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Keywords
Cryopreservation, Dormant buds, Grafting, Liquid nitrogen, Populus, Rooting, Salix

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CRYOPRESERVATION OF *Populus Trichocarpa* AND *Salix* DORMANT BUDS WITH RECOVERY BY GRAFTING OR DIRECT ROOTING

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Abstract

BACKGROUND: Methods are needed for the conservation of clonally maintained trees of *Populus* and *Salix*. In this work, *Populus trichocarpa* and *Salix* genetic resources were cryopreserved using dormant scions as the source explant. **OBJECTIVE:** We quantified the recovery of cryopreserved materials that originated from diverse field environments by using either direct sprouting or grafting. **MATERIALS AND METHODS:** Scions (either at their original moisture content of 48 to 60% or dried to 30%) were slowly cooled to -35°C, transferred to the vapor phase of liquid nitrogen (LNV, -160°C), and warmed before determining survival. **RESULTS:** Dormant buds from *P. trichocarpa* clones from Westport and Boardman, OR had regrowth levels between 42 and 100%. Direct rooting of cryopreserved *P. trichocarpa* was also possible. Ten of 11 cryopreserved *Salix* accessions, representing 10 different species, exhibited at least 40% bud growth and rooting after 6 weeks when a bottom-heated rooting system was implemented. **CONCLUSION:** We demonstrate that dormant buds of *P. trichocarpa* and *Salix* accessions can be cryopreserved and successfully regenerated without the use of tissue culture.

Keywords: dormant buds, *Salix*, *Populus*, grafting, rooting, cryopreservation, liquid nitrogen

INTRODUCTION

Both *Populus* and *Salix* are woody genera adapted to the winters of harsh northern hemisphere climates. These species also have economic value, particularly for timber, plywood, pulp, paper, ornamental, and revegetation purposes (1, 16, 22). In addition, some *Salix* species have been identified as heavy metal accumulators that may prove useful in land-reclamation projects (2, 10). Neither *Populus* nor *Salix* seeds are long-lived using conventional storage techniques.

Populus is a key model tree species for genomics research due to its small genome size, rapid growth rate, and short time to maturity (6). The *Populus trichocarpa* (Torr. & Gray) genome has been sequenced, and transgenic methods, as well as knock out lines, have been established (3, 21, 23). Breeding programs have identified key cultivars, or clones, that have desirable pest resistance, growth rate, and wood quality

traits (22), but only a few clones are widely propagated and available.

Field, greenhouse, and *in vitro* conservation of valuable clones is expensive (6). Field collections of woody plants require large amounts of land and labour for maintenance and are vulnerable to environmental disasters and biotic infestations. One such field collection is at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA where most of the 55 *Salix* clones in the USDA-ARS National Plant Germplasm System are maintained.

Populus and *Salix* are winter-hardy; cold hardening occurs in response to environmental signals such as photoperiod and temperature changes (8). In these genera, ice forms in the extracellular spaces between the cell walls. During the cooling process, water is drawn out of the cells and into extracellular spaces, thus dehydrating cells and preventing lethal intracellular ice formation (4). Successful dormant bud cryopreservation relies on this phenomenon. Dormant, cold-hardy budwood is sampled, then slow cooled to temperatures between -30 and -40°C, either with or without prior desiccation. Once cooled and equilibrated, twig sections are placed into liquid nitrogen (LN) vapour for long-term preservation. Hardy twigs can survive immersion in LN if they are sufficiently dehydrated by extracellular freezing during slow cooling (15).

Some of the original cryopreservation research was performed by using *Populus sieboldii* Miq. and *Salix koriyanagi* Kimura. Budwood (15 cm length, 0.8 cm diameter) was cooled to -30°C, kept in LN for 24 h, then sprouted and rooted in the greenhouse (14). In addition, Sakai (14) cooled non-desiccated willow twigs to -30°C, and they developed into entire plants after LN exposure and warming. Medium term storage (-80°C) of *Populus* buds has also been demonstrated (1). A cryopreservation method for willow published by Towill and Widrlechner (20), demonstrated successful shoot formation (bud break) after dormant budwood was slowly cooled to -35°C, transferred to the vapor phase of LN (-160°C), and later warmed.

In this work, we quantified recovery of cryopreserved materials that originated from diverse field environments by using either direct sprouting or grafting. We selected dormant bud methods because many poplar and willow genetic resources are maintained as field plants and are also known to be winter hardy. These methods could also be implemented within the *Populus* and *Salix* genomics, breeding, and gene bank communities.

MATERIALS AND METHODS

Materials

In 2010 and 2011, dormant *P. trichocarpa* scions of accessions GS-040-10 and GS-049-04 (between 5 and 15 mm diameter) were harvested from Westport, OR, USA and were sent overnight to the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO for cryopreservation experiments. These clones were originally selected from trees in Adair and Harrisburg, OR. Westport, OR averages 191 cm of rain and 23 cm of snow each year, with 191 precipitation days. The average January low temperature is 2.4°C. Budwood from *P. trichocarpa* clones (identified as 011-066, 011-010, 015-004, 015-011, 022-007, 023-001, 024-001, 041-007, 041-014, and 041-023) was harvested from Boardman, OR. Boardman has an average of 21 cm of rain and 19 cm of snow each year, with 64 precipitation days. The average January low temperature is -2.8°C. These ten clones were originally from Bend, OR.

In January 2006, dormant *Salix* scion wood (between 4 and 10 mm diameter) containing vegetative buds from field grown trees was shipped overnight from the NCRPIS to NCGRP. In Ames, IA, the average January low temperature is -11°C, with 86.5 cm of precipitation annually. NPGS accessions were selected to represent 11 accessions of ten diverse *Salix* species.

Populus and *Salix* scions were stored at -5°C in large plastic bags until use (within 4 to 5 months).

Cryopreservation and recovery

Before use, initial *Populus* moisture contents were determined by drying 3 cm single-node cuttings for 3 days. Moisture contents were calculated on a fresh weight basis ((FW-DW)/FW). Most of the dormant-bud cryopreservation experiments were performed on materials that were not dried; however, a subset of budwood was cut into 3 cm nodal sections and dried to approximately 30% moisture content at -5°C in a walk-in cooler at ambient relative humidity. Nodal sections were then placed into heat shrink polyolefin tubing (~2 cm diameter × 30 cm length; Newark Electronics, Arvada, CO) and cooled at -1°C h^{-1} or -5°C d^{-1} (-1°C h^{-1} for 5 h each day) to -35°C by using either a controlled rate freezer (Sigma Systems Model CC-3, San Diego, CA) or chest freezer (ScienTemp Model 51-12, Adrian, MI), respectively. Materials were then held for 24 h at -35°C and quickly transferred to LN vapor (-160°C). Cryopreserved sections were warmed to 5°C for 24 h and either grafted or rooted to determine viability. Buds were grafted by chip budding onto first-year *P. trichocarpa* seedling rootstocks (approximately 10 mm diameter) purchased from Lawyer Nursery (Plains, MT). Seedling trees were topped after 3 weeks to force bud growth. Either one or two cryopreserved buds were grafted onto each rootstock. Grafted buds were considered viable if they grew into a shoot that had a minimum length of 2 cm.

For rooting, the section base of the cryopreserved material was recut at a 45 degree angle and then dipped into a 20:1 dilution of Dip N' Grow rooting hormone solution (Clackamas, OR; final concentration 500 ppm indole-3-butyric acid, 250 ppm 1-naphthaleneacetic acid). Cuttings were placed in well-drained 1:1 sand: potting soil mixture in a plastic container with the lid slightly open. Containers were placed on a heat mat within a 3°C cold room. Temperatures were stabilized to provide 13°C in the root zone and 4°C above the medium in the container. Low light (25 μmol m^{-2} s^{-1}) was provided by a single cool-white fluorescent bulb (10 h day^{-1}) for 4 weeks, and then cuttings were transferred to a growth chamber maintained at 30°C with HID lighting for 2 weeks. At this time, rooting and shoot elongation data were collected on each nodal cutting.

For *Salix* cryopreservation, scions were cut into 4 to 6 cm nodal sections with two to three buds per section. Ten sections were stacked and sealed into polyolefin tubing. Tubes were cooled in the controlled-rate freezer to –35°C at –5°C day^{-1} (-1°C h^{-1} for 5 h per day). Tubes were held at -35°C for 24 h and then placed in LN vapour for between 8 and 11 days. Tubes were warmed in a cold room set at 5°C for 24 h and then brought to 22°C, where the tubes were opened and the nodal sections were cut at 45 degree angles at the basal end. For hormone treatments, scions were dipped into 1:20 dilution of Dip-N-Grow for 3-5 s before putting into a sterilized medium (1:1:1:1 perlite: vermiculite: peat moss: sand) saturated with sterile water. This medium was selected after preliminary experiments revealed a higher percent bud expansion when using this medium as compared to 100% peat moss as previously reported (20). Scions that were not treated with hormones were cut and immediately placed into the sterilized medium. The 10 scions for each accession were inserted 2-3 cm into the 3-4 cm deep medium in rows with 2 cm spacing within loosely covered polystyrene plastic containers (26 × 32 cm). Rooting environmental conditions were the same as described for *P. trichocarpa*, except cuttings were kept at the reduced temperature for 6 weeks, at which time data were collected. Two replicates (in time) were performed for each treatment. A cutting was considered rooted when it developed one or more roots ≥ 2 mm in length. Bud break was scored as any buds that had opened with visible leaves and shoots forming. For *Salix*, the entire

experimental protocol was performed in April and then replicated in May, 2006.

Statistical analyses were performed with the JMP software package (SAS Institute Inc., Cary, N.C., USA). All experiments included at least 10 replicate budwood sections per treatment and were replicated at least twice.

RESULTS

Populus

In 2010, the two *P. trichocarpa* clones from Westport (GS-040-10 and GS-049-04) were either cooled with an initial moisture content (MC) of 48% (undried) or 31.5% (dried), at rates of either -1°C h^{-1} or -5°C d^{-1} prior to exposure to LN vapour. In 2010, clones from Westport had regrowth levels between 70 and 86%, with no significant differences among treatments (Table 1). The undried vs dried and -1°C h^{-1} and -5°C d^{-1} experiments were repeated in 2011 using the same two clones and in these experiments, higher recoveries were obtained with the undried (50% MC) budwood sections, cooled at either -1°C h^{-1} or -5°C d^{-1} (67% and 77%, respectively; Fig. 1).

Budwood from ten *P. trichocarpa* clones from Boardman, OR was received in both 2010 and 2011. Undried materials (average of 51% MC) had higher average viabilities as determined by grafting, after LN vapour exposure in 2010, than those that were dried before cooling to -35°C at a rate of -1°C h^{-1} (Table 2). In 2010, cryopreserved budwood was also directly rooted to assess the feasibility of this method for *P. trichocarpa* recovery after cryopreservation. In our experiments, 100% of the undried, untreated controls rooted, and 48% of the bud sections formed shoots. In contrast, between 54 and 66% of the cryopreserved bud sections (cooled at either -1°C h^{-1} or -5°C d^{-1}) formed roots and fewer than 40% of these sections formed shoots (Table 3).

Figure. 1. Dormant budwood sections of *Populus trichocarpa* were cryopreserved in an undried state and cooled to -35°C at a rate of -1°C h^{-1}, then plunged into LN vapour. Buds of clone GS-049-04 (top) and GS-040-10 (bottom) were warmed and grafted onto a rootstock for viability assessment.

Overall, *P. trichocarpa* buds could be successfully cryopreserved in their dormant state, without additional desiccation prior to cooling. Bud recovery levels, as determined by grafting, averaged between 62 and 82% when cooled to -35°C at -1°C h^{-1} (Table 1, 2). Buds from trees grown in a more temperate climate in Westport, OR had survival levels comparable to buds collected from clones from the more severe Boardman, OR location.

Salix

In 2006, several experiments were performed to determine the levels of root and shoot formation in *Salix* species after cryopreservation. Overall, 59% and 62% of the accessions exhibited root growth either with or without rooting hormone treatment, respectively (Table 4). Bud growth occurred in 59 and 84% of the accessions, either with or without rooting hormone treatment, respectively (Table 4). Both of the two buds on the scion usually elongated when bud growth occurred.

Ten of the 11 *Salix* species had roots 3 to 60 mm in length and bud growth on more than 40% of the budwood sections under our conditions after LN vapor exposure. *Salix purpurea* (PI 487629) was the only species that did not have rooting or bud growth on more than 40% of the sections (Table 2). Similarly, this species exhibited poor bud growth under conditions used by Towill and Widrlechner (20). Towill and Widrlechner (20) also reported poor bud growth in *S. lemmonii* (PI 573104). Under

Table 1. Dormant budwood of *Populus trichocarpa* clones was received from Westport, OR in 2010 -35°C at either -1°C h^{-1} or -5°C d^{-1}, either without or with desiccation (at -5°C) prior to the cooling tre overnight and transferred to LN vapour. Recovery was assessed by grafting and results are present Letters denote significant differences within a row using Tukey's mean separation test (alpha<0.05).

Clone	Year	Moisture content (%)	Cooled to -35°C at -1°C h^{-1}, LN	Cooled to -35°C at -5°C d^{-1}, LN	Moisture content after desiccation (%)	G c un
GS-040-10	2010	48	80 ± 20 a	86 ± 13 a	31	100
	2011	51	83 ± 3 a	77 ± 9 a	34	90
GS-049-04	2010	48	83 ± 3 a	70 ± 3 a	32	64
	2011	49	55 ± 10 a	77 ± 7 a	32	90
Mean	2010	48	82 ± 8 a	78 ± 7 a	32	82
	2011	50	67 ± 8 ab	77 ± 5 a	33	90

Table 2. Dormant budwood of *Populus trichocarpa* clones was received from Boardman, OR in 2010 and 2011. Bud sections were cooled to -35°C at -1°C h^{-1}, either without or with desiccation (at -5°C) prior to the cooling treatment. Sections were held at -35°C overnight and plunged into LN vapour. Recovery was assessed by grafting and results are presented as % bud regrowth (± standard error). Letters denote significant differences within a row using a t-test.

		Moisture content (%)		Bud regrowth (%)	
				Undried, cooled to -35°C at -1°C h^{-1}, LN	Dried, cooled to -35°C at -1°C h^{-1}, LN
Clone	Year	Initial	After desiccation		
011-066	2010	51	32	77 ± 10 a	65 ± 5 a
	2011	52	34	75 ± 5 a	58 ± 2 a
011-010	2010	53	32	60 ± 27 a	55 ± 5 a
	2011	54	37	85 ± 5 a	62 ± 8 a
015-004	2010	52	34	100	90
	2011	53	34	90 ± 10 a	100 ± 0 a
015-011	2010	54	28	93	65 ± 15
	2011	56	34	70 ± 30 a	71 ± 1 a
022-077	2010	60	31	70 ± 20 a	46 ± 3 a
	2011	58	34	60 ± 10 a	70 ± 10 a
023-001	2010	59	32	82 ± 7 a	32 ± 14 a
	2011	56	36	75 ± 5 a	45 ± 5 a
024-001	2010	63	30	45	20
	2011	59	31	70 ± 10 a	75 ± 5 a
041-007	2010	56	27	95 ± 5 a	79 ± 9 a
	2011	58	36	57 ± 13 a	36 ± 14 a
041-014	2010	60	33	33	11
	2011	58	34	74 ± 14 a	43 ± 18 a
041-023	2010	58	31	25	46 ± 13
	2011	58	37	85 ± 5 a	75 ± 5 a
Mean	2010	51	28	62 ± 8 a	46 ± 8 b

Table 3. Dormant budwood of *Populus trichocarpa* clones was received from Westport, OR in 2010. Bud sections were cooled to -35°C at either -1°C h-1 or -5°C d^{-1} without desiccation prior to the cooling treatment. Sections were held at -35°C overnight and plunged into LN vapour. Recovery was by direct rooting and shoot elongation and was measured in % (± standard error). Letters denote significant differences within a row using Tukey's mean separation test (alpha<0.05).

		Rooting (%)			Shoot elongation (%)		
Clone	Initial moisture content (%)	Control, uncooled	Cooled to -35 °C at -1°C h^{-1}	Cooled to -35 °C at -5°C d^{-1}	Control, uncooled	Cooled to -35 °C at -1°C h^{-1}	Cooled -35 °C at -5°C d^{-1}
GS-040-10	48	100 ± 0 a	66 ± 7 b	63 ± 4 b	50 ± 10 a	13 ± 4 b	8 ± 0 b
GS-049-04	48	100 ± 0 a	54 ± 4 b	61 ± # ab	46 ± 4 a	36 ± 6 a	22 ± 3 a
Mean	48	100 ± 0 a	60 ± 6 b	62 ± 1 b	48 ± 2 a	25 ± 12 a	15 ± 7 a

Table 4. Dormant budwood of *Salix* species was received from Ames, IA in 2006. Bud sections we desiccation prior to the cooling treatment. Sections were held at -35°C overnight and plunged into L and shoot elongation (% ± standard error), either with or without a hormone treatment. Letters deno columns using a t-test.

Accession number	Salix species	Common name	Rooting (%)	
			+ hormone	- ho
Ames 7658	*S. alba* L.	White willow	50 ± 0 a	89 ±
PI 502252	*S. alba* L.	White willow	100 ± 0 a	88 ±
PI 505945	*S. eriocephala* Michx.	Heart-leaf willow	100 ± 0 a	95 ±
Ames 13709	*S. integra* Thunb.		70 ± 10 a	40 ±
PI 573104	*S. lemmonii* Bebb.	Lemmon's willow	15 ± 15 a	40 ±
PI 487629	*S. purpurea* L.	Basket willow	20 ± 20 a	0 ±
PI 505949	*S. triandra* L.	Almond willow	35 ± 5 a	45 ±
PI 502255	*Salix* sp.		70 ± 10 a	75 ±
PI 505952	*Salix x calodendron* Wimm.	Holme willow		60 ±
PI 502250	*Salix x rubens* Schrank	Hybrid crack willow	85 ± 15 a	90 ±
PI 487627	*Salix x rubra* Huds.	Green leaf willow	65 ± 5 a	65 ±
Mean			59 ± 8 a	62 ±

our rooting conditions, rooting and bud-growth levels of *S. lemmonii* were acceptable.

These data demonstrate that cryopreserved *Salix* budwood could be successfully recovered via direct rooting for most of the accessions tested. These recovery levels were higher than those that were obtained for cryopreserved *P. trichocarpa*, when using our rooting conditions.

DISCUSSION

There is a need for reliable cryopreservation methods for clonally maintained trees, such as *Populus* and *Salix*. Critical materials produced in breeding programs, as a result of advanced genetic and genomic techniques, as well as those conserved in gene banks, are often maintained vegetatively. These collections are expensive to maintain and back-up under field conditions. We focused on the use of dormant buds to preserve *P. trichocarpa* and *Salix* and to determine the feasibility of cryopreservation and recovery by either grafting and/or rooting.

We found that dormant winter buds of *P. trichocarpa* originating from either temperate Westport, OR or a more continental Boardman, OR location could be successfully cryopreserved without an initial desiccation step, although some desiccation may have occurred while the scions were held at -5°C before use. These buds had the highest regrowth levels when grafting was used for viability assessment; however, some cuttings could be successfully propagated by direct rooting. In contrast, cryopreserved *Salix* accessions could be successfully recovered by rooting them either with or without commercially available rooting hormones.

Numerous woody genera can be cryopreserved by using dormant buds. These include, but are not limited to, *Malus*, *Prunus cerasus*, *Salix*, *Fraxinus*, *Diospyros*, and *Morus* (7, 12, 13, 18, 19, 24). The standard protocols for *Malus* and *Prunus cerasus* cryopreservation require dormant scion sections to be dried to optimal moisture contents (approximately 30% MC) prior to slow cooling (18, 20). However, this drying step is not mandatory in *Malus* or *Salix* (17, 20).

Methods for *Populus* and *Salix* cryopreservation have been previously published. Some of the original work on dormant bud cryopreservation was performed on these two genera (14). Successful cryopreservation of hybrid aspen (*Populus tremula* L. x *Populus tremuloides* Michx.) was previously reported by Jokipii et al. (9). Dormant sections were slowly cooled from 0 to -38°C at a rate of -10°C h^{-1} and then kept at that temperature for 24 h prior to immersion in LN. Buds were warmed at 37°C for 2 min, then shoot tips were extracted and recovered on medium. In contrast, we use grafting and rooting to assess recovery after LNV exposure.

Towill and Widrlechner (20) reported on the successful cryopreservation of *Salix* dormant buds, with recovery by direct rooting of cuttings. In this work, we have modified the previously reported rooting methods and have achieved overall higher levels of shoot regrowth.

We demonstrate improved rooting methods for cryopreserved willow scions by changing the substrate for the cuttings from the peat moss described by Towill and Widrlechner (20) to a 1:1:1:1 perlite: vermiculite: peat moss: sand mixture and by performing the rooting in a bottom-heated system in which the roots were kept at 13°C and the air temperature was maintained at 4°C. For some *Salix* species, it was beneficial to treat scions with rooting hormones before rooting.

Some accessions (*S. lemmonii* PI 573104, *S. purpurea* PI 487629, and *S. triandra* PI 505949) were sensitive to hormone exposure and exhibited poor bud growth and rooting when treated with hormones. These three accessions had higher rooting and bud growth when not treated with hormones before transfer to the rooting medium.

In some cases, *in vitro* materials may be the preferred explant for *Populus* cryopreservation. Vitrification methods have been published for cryopreserving shoot tips of *Populus alba* (5, 11) and hybrid aspen (22). Although these methods may be necessary for materials that are maintained *in vitro*, or under field conditions in which adequate levels of winter dormancy are not achieved, dormant buds are often the most economical method for cryopreservation when adequate quantities of appropriate source materials are available.

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