth outcomes were classified into one of five outcome groups: no growth, cracking near the bud, bud swelling, bud tip greening, and leaf or shoot production. This rating system is described in detail in a previous report of AFBD [16].

For direct TC recovery, the outer bud scales were carefully removed with a scalpel to reduce the incidence of contamination. After peeling, buds were excised from the twig segment by cutting through the bud axis to allow a small piece of basal tissue underneath the bud to remain during

surface sterilization. Surface sterilization was accomplished by submersing peeled and excised buds in 70% (vol./vol.) isopropanol for 45 seconds before transferring to 0.825% sodium hypochlorite solution containing two drops of Tween 20 surfactant (Sigma Aldrich, St. Louis, Missouri) for 10 minutes. After surface sterilization, buds were rinsed three times with sterile, deionized water inside a laminar flow hood. Buds were then given a fresh cut at the base to remove tissue damaged by surface sterilization before plating on sterile species-specific media.

For AFBD-TC, the same initiation steps were used as the material in the AFBD viability assessment. Buds were monitored daily for growth and were excised as soon as leaf and shoot development became apparent. These shoots underwent the same surface sterilization, plating process, and media type as the material used in direct TC recovery.

For both TC recovery groups, material was replated onto fresh media after browning appeared due to phenolic oxidation, 24 hours after planting. This replating was repeated as necessary every 24 hours until discoloration from oxidized phenolic compounds was no longer observed. Cultures remaining free from microbial contamination were subcultured onto fresh media after four weeks. Material was evaluated as either contaminated, clean, or clean and growing eight weeks after TC initiation.

MEDIA

Apple and pear accessions were cultured on full strength Murashige & Skoog basal medium with vitamins supplemented with 30 g sucrose, 0.5 mg 6-benzylaminopurine, 0.1 mg indole-3-butyric acid, and 7.5 g agar per liter of media. The pH was adjusted to 5.8 before autoclaving. This media formulation was developed at NLGRP. For peach and sour cherry, plant material was cultured on media containing full strength Murashige & Skoog basal medium with half-strength macronutrients and full-strength micronutrients and supplemented with 30 g sucrose, 0.25 mg 6-

benzylaminopurine, 0.25 mg kinetin, 0.05 mg gibberellin A_3, and 7.5 g of agar per liter of media. The pH was adjusted to 6.0 before autoclaving. This media formulation was developed at NLGRP. Apricot was cultured on full strength Murashige & Skoog basal medium with vitamins supplemented with 30 g sucrose, 0.5 mg 6-benzylaminopurine, 0.01 mg 1-naphthaleneacetic acid, 0.5 mg gibberellin A3, and 7.5 g of agar per liter of media [10].

STATISTICAL ANALYSIS

Statistical analyses were conducted using GraphPad Prism 8.3. Individual observations of growth in AFBD were converted to show percent of total (%). Two-way ANOVA was conducted on AFBD data to identify sources of significant variation. Analysis of correlation was preformed using Pearson correlation coefficients to investigate the relationship to growth outcomes observed in AFBD and TC establishment from AFBD sprouted buds. Contamination rates in culture establishment form direct TC and AFBD-TC were analyzed using a paired, two-tailed, T-test. All graphs were produced using GraphPad Prism 8.3.

RESULTS

SPROUTING IN ANTIMICROBIAL FORCED BUD DEVELOPMENT

All species tested were capable of sprouting and growing in AFBD after storage in LN (Figure 4.1). Shoot growth was observed in all six apple accessions tested. Of the apple, A4 had the lowest regrowth in AFBD with only 24% of dormant buds forming shoots with leaves, while A2 and A6 showed the highest regrowth potential at 93% shoot and leaf formation (Figure 4.2). Six of the eight sour cherry accessions were able to produce shoots with leaves after LN exposure. The two

that did not sprout in AFBD, P08 (*P. cerasus* cv. 'Studencheskaya o.p. IV-6-15') and P10 (*P.* sp. Cv. 'Stevns'), along with one that did, P12 (*P. cerasus* cv. 'Early Ludwig'), were exposed to 34 °C just prior to slow cooling due to equipment malfunction. The highest regrowth potential observed in sour cherry was 90% in both P06 and P22 (Figure 4.2).

For apricot, 67% of buds were able to produce shoots with leaves (Figure 4.2). As the buds of apricot are located close together, each 3.5 cm segment had several buds. On surviving segments, several buds developed into shoots, increasing the number of shoots available for culture induction. Pear had the highest rate of growth potential observed in this study with 100% of buds forming shoots with leaves (Figure 4.2). For peach, 33% of DB formed a shoot with leaves in AFBD (Figure 4.2).

Along with new vegetative shoots, several accessions produced flowers in AFBD including apple A1, A5, and A6, and cherry P03 and P22 (Figure 4.3). Floral buds are developed on budwood during the previous season and are more cold-sensitive than vegetative buds. Flowers come from mixed buds in apple and cherry, with shoots coming from lateral meristems below the apical flower buds. Not all buds which flowered produced shoots. Flower buds were not used for establishing shoot cultures. Whenever present, flowers were removed from shoots before shoot culture establishment. The ability to obtain shoots from flower buds was not tested.

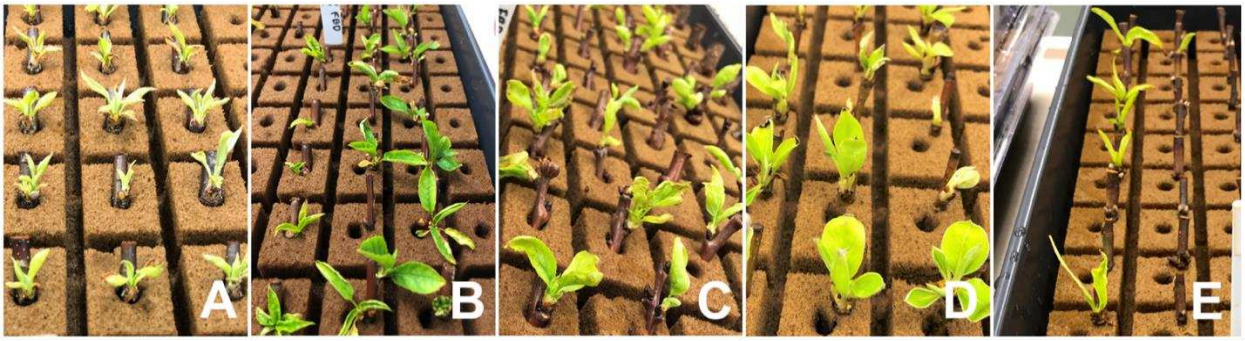

FIGURE 4.1. Sprouting LN-exposed dormant buds of A) apple, B) sour cherry, C) apricot, D) pear, and E) peach buds in antimicrobial forced bud development.

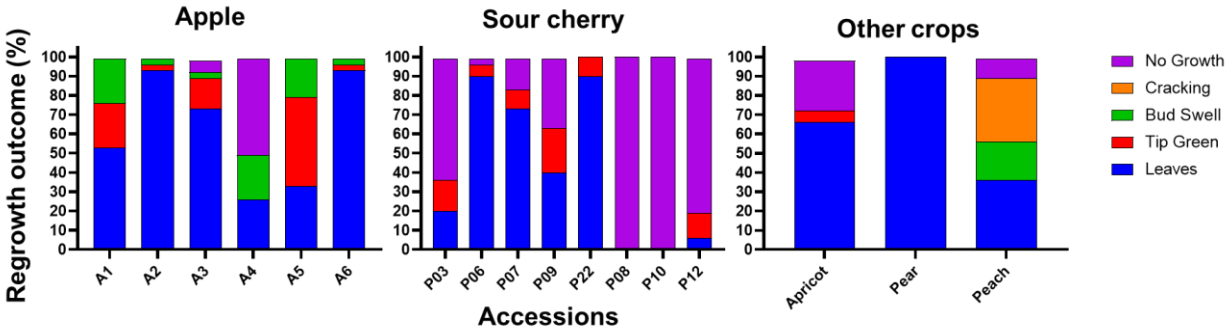

FIGURE 4.2. Growth outcomes LN-exposed dormant buds recovered by antimicrobial forced bud development. Final assessment of shoot development was conducted 6 weeks after planting. n=30.

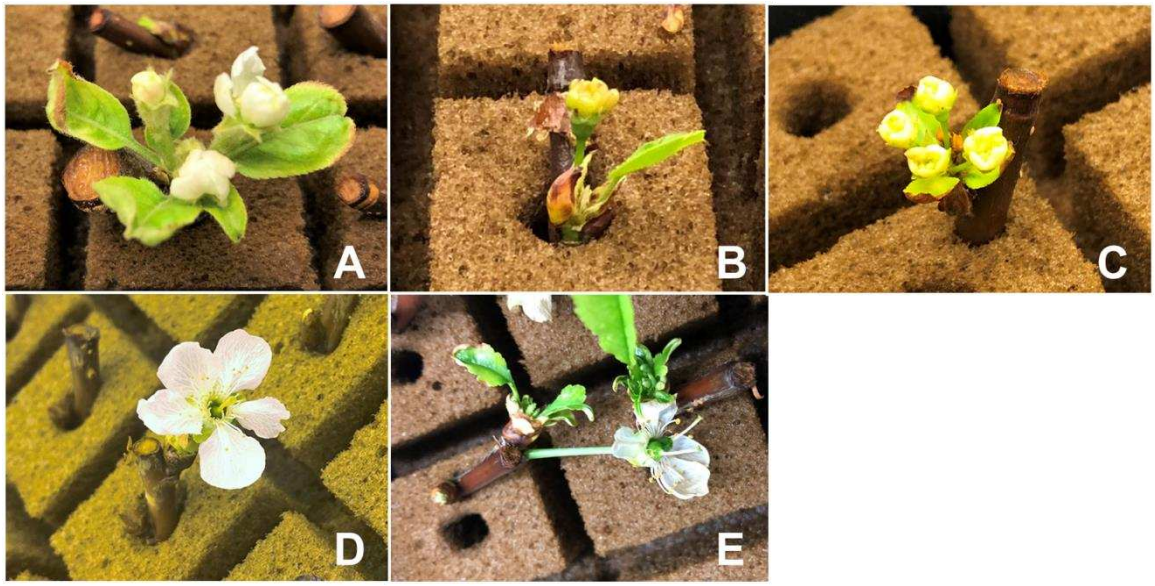

FIGURE 4.3. Flower growth in LN-exposed dormant buds under antimicrobial forced bud development in three apple and two sour cherry accessions. A) A1, *M. domestica* cv. 'Antonovka 1.5 pounds', B) A5, *M. coronoria* 'GMAL 2892', C) A6, *M. sargentii* 'GMAL 397.1', D) P03, *P.* sp. cv. 'Rosi de Bistrista', and E) P22, *P. cerasus* cv. 'Tamaris'.

CULTURE ESTABLISHMENT

Two culture initiation methods were attempted, direct culture after thaw and culture of sprouted shoots produced using AFBD. Using direct culture after cryostorage, the establishment of sterile actively growing shoot cultures was not possible. Although a few buds did begin growing after surface sterilization shoot cultures were not able to be established and maintained through direct TC (Table 4.2). Many buds began early development, showing bud swelling and tip greening development stage before ceasing further growth. Contamination also contributed to a lack of establishment of shoot cultures (Figure 4.4). Contamination was higher for buds directly cultured compared to AFBD shoots in all species tested except *Malus,* in which contamination level was not significantly different in either method.

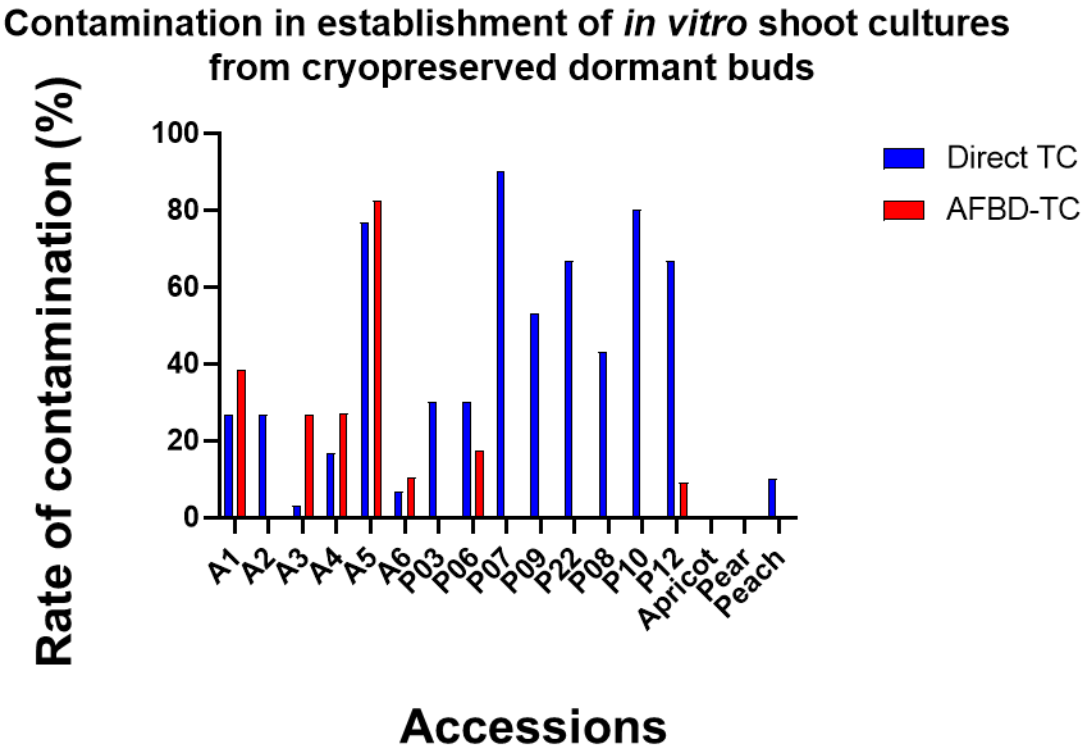

FIGURE 4.4. Contamination in shoot cultures from LN-exposed dormant buds. Cultures were induced from dormant buds that were either cultured directly after thawing (Blue) or cultured after sprouting using antimicrobial forced bud development preconditioning (Red).

Compared to the direct TC approach, buds that underwent and AFBD preconditioning stage were much more capable of producing clean cultures that could be maintained through several culture cycles. Fourteen out of the seventeen accessions tested in this study were capable of producing sterile, actively growing plants through the AFBD-TC recovery method (Table 4.2). Sterile actively growing plants were distinguished from plants that were contaminated with microbial growth or had died and were no longer capable of shoot growth which could be used for micropropagation.

TABLE 4.2. Percentages of clean cultures and growing cultures established from LN-exposed dormant buds. Direct culture of thawed buds compared to culture induction of shoots produced in antimicrobial forced bud development. 30 DB segments were recovered for each group, however, only buds sprouted in AFBD were initiated into culture in the AFBD-TC method.

		Direct TC			AFBD-TC		
ID code	**PI number**	**Total number of buds**	**Clean (%)**	**Growing + Clean (%)**	**Total number of buds**	**Clean (%)**	**Growing + Clean (%)**
A1	107196	30	73.3	0.0	26	61.5	3.8
A2	588880	30	73.3	0.0	29	100.0	10.3
A3	588943	30	96.7	0.0	30	73.3	6.7
A4	590185	30	83.3	0.0	11	72.7	18.2
A5	589976	30	23.3	0.0	23	17.4	0.0
A6	613813	30	93.3	0.0	29	89.7	82.8
P03	657712	30	70.0	0.0	11	100.0	9.1
P06	592860	30	70.0	0.0	23	82.6	4.3
P07	592862	30	10.0	0.0	24	100.0	54.2
P09	592878	30	46.7	0.0	9	100.0	33.3
P22	657743	30	33.3	0.0	19	100.0	94.7
P08	592872	30	56.7	0.0	-	-	-
P10	657730	30	20.0	0.0	-	-	-
P12	657733	30	33.3	0.0	11	90.9	27.3
Apricot	290818	30	100.0	0.0	30	100.0	0.0
Pear	267940	30	100.0	0.0	30	100.0	100.0
Peach	673787	30	90.0	0.0	10	100.0	30.0

While the number and maturity of shoots varied by accession, all six apple produced shoots in AFBD that could be used for TC (Figure 4.5). Five of the six apple accessions were successfully established and maintained in TC (Figure 4.6). The only apple accession not possible to maintain was A5 (*M. coronoria* 'GMAL 2892'). As a wild species of Malus, A5 may not have been adequately supported by the growth medium, resulting in shoot collapse and dieback of uncontaminated shoot cultures. Establishment rates in apple ranged from 0% for A5 to 82% for A6 (*M. sargentii* 'GMAL 397.l') (Table 4.2).

FIGURE 4.5. Shoot development of LN-exposed apple DB under antimicrobial forced bud development four weeks after planting. All apple tested developed shoots with leaves. New growth from sprouted buds used to establish *in vitro* cultures. A) A1, *M. domestica* cv. 'Antonovka 1.5 pounds', B) A2, *M. domestica* cv. 'Granny Smith', C) A3, *M. domestica* cv. 'Liberty', D) A4, *M. domestica* cv. 'Jonathan', E) A5, *M. coronoria* 'GMAL 2892', and F) A6, *M. sargentii* 'GMAL 397.1'.

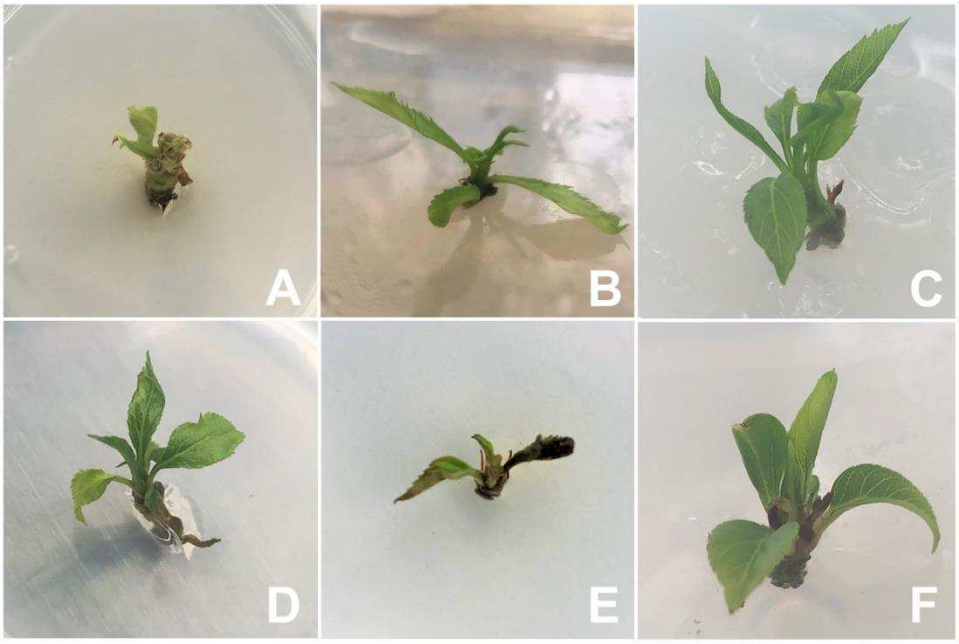

FIGURE 4.6. LN-exposed apple buds sprouted through AFBD and induced into tissue culture. All apple accessions successfully established uncontaminated and actively growing shoot cultures through AFBD-TC. A) A1, *M. domestica* cv. 'Antonovka 1.5 pounds', B) A2, *M. domestica* cv. 'Granny Smith', C) A3, *M. domestica* cv. 'Liberty', D) A4, *M. domestica* cv. 'Jonathan', E) A5, *M. coronoria* 'GMAL 2892', and F) A6, *M. sargentii* 'GMAL 397.1'.

Six of the eight sour cherry accessions produced shoots in AFBD that were suitable for TC induction (Figure 4.7). The two that did not sprout in AFBD, P08 and P10, may have experienced reduced survival due to exposure to 34 °C during cryoprocessing. All six sour cherry accessions that produced shoots in AFBD established sterile shoot cultures (Figure 4.8). TC establishment rates ranged from 4.4% for P06 (*P. cerasus* cv. 'Csengodi Csokros') to 94.7% for P22 (*P. cerasus* cv. 'Tamaris') (Table 4.2).

FIGURE 4.7. Shoot development of LN-exposed sour cherry DB under antimicrobial forced bud development four weeks after planting. Six accessions developed shoots with leaves. New growth from sprouted buds used to establish *in vitro* cultures. A) P03, *Prunus* sp. cv. 'Rosi de Bistrista', B) P06, *P. cerasus* cv. 'Csengodi Csokros', C) P07, *P. cerasus* cv. 'Maliga Emleke', D) P09, *P. cerasus* cv. 'Balaton', E) P12, *P.* sp. cv. 'Early Ludwig', and F) P22, *P. cerasus* cv. 'Tamaris'.

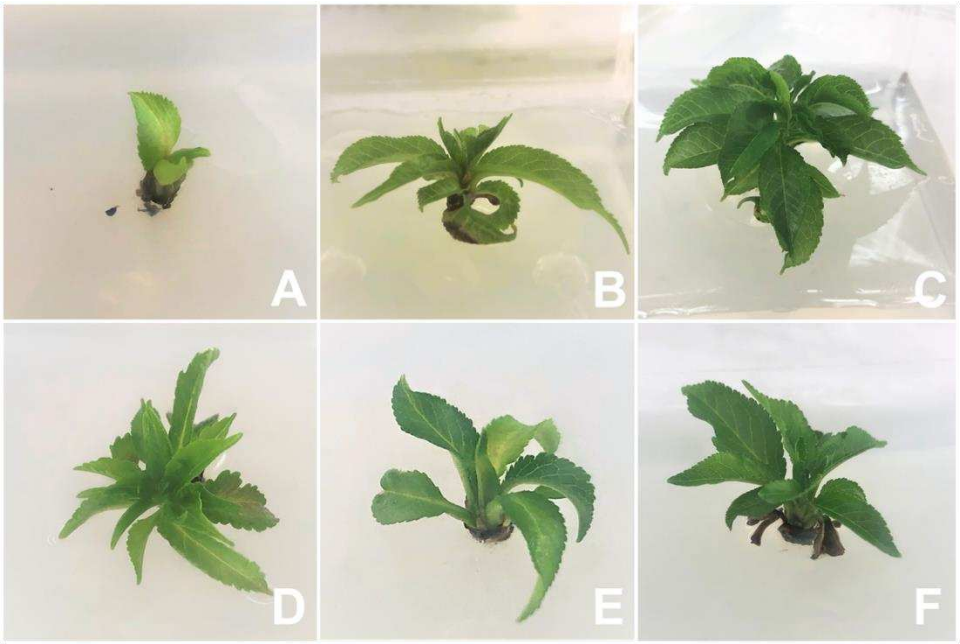

FIGURE 4.8. LN-exposed sour cherry buds sprouted through AFBD and induced into tissue culture. All sour cherry accessions that grew in AFBD successfully established uncontaminated and actively growing shoot cultures through AFBD-TC. A) P03, *Prunus* sp. cv. 'Rosi de Bistrista', B) P06, *P. cerasus* cv. 'Csengodi Csokros', C) P07, *P. cerasus* cv. 'Maliga Emleke', D) P09, *P. cerasus* cv. 'Balaton', E) P12, *P. sp.* cv. 'Early Ludwig', and F) P22, *P. cerasus* cv. 'Tamaris'.

All LN-exposed buds of apricot, pear, and peach produced shoots under AFBD (Figure 4.9). In apricot (*P. armeniaca* cv. 'Tilton'), uncontaminated and actively growing shoot cultures were initially established (Figure 4.10) but could not be maintained long-term on the culture medium that was used. Dieback occurred during the two four-week culture cycles, resulting in 0% apricot after eight weeks (Table 4.2). All segments of pear (*Pyrus communis* cv. 'Bartlett') produced shoots under AFBD, and all 30 shoots produced clean and actively growing cultures (Table 4.2, Figure 4.10). This was the highest establishment rate seen in this study for all accessions tested. In peach (*P. persica* cv. 'Cresthaven'), uncontaminated shoot culture establishment was initially high, however, shoot growth was slow and dieback was observed in several actively growing explants, suggesting a suggesting a suboptimal medium formulation for this cultivar. After two culture cycles 30% of peach were established in TC (Table 4.2, Figure 4.4).

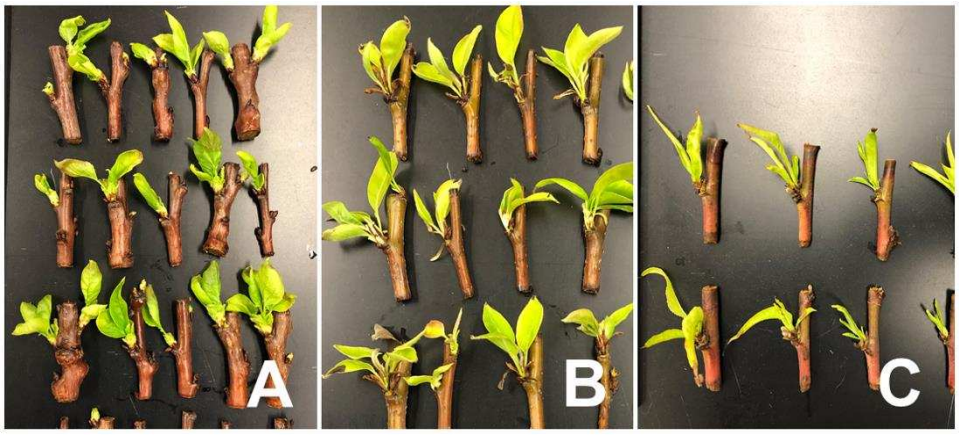

FIGURE 4.9. Shoot development of LN-exposed A) apricot, *Prunus armeniaca* cv. 'Tilton', B) pear, *Pyrus communis* cv. 'Bartlett', and C) peach, *Prunus persica* cv. 'Cresthaven' under antimicrobial forced bud development four weeks after planting. All three species developed shoots with leaves. New growth from sprouted buds used to establish *in vitro* cultures.

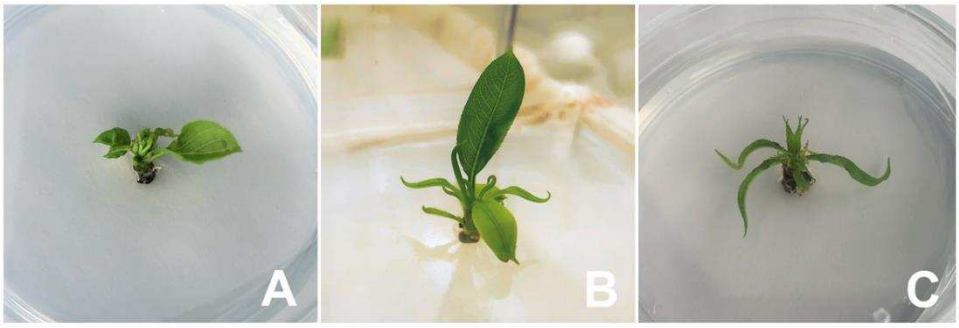

FIGURE 4.10. LN-exposed buds sprouted through AFBD and induced into tissue culture: A) apricot, *Prunus armeniaca* cv. 'Tilton', B) pear, *Pyrus communis* cv. 'Bartlett', and C) peach, *Prunus persica* cv. 'Cresthaven'. Apricot, pear, and peach accessions successfully established uncontaminated and actively growing shoot cultures through AFBD-TC.

Establishment of shoot cultures through AFBD-TC recovery was significantly ($P < 0.05$) correlated with the development of shoots with leaves in the AFBD viability assessment (Table 4.3). Buds that only developed to tip green, bud swell stage, or did not develop were not significantly correlated with establishment of *in vitro* shoot cultures.

Shoot culture contamination rate was significantly ($P < 0.01$) less in AFBD-TC compared to direct TC for sour cherry and peach. Both apricot and pear were free from contamination in both methods.

For apple, there was no significant difference in contamination rate. Contamination rates for both recovery treatments are shown in Figure 4.10; rates are reported as a percentage of the total number of samples initiated into TC (n=30 for direct TC, variable from 0 to 30 in AFBD-TC).

TABLE 4.3. Correlation between establishment of shoot cultures and growth outcomes in antimicrobial forced bud development. Data from 17 accession of cryopreserved pom and stone fruit budwood recovered using antimicrobial forced bud development. For each accession 60 twig segments were used for sprouting; 30 to evaluate regrowth potential of shoots at six weeks after planting, and 30 for culture induction four weeks after planting.

	AFBD-TC Est % vs. Leaves	AFBD-TC Est % vs. Tip Green	AFBD-TC Est % vs. Bud Swell	AFBD-TC Est % vs. No Growth
Pearson r				
r	0.5374	-0.2289	-0.2288	-0.4446
95% confidence interval	0.07653 to 0.8091	-0.6392 to 0.2828	-0.6391 to 0.2830	-0.7623 to 0.04590
R squared	0.2888	0.05241	0.05234	0.1976
P value				
P (two-tailed)	0.0261	0.3768	0.3771	0.0738
P value summary	*	ns	ns	ns
Significant? (alpha = 0.05)	Yes	No	No	No
Number of XY Pairs	17	17	17	17

DISCUSSION

Traditionally, cryopreserved DB are recovered by grafting onto appropriate rootstocks [2,5,17]. This is a labor and resource intensive process. Directly inducing dormant buds into culture after thawing seems like a good alternative, especially for facilities that already possess tissue culture laboratories; however, this study reveals many challenges that precluded successful regeneration through this approach. Lower rates of TC establishment have been reported for DB compared to *in vitro* derived shoot tips [18]. Similarly, our study was not successful in establishing shoot cultures directly from cryopreserved dormant buds.

Direct TC initiation of cryopreserved DB results in high contamination rates, even with the additional step of removing the outer layer of bud scales. Removal of bud scales was deemed necessary for this research, as spaces in between bud scales may protect microbes from sterilization. An initial induction trial in pear showed less contamination in buds with outer scales removed than buds left intact (Unpublished data).

The AFBD-TC method did however, yield cultures that were actively growing and free from contamination (Table 4.2, Figures 4.6,4.8,4.10). As DB are produced on field trees, they may harbor more microbial inoculum than shoots produced inside a climate-controlled growth chamber under AFBD. By combining forced bud development with an antimicrobial forcing solution, recovering DB can produce new shoots without the high microbial pressure associated with freeze-induced stress [16]. Our results, particularly in sour cherry, suggest that the added step of AFBD may lead to more reliably clean cultures. Bud scale surface anatomy might also contribute to

contamination rates and could explain some of the differences in contamination levels observed for different species.

Improvement in culture establishment using AFBD could be explained in part by the isolation of healthy shoot tissue since the xylem of budwood is likely to have severe damage after cryopreservation [14]. Shoots must be excised from budwood in AFBD, surface sterilized and plated onto fresh media before visual observation of decline in the sprouting buds. The amount of shoot development attained before decline was observed to varied by species and cultivar and is highlighted in Figures 4.4, 4.6, and 4.8. The correlation between the development of shoots with leaves and the ability to establish shoot cultures suggest that there is a reliable relationship between viability assessment in AFBD and recovery results in AFBD-TC, making the former a suitable indicator of the latter.

After surface sterilization and plating, explants from apple, sour cherry, and apricot exhibited a burst in the production of phenolic compounds from the cut tissue into the media surrounding the explant as observed by a yellowish discoloration diffusing from the base of the explant into the media. Addressing phenolics by subculturing required more labor and materials to transfer explant onto fresh media as needed. Excised, surface sterilized buds were replated every 24 hours until discoloration in media was not observed. For the extreme cases as with A1 (*M. domestica* cv. 'Antonovka 1.5 pounds') and A3 (*M. domestica* cv. 'Liberty') explants were transferred four times before the media would remain clear from discoloration 24 hours later. In the future, the addition of an antioxidant, such as ascorbic acid, may improve culture establishment in accessions releasing phenolics *in vitro* [4]. Phenolics production was more persistent in buds directly initiated in culture as compared to the green shoot tips initiated from AFBD. It is reasonable to assume that phenolics released from explants into media may have been responsible for the inhibition and stalling

observed directly culture DB from cryostorage. Stalling was a major issue observed in all buds directly initiated into culture after storage but was also observed in a few shoot cultures from AFBD-TC as well. Stalling of explants may also be caused by deficiency of growth media formulations in supporting the development of shoots from diverse genotypes, some of which may respond poorly to any given formulation.

Both recovery methods in this study may have been affected by the use of suboptimal media for the individual genotypes. The use of optimized shoot culture media will enhance the success of establishment as was observed in pear (Table 4.2). The reliance on a single media formulation for the development and growth of a species with diverse genotypes presents a major disadvantage to *in vitro* recovery systems. In this study, several accessions developed well under AFBD but struggled to grow in TC, particularly A1 (*M. domestica* cv. 'Antonovka 1.5 pounds'), A4 (*M. domestica* cv. 'Jonathan'), A5 (*M. coronoria* GMAL 2892), P03 (*Prunus* sp. cv. 'Rosi de Bistrista'), apricot (*Prunus armeniaca* cv. 'Tilton'), and peach (*Prunus persica* cv. 'Cresthaven'). In the future, variations on the media may be needed to cater to needs of specific plants.

An advantage of the AFBD-TC system is flexibility. When the intent is to quickly estimate viability of cryopreserved DB germplasm in order determine whether the material is successfully preserved, AFBD can provide insight into growth potential at reduced labor expense compared to grafting or TC. AFBD regrowth after LN exposure can even be conducted without a tissue culture facility. When reestablishment of an accession is required, AFBD provides a preconditioning step to increase successful TC establishment. The results of this study confirm a previous report of culture establishment after cryopreservation using AFBD [16].

An added benefit of AFBD-TC is that it allows for efforts to be focused on material showing signs of life, unlike initiating DB directly. In this study, P08 and P10 were two accessions that did not

grow through either recovery method. This material was likely irreparably damaged prior to slow cooling. Through direct TC, media was prepared and 30 buds from each accession were peeled, sterilized, plated, and replated. Through AFBD-TC, 30 segments from each accession were placed in Oasis Horticube media and forcing solution, then left to grow in a monitored growth chamber. Because the material did not sprout, no further labor was needed to report a survival of zero.

The use of AFBD and AFBD-TC allows for efficient viability and recovery of cryopreserved dormant buds. Shoot recovery from forced buds can have a higher efficiency than one shoot per bud in compound bud structures, as was observed in some sour cherry, or may have several buds developing shoots from a single twig segment, as was observed with apricot. By effectively recovering all surviving material, more germplasm may be conserved in storage for later use in cultivation, breeding, or research.

CONCLUSION

Preservation of fruit tree germplasm can be efficiently accomplished using dormant bud cryopreservation. This approach can be used to preserve important cultivars at a large scale and easily provide a security back-up to safeguard against loss in field collections. By using AFBD as a preconditioning treatment before recovery as *in vitro* culture, uncontaminated shoot cultures can be established for a variety of genotypes of woody deciduous fruit trees. The AFBD-TC approach holds promise as a recovery method for apple, sour cherry, apricot, pear, and peach dormant buds, allowing for crucial varieties of these crops to be cryoprocessed faster and evaluated more easily in laboratory settings. While more study is needed to investigate the utility of AFBD and AFBD-TC as a means of recovering other taxa, the reduced rates of contamination and increased rates of establishment shown in this study indicate that antimicrobial forced bud development is a suitable path to complete plant recovery for cryopreserved dormant buds.

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