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CRYOPRESERVATION OF DORMANT BUDS FROM DIVERSE *Fraxinus* SPECIES

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Abstract

Ash (*Fraxinus*) is an economically important tree genus in the landscape industry, as well as a key component of North American forests, especially in the North Central United States and adjacent regions in Canada. In recent years, the Emerald Ash Borer beetle (*Agrilus planipennis)* has significantly threatened the survival of native North American *Fraxinus* species. A dormant-bud cryopreservation technique has been developed as a method to conserve specific clones of ash. Dormant buds of three ash species were successfully cryopreserved when desiccated on their stem sections to 30% moisture content (w/v) and then cooled at rates of either -1ºC/h or -5ºC/day to either -30 or -35ºC before immersion in liquid nitrogen vapor (LNV). Stem sections were removed from LNV, warmed, and rehydrated, and their buds grafted onto rootstocks to evaluate survival. Recovery percentages ranged from 34 to 100% after LNV exposure and were dependent upon accession and cooling rate. The cryopreservation methods proposed herein can complement seed-collection efforts aimed at conserving diversity, supplementing *ex situ* genebank and botanic-garden collections.

Keywords: ash, genetic resources, genebank, *Fraxinus pennsylvanica*, *Fraxinus mandshurica*, *Fraxinus chinensis*

INTRODUCTION

Ash (*Fraxinus* L.) species are important components both of the native forests of eastern North America and of managed landscapes (reviewed by 9). Diverse forest products are harvested from ash, ranging from lumber for furniture, tool handles, and baseball bats to splints for basketry (13). In urban areas, ash species are particularly important as street trees, based on a relatively small number of widely marketed (primarily staminate) clones (5, 9, 24) that are budded onto seedling rootstocks (4).

Over one billion native ash trees in the North Central United States and adjacent Canada are anticipated to become damaged or killed by the spread of the Emerald Ash Borer (EAB; *Agrilus planipennis* Fairmaire), a wood-boring beetle introduced from Northern Asia (2). Upon infestation, susceptible trees are girdled and killed within one to four years. *Fraxinus* susceptibility to EAB varies among species, with all species native to North America that have been tested showing no tolerance to this pest. In contrast, *Fraxinus* species native to Asia

that have co-evolved with EAB have varying degrees of susceptibility (1, 11). Recently, EAB was also found in European Russia (7), which threatens native European *Fraxinus* species. Wild ash populations in Europe are also under threat from deforestation, climate change, air pollution, and invasive-species competition (10). *Fraxinus* is a key component of the European Forest Genetic Resources Programme, which manages thousands of clones and seed orchards in *ex situ* collections (10).

Ash is also of interest from a phytochemical standpoint. Extracts from *Fraxinus* species have been identified with anti-inflammatory, anti-allergenic, and antimicrobial properties (8). Two new coumarins were recently identified in the bark of *Fraxinus chinensis* Roxb. (25).

It is possible to cryopreserve the dormant budwood of many temperate woody genera (12). Dormant-bud cryopreservation has been successfully implemented in genebanking programs for economically important fruits, such as *Malus* Mill. and *Prunus cerasus* L. (6, 16, 17, 20), and method development is ongoing for *Salix* L. and other genera (18, 21). When possible, dormant-bud cryopreservation is often preferred over shoot-tip protocols that are dependent upon tissue-culture systems and cryoprotectant treatments (21). Although the buds of some species can be cryopreserved without prior desiccation (15), most species are more reliably preserved after being desiccated to 20-30% (14) and then cooled slowly to between -30 and -40ºC before exposure to liquid nitrogen vapor (LNV) (12, 19).

Shoot-tip regeneration after cryopreservation of *F. excelsior* L. zygotic embryos excised from imbibed seeds was as high as 33% after LN exposure (3). In this report, we have identified a method to cryopreserve genotypes of three *Fraxinus* species by using dormant vegetative buds as source material.

MATERIALS AND METHODS

Fraxinus pennsylvanica Marshall, *F. mandshurica* Rupr., and *F. chinensis* were selected as representative species of *Fraxinus* (22, 23). In January 2007 and 2008, dormant budwood of *F. pennsylvanica* (PI 469226), *F. mandshurica* (Ames 19150), and *F. chinensis* (Ames 22266) was collected from the USDA-ARS North Central Regional Plant Introduction Station in Ames, IA, USA and was shipped overnight to the USDA-ARS-National Center for Genetic Resources Preservation in Fort Collins, CO, USA for processing. In January 2008, three additional accessions were collected: *F. mandshurica* (Ames 24101 and 26081) and *F. chinensis* (Ames 22267). Budwood was held at -5ºC (20-30% relative humidity) for up to five months until desiccation treatments began. Budwood was then cut into one-node sections (each 30 mm long, containing one pair of oppositely arranged vegetative buds) and sections were dried 4-7 d on trays in a -5ºC cooler to achieve a moisture content of 30%. Fresh weights of five sample twigs were measured daily and compared with dry weights of twigs with comparable initial moisture contents.

After desiccation, ten budwood sections were placed into 280 mm long and 19 mm diameter, clear, heat-shrinkable flexible polyolefin tubes (3M, Austin, TX), heat-sealed, and placed in aluminum cryoboxes (64 mm × 64 mm × 285 mm) for slow-cooling. Cryoboxes were cooled from -5ºC to -30 or -35ºC at -5ºC/day (ramped at -1ºC/h) in a super-cold temperature freezer (ScienTemp, Adrian, MI) as previously described (20). Cryoboxes were then transferred to LNV within a stainless steel cryotank (Cryenco, Biostat, Denver, CO). A -1ºC/h cooling rate was attained by placing cryoboxes in a liquid nitrogen (LN) cooled programmable freezer (Sigma, Model CC-3, San Diego, CA) for cooling to -30 or -35ºC before transfer to LNV. Three replicate tubes were processed independently for each LNV treatment. For some control treatments, only one tube was processed.

Bud sections were warmed after two to five months of LNV exposure. A limited number of sections collected in 2007 were also removed after 18 months and compared to earlier

removal events to determine if there was any loss of viability over time. For warming, tubes were placed at +3ºC overnight in a cold-room, and sections were then removed from the tubes and subsequently placed in damp peat moss at +3ºC in the dark for two weeks prior to budding. Dormant seedling *F. pennsylvanica* rootstocks (Lawyer Nursery, Plains, MT) were potted in conical tree-seedling nursery containers ("Deepots," Stuewe and Sons, Corvallis, OR) containing growth medium (Metromix 200, Scotts-Sierra Horticultural Products, Marysville, OH) supplemented with slow release fertilizer (Osmocote Plus 15-9-12, Scotts-Sierra Horticultural Products, Marysville, OH) and grown in the greenhouse for three to four weeks before budding. One of the hydrated buds from each one-node section was chip-budded onto rootstocks, which were then topped and rubber bands were removed after 17 d. Buds were evaluated for viability three weeks after topping. At the time of budding, the remaining bud and bark of each one-node section was sliced with a surgical razor blade for a visual rating of tissue color. The inner and outer regions and vascular tissue of the remaining buds, as well as the stem phloem, cambium, and xylem, were rated as appearing green, yellowish-green, or brown.

Mean percentages of bud survival after grafting and standard errors were calculated across experimental replicates within species. Due to limited quantities of samples available, fewer treatments were performed in 2007; statistical comparisons within accessions and among treatments were performed for treatments that were replicated, data without statistics or standard errors are provided for information only. Statistical comparisons (ANOVA, Tukey means separation tests, $P<0.05$) were made with the JMP software package (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Fraxinus buds representative of three diverse species were successfully cryopreserved by using dormant budwood techniques. Survival levels based on grafting after LNV exposure, ranged from 34 to 100% (Tables 1, 2). These levels that are higher than the survival levels published for *Fraxinus* zygotic embryos after cryopreservation (3). In 2008, buds from Ames 19150 and Ames 22267 had nearly 100% survival after LNV exposure, regardless of whether buds were cooled at a rate of 1ºC/h or -5ºC/day to either -30 or -35ºC prior to submersion in LNV. Survival levels after LNV exposure ranged from 54 to 92% for PI 469226 and between 42 and 59% for Ames 22266. Overall, the highest survival was attained in 2008 when buds were cooled to -35ºC at the slower cooling rate of -5ºC/day.

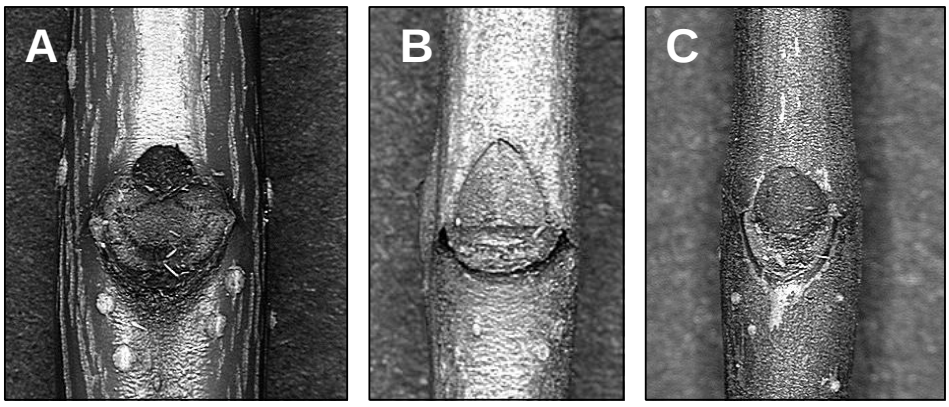

Figure 1. Dormant buds of A) *F. mandshurica*, B) *F. chinensis*, and C) *F. pennsylvanica*.

Table 1. Effect of cryopreservation method on recovery by grafting (mean %±SE) of pretreated (-LNV) or cryopreserved (+LNV) ash vegetative buds. Buds were desiccated and then cooled at the indicated rate to the indicated temperature before LNV treatment. Significant differences are indicated by letter within accessions and among treatments by year when three replicate tubes of ten buds each were evaluated.

		Cooling	Temp	2007				2008		
Species	Accession	Rate	°C	-LNV		+LNV		-LNV	+LNV	
F. mandshurica	Ames 19150	None	-4	97±3	b			100		
F. mandshurica	Ames 19150	-1°C/h	-35					100	96±4	a
F. mandshurica	Ames 19150	-5°C/day	-35	33±12	a	34±9		100	100±0	a
F. mandshurica	Ames 19150	-1°C/h	-30					80	97±3	a
F. mandshurica	Ames 19150	-5°C/day	-30					100	100±0	a
F. pennsylvanica	PI 469226	None	-4	93±4	a			100		
F. pennsylvanica	PI 469226	-1°C/h	-35	92±0	a	34±9	b	100	56±18	A
F. pennsylvanica	PI 469226	-5°C/day	-35	70±6	b	74±6	a	100	92±3	A
F. pennsylvanica	PI 469226	-1°C/h	-30					100	54±18	A
F. pennsylvanica	PI 469226	-5°C/day	-30					100	84±6	A
F. chinensis	Ames 22266	None	-4	83±3	a			40		
F. chinensis	Ames 22266	-1°C/h	-35	34±3	b	34±9	a	20	52±27	A
F. chinensis	Ames 22266	-5°C/day	-35	47±9	b	35±10	a	18	59±9	A
F. chinensis	Ames 22266	-1°C/h	-30					20	42±8	A

Table 2. Effect of cryopreservation method on recovery by grafting (mean %±SE) of pretreated (-LNV) or cryopreserved ash vegetative buds. Buds were desiccated and then cooled at the indicated rate to the indicated temperature prior to LNV treatment. Significant differences are indicated by letter among treatments within accession when three replicate tubes of ten buds each were evaluated. All buds were treated and grafted in 2008.

		Cooling	Temp			
Species	Accession	rate	°C	-LN	+LN	
F.chinensis	Ames 22267	none	-4	100		
F.chinensis	Ames 22267	-1°C/h	-35	100	100±0	a
F.chinensis	Ames 22267	-5°C/day	-35	90	100±0	a
F.chinensis	Ames 22267	-1°C/h	-30	100	100±0	a
F. mandshurica	Ames 24101	-1°C/h	-35		95±5	
F. mandshurica	Ames 26081	-1°C/h	-35	100	90±1	

Buds processed in January 2007 by cooling at -5ºC/day to -35ºC and then thawed from LNV 18 months later (as opposed to 2 to 5 months) in July 2008 for PI 469226 and Ames 22266 had 82±3 and 79±7% survival, respectively. These levels were equal to or higher than those obtained from the same treatments that were thawed in 2007 (Table 1).

Visual color ratings were performed to determine if tissue coloration could serve as a predictor for bud survival, as an alternative to the labour-intensive grafting process. In all cases, the xylem was brown after LNV exposure. However, this did not inhibit bud growth.

In many cases, the entire bud as well as the phloem and cambium of the stem remained green after LNV exposure. In some cases, there was a correlation between yellow-green coloration of the meristem and first leaf primordia, outer leaf primordia, and vascular cambium and a lack of growth after grafting, although there were also instances where the buds appeared discolored but they survived the grafting process (data not shown). We conclude that buds that were brown after LNV exposure typically did not survive, but a lack of brown coloration was not a reliable indicator of survival. Successful shoot elongation after grafting served as the most reliable test of survival after cryopreservation.

These results suggest that *Fraxinus* clones can be successfully cryopreserved when dormant budwood is collected mid-winter and subjected to slow-cooling protocols. By following this approach, vegetative buds can be conserved in LNV and then grafted onto ash rootstocks for recovery.

Fraxinus dormant-bud cryopreservation is intended to complement ongoing seed-conservation efforts. The successful results reported herein provide necessary information so that more extensive cryopreservation experiments to develop suitable protocols for all key North American *Fraxinus* species can be initiated. In this work, *F. pennsylvanica*, *F. chinensis*, and *F. mandshurica* were successfully grafted onto *F. pennsylvanica* rootstock. Further research is needed to confirm rootstock compatibility for other species.

The proposed method can be applied to at-risk ash populations endangered by EAB or other threats, as well as to individual trees or clones of high cultural or economic value and to staminate trees that produce no seeds. The successful results obtained when the dormant-bud cryopreservation methods are applied to diverse *Fraxinus* taxa suggest that this method could be applied across a broad range of cold-acclimated species.

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