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Keywords
Salix, willow, cooling rate, warming rate, regrowth, shoot formation, root formation

Disciplines
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CRYOPRESERVATION OF *Salix* SPECIES USING SECTIONS FROM WINTER VEGETATIVE SCIONS

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Abstract

Twigs of *Salix* species are candidates for cryopreservation procedures because they become tolerant of freezing temperatures during mid-winter. We examined several variables in developing a two-step cryopreservation procedure for sections from these twigs. Samples of *Salix triandra* cooled to -30ºC or -35ºC and then transferred to the vapor phase over liquid nitrogen gave the greatest percent shoot formation. Cooling rate to -35ºC had a major influence on shoot formation. Samples cooled at rates greater than 10ºC/hr showed no shoot formation. The highest percent of shoot formation was achieved by cooling at 0.21ºC/hr. Cooling rate from -35ºC to liquid nitrogen did not influence shoot formation. Warming procedures affected shoot formation. Transferring samples from -160ºC either to a +2ºC cold room or to -3ºC methanol gave similar levels of shoot formation. No shoot formation occurred either with warming in +40ºC water or very slowly in a Styrofoam box. Eight of eleven *Salix* taxa tested using the established protocol had significant levels of shoot formation after cryogenic treatment.

Keywords: *Salix*, willow, cooling rate, warming rate, regrowth, shoot formation, root formation.

INTRODUCTION

The genus *Salix* comprises 400-500 species and is distributed widely from subtropical to boreal climates. Several species have been selected for soil stabilization, basketry, biomass or ornamental value and are maintained as clones. As part of the U.S. National Plant Germplasm System (NPGS), the North Central Regional Plant Introduction Station in Ames, Iowa maintains 48 accessions, representing about 20 species. These are held as field plantings, but maintenance is expensive and individual accessions are at risk of loss. A cryogenic backup is desired.

The USDA-ARS National Center for Genetic Resources Preservation (NCGRP, formerly National Seed Storage Laboratory) in Fort Collins, CO, is the long-term genebank for NPGS germplasm. Species maintained as clones at several repositories are placed into cryogenic storage in Fort Collins using a variety of methods. A method using winter vegetative buds has been useful for the cryopreservation of *Malus* (3) and *Prunus cerasus* (12) germplasm. For these, nodal sections from winter-hardy scions are first desiccated at -5ºC to 30% moisture

(fresh weight basis) and then cooled using a two-step procedure. Samples are budded to rootstock to generate the plant. The system, however, is time consuming.

Shoot tips from *in vitro* plants of *Salix* have been cryopreserved with an encapsulation/dehydration procedure (2), but application at NCGRP would require first establishing the stock plant *in vitro*, retrieving the cryo-treated shoot tip in culture and eventually recovering a potted plant. This method is also time consuming and labor intensive.

Species of *Salix* exhibit a wide range of cold tolerances depending upon whether their geographic origin is from temperate or tropical areas (8). *Salix* species from temperate areas are planted in Ames, IA and experience an average low temperature of -28ºC. Most cells within *Salix* vegetative buds and cambium are believed to tolerate subfreezing temperatures through freeze desiccation (1). Some evidence from cryo-scanning electron microscopy suggests that xylem ray parenchyma of *S. sachalinensis*, a species that survives temperatures well below -40ºC, only partially dehydrates during cooling and then forms intracellular ice, but questions remain about cell viability (5). After non-lethal cooling to between -20ºC and -30ºC, twigs from cold hardy *Salix* species survive rapid cooling to LN (8,9). Presumably this treatment allows water remaining in cells following the freeze dehydration step to vitrify as reported for wood of the related genus *Populus* (4).

We wished to examine whether sections from winter scions of *Salix* in the NPGS collection can be cryopreserved without the use of tissue culture techniques. Such a system would simplify application to the *Salix* collection.

MATERIALS AND METHODS

Materials

Scions consisting of the current season's growth were pruned from field accessions in Ames, Iowa. Twigs of *Salix triandra* (PI 505949) were harvested on 7 January 2002 (air temperatures: high 1.1ºC, low –16.1ºC) and were used for most of the tests. Five other species (*S. alba* Ames 7658, *S. eriocephala* 'American Mackay PI 505945, *S. integra* 'Albomaculata' Ames 13709, *S. sitchensis* 'Plumas' PI 508558, *S.* x *rubra* 'Mawdesley Seedling G' PI 487627) were also collected on 7 January 2002 and shipped to Fort Collins on 8 Jan. 2002. Five additional taxa (*S. lemmonii* 'Palouse' PI 573104, *S. purpurea* 'Streamco' PI 573104, *S.* sp. 'Korso' PI 502255, *S. viminalis* 'Bowles Hybrid' PI 502256 and *S.* x *rubens* 'Hutchinson Yellow Barked' PI 502250) were collected on 11 February 2002 (air temperatures: high 4.8ºC, low –6.2ºC) and shipped to Fort Collins on 12 February 2002. Twigs of *Salix triandra* (PI 505949) were also harvested and shipped to Fort Collins on 15 March 2002 (air temperatures: high 2.9ºC, low –2.8ºC). Samples were stored at +4ºC prior to overnight shipment (without ice). At Fort Collins, all scions were wrapped in plastic to minimize desiccation and stored at -3.5ºC until used. Experiments were performed over five months. Although not tested with *Salix*, cold hardiness of apple, peach and pear scions did not change over six months when stored at –3.5ºC (Towill, unpublished).

Samples

The growing season of 2001 was fairly dry and the season's growth was reduced. The scions shipped varied in length and diameter. Internode length varied considerably. Sections about 4 to 6 cm, containing 2-4 buds, were cut from the scions and randomly packaged in 20 x 125 mm test tubes for cooling.

Cooling

Scions were cooled to liquid nitrogen (LN) temperatures using a generalized two-step cooling procedure. In the first step, samples were cooled from –5ºC to temperatures between

-20ºC to -40ºC at rates ranging from 0.21ºC/hr to 150ºC/hr. Rates were controlled using a programmable cooler (Sigma Systems Model CC-3, San Diego CA) attached to a LN supply line (rates equal to or greater than 1ºC/hr); a chest freezer (Scientemp, Adrian MI), manually adjusted each day, was used for cooling at rates of 0.21ºC/hr (5ºC/day). Samples cooled to various temperatures were held for 24 hrs before transfer to cryogenic temperatures. In the second step of the 2-step procedure, samples were cooled to LN temperatures in two ways. Test tubes were transferred to the vapor phase over LN (LNV, -160ºC to -185ºC). Alternatively, sections were quickly removed from the test tube at the transfer temperature and immersed in partially solidified nitrogen (PSN). PSN was produced by placing LN within a Styrofoam cup in a vacuum desiccator and applying a vacuum for about 5 minutes. Sections were held in PSN for 1-2 min and then transferred to LNV. All samples remained in LNV for at least one day prior to warming. Cooling rates were measured with a 30 gauge copper-constantan thermocouple attached to a section using a thin strip of Parafilm. Data were recorded using Visual Designer software interfaced with Intelligent Instrumentation hardware. Unless otherwise indicated, scions were cooled to -30ºC or -35ºC at 0.21ºC/hr and transferred into LNV.

Warming

Samples within test tubes were warmed from the transfer temperature in a +2ºC room and held overnight. Samples for determining the transfer temperature to LN (Figure 1) and for the effect of cooling rate to the transfer temperature (Figure 2) were within test tubes and were transferred to +2ºC for warming. To determine the effect of warming procedure (Table 2), samples within LNV were warmed with five different protocols: a.) sample tubes were placed in a +2ºC room, b.) at ca. –170ºC sections were transferred from the tube to a plastic bag; bag was immersed in +40ºC water until the section temperature reached about +25ºC, c.) sample tubes were placed within –3ºC crushed ice, d.) sample tubes were placed within vermiculite in a Styrofoam box equilibrated at –180ºC; box was placed at +25ºC, e.) sections were removed from sample tubes at ca. –170ºC and immersed in –3ºC methanol for one min, blotted, held 1 hr in air at –3ºC, then transferred to plastic bags at +2ºC. In preliminary tests, exposure of bare sections to methanol for 1 min did not influence survival. After warming, all samples except those in condition 'b', were held overnight at +2ºC prior to the recovery test.

Recovery

Sections were inserted vertically into moistened peat moss contained in 27 x 19 x 9 cm plastic boxes. Boxes were partially covered to retain humidity and incubated at 22-25ºC for approximately 6 weeks after which sprouting and root formation were recorded. Water was periodically added to keep the peat moist. Buds from untreated scion sections containing 2-4 buds sprouted in about 2-4 weeks and developed roots concurrently. Preliminary tests demonstrated that low temperature-treated samples also developed shoots under the same growth conditions so shoot formation within peat moss was the principal viability assay for this study. The extent of root formation among sections was variable. Untreated sections from some *Salix* species did not form roots as readily or consistently as those from *S. triandra*, so rooting behavior of cryo-treated sections from these species was not scored. Cambium health was estimated by sectioning with a scalpel. A green, firm cambium, similar to that from untreated sections, was judged to be viable.

Data were analyzed by chi-square comparisons using the SAS statistical software program (SAS Institute, Inc.). Figures were prepared using SigmaPlot 8.02 (SPSS Inc.)

RESULTS AND DISCUSSION

A protocol was developed for the cryopreservation of sections from winter hardy *Salix* species using a two-step cooling procedure. The initial test determined the temperature to transfer scions to LN and was measured from scions cooled to -20ºC to -40ºC at 0.21ºC/hr (Fig. 1).

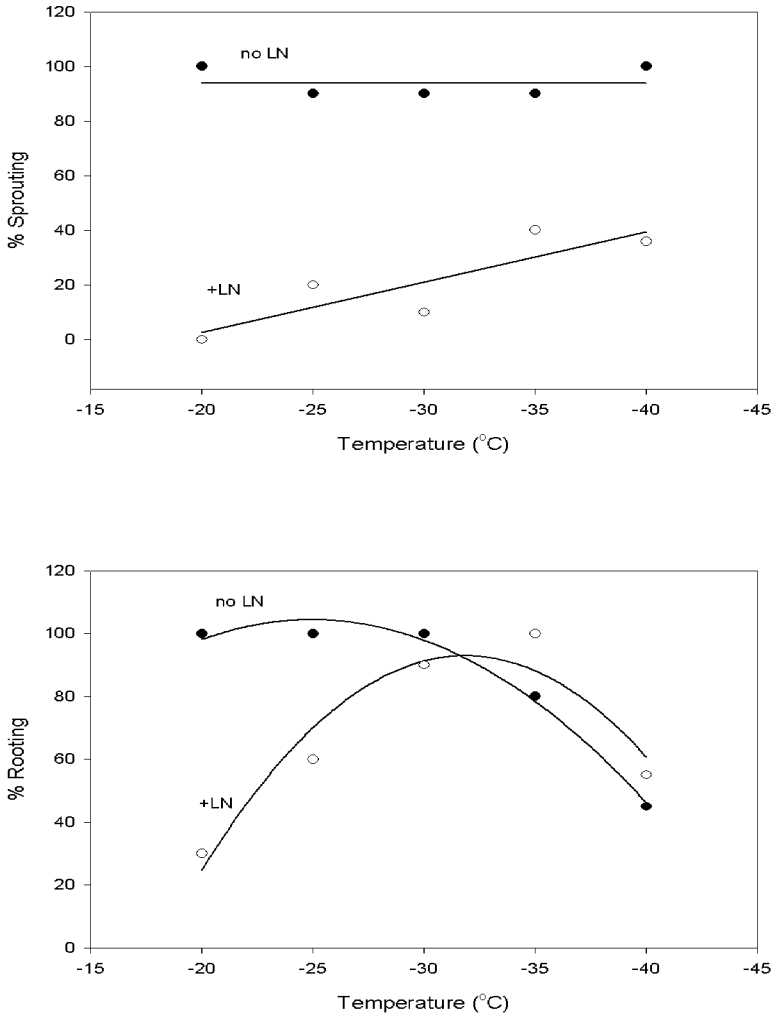

Figure 1. Effect of cooling to temperatures as low as –40ºC with (o) and without (●) transfer temperature to LN on percent of *Salix triandra* sections that formed shoots (A) and roots (B). Sections were in test tubes and were cooled at 0.21ºC/hr to various temperatures then warmed at +2ºC (no LN) or transferred to the vapor phase above LN then warmed at +2ºC (+LN). Curves were calculated from linear (A) and 2nd order polynomial (B) regressions and are intended as an aid to the eye.

Temperatures lower than –40ºC were not examined because some of the species tested were not cold hardy below about –38ºC (data not shown) and we wished to develop a method that might be useful for all the lines in the collection. S*alix triandra* twig sections exposed to temperatures as low as –40ºC showed no differences in shoot formation (Figure 1a), though

root formation was reduced when sections were exposed to temperatures less than –30ºC (Figure 1b). Generally, decreasing the temperature at which scions were transferred to LN increased both shoot and root formation (Figure 1, open symbols). However, cooling below -35ºC decreased root development. The highest shoot and root formation percent occurred with transfer at –30ºC and –35ºC to LNV. Samples cooled to temperatures as low as –40ºC and warmed had a healthy cambium. The cambium was brown for samples transferred to LNV from –20ºC and –25ºC. At transfer temperatures of -30ºC and -35ºC, LNV exposed sections exhibited a predominantly healthy cambium. Subsequent experiments used either –30ºC or –35ºC as the temperature of transfer to LN. These data are consistent with other studies whereby transfer temperatures in the

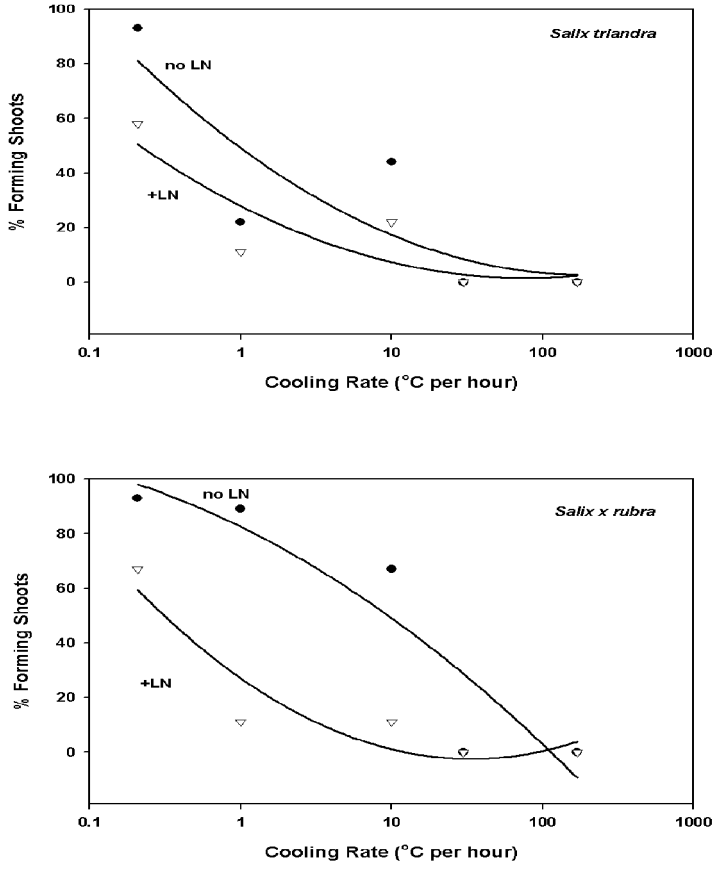

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Figure 2. Effect of cooling rate to –35ºC on percent of sections forming shoots for *Salix triandra* and *Salix* x *rubra*. Samples in test tubes were cooled at rates ranging from 0.21ºC/hr to 125ºC/hr to –35ºC then either warmed (no LN) at +2ºC or transferred to the vapor phase above LN and then warmed at +2ºC (+LN). Curves were 2nd order polynomial regressions with rates expressed as a logarithm and are intended as an aid to the eye.

range of –25ºC to –40ºC are used in two-step cooling procedures (13). Cold-tender species or species not acclimated to their full extent require a lower transfer temperature than hardy species or fully- cold hardened samples for maximum survival at cryogenic temperatures (7).

The importance of cooling rate for cryopreservation is well known. The effect of cooling rate to the transfer temperature on shoot formation was measured for *S. triandra* and *S.* x

rubra (Figure 2). Cooling rates affected regrowth of sections exposed to –35ºC with or without transfer to LNV. Samples cooled to -35ºC faster than 10ºC/hr did not form shoots. Shoot formation at –35ºC and -35ºC with transfer to LNV increased with slower cooling rates and for both lines shoot development of sections was greatest for samples cooled at 0.21ºC/hr, the lowest rate tested. This rate is beneficial for several woody species in which large sections are cooled (10). There is sparse information on water permeability characteristics of plant cells and even less for the kinetics of change at lower temperatures and on differences among cell types. This lack of information makes it difficult to predict what cooling rates are optimal. Indeed, optima may differ among cell types, as is well recognized for preserving animal organs. It is uncertain whether higher survival would occur with cooling rates slower than 0.21ºC/hr.

The cooling rate of the second step, that from –30ºC to LN, might influence viability since some liquid water would still be within cells at –30ºC and the amount would depend upon how much was removed by freeze desiccation. The effect of cooling rate from the transfer temperature to LN was measured for *S. triandra* harvested on 2 dates (Table 1).

Table 1. Effect of cooling procedure from -30ºC to liquid nitrogen on percent of *Salix triandra* twig sections showing growth.

Collection date	Cooling to LN[1]	Rate	% Sections with bud growth[2]
7 January 2002	no	--	94 (8)a
	LNV	62ºC/min	40 (16)b
	PSN	2591ºC/min	53 (25)b
13 March 2002	No	--	100 (0)a
	LNV	62ºC/min	73 (9)a
	PSN	2591ºC/min	60 (28)a

[1] Samples were cooled from –5ºC to –30ºC at 0.21ºC/hr: cooling from –30ºC to LN was by transfer of test tubes directly into the vapor phase above LN (LNV) or by removing the sections from the test tube at –30ºC and immersing them in partially solidified LN (PSN) for 1-2 min then removal to LNV. Warming is by transfer of the twigs within a test tube to +2ºC.

[2] Mean percent of sections showing bud growth (standard error). Within collection date, different letters signify statistical significance (P=0.05).

Sections collected from the field on 7 January or 15 March were cooled at two rates, 62ºC/min and 2591ºC/min, from –30ºC to LN. There were no statistical differences for shoot formation between the two cooling rates for either collection date. Rapid cooling to LN may allow survival in samples with somewhat higher water contents (14). Samples were transferred at –30ºC rather than –35ºC with the argument that if an effect of rate to LN occurred it might be more noticeable when there was slightly more water in the cell. However, viability was not enhanced by the faster cooling rate from –30ºC to PSN, suggesting that the sample was extensively freeze-desiccated. Direct immersion of the unpackaged sections in PSN gave the fastest cooling rate we could easily obtain.

To determine effects of warming rate from LN, we used several warming protocols that gave a variety of rates ranging from 0.6ºC/min to 2300ºC/min and final temperatures between -3ºC and 25ºC (Fig. 3).

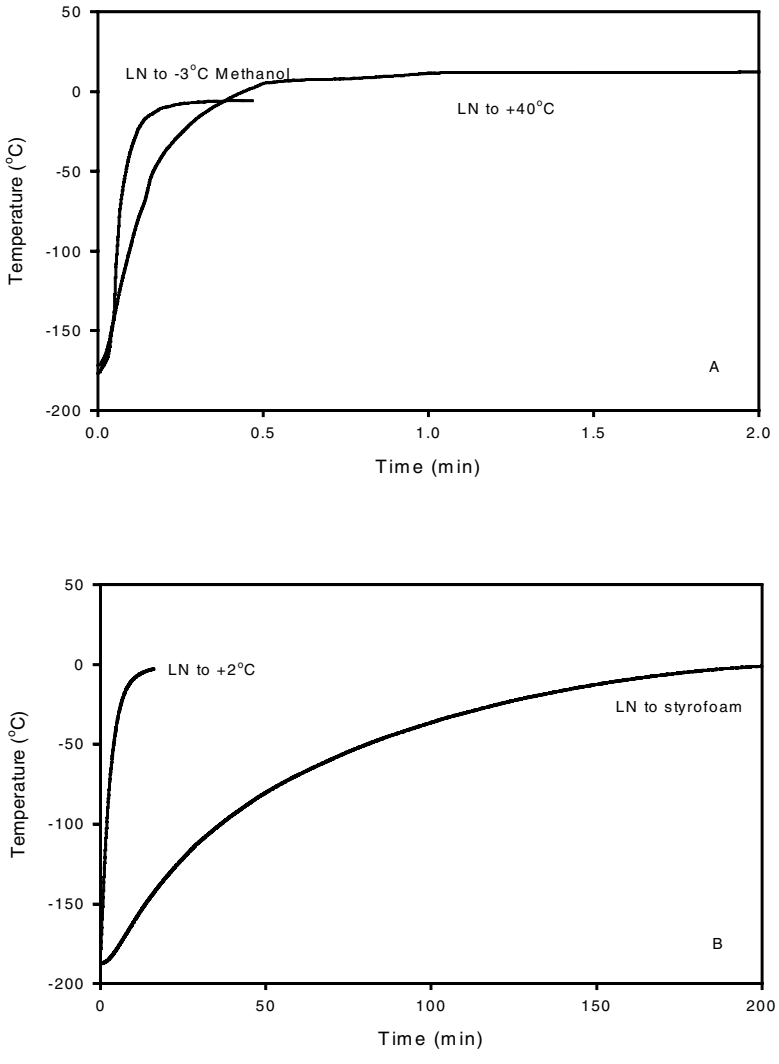

Figure 3. Rates obtained by different warming procedures. A. Transfer of bare twig sections into –3ºC methanol (average rate from –180ºC to –50ºC: 2300ºC/min; average rate from –50ºC to –10ºC: 317ºC/min). Transfer of sections within plastic bags to +40ºC water (average rate from –180ºC to –50ºC: 734ºC/min; average rate from –50ºC to –10ºC: 453ºC/min). B. Transfer of sample tubes into +2ºC air: (average rate from –180ºC to –50ºC: 33ºC/min; average rate from –50ºC to –10ºC: 7ºC/min). Transfer of sample tubes into Styrofoam chest containing vermiculite, chest placed at room temperature (average rate from –180ºC to –50ºC: 1.6ºC/min; average rate from –50ºC to –10ºC: 0.5ºC/min)

Samples that had been transferred to LNV from –35ºC and then rapidly warmed (+ 40ºC water) or very slowly warmed (Styrofoam chest) did not sprout. Percentages of shoot formation were not statistically different for sections warmed from LNV into air at + 2ºC or into crushed ice at -3ºC. Shoot formation of samples that had been transferred to PSN or LNV at –30ºC was not statistically different when warmed at +2ºC. Likewise, shoot formation in samples transferred from -30ºC to LNV and then warmed at either +2ºC or in methanol at –3ºC was not statistically different.

Table 2. Effect of warming rate from liquid nitrogen vapor on percentage of sections from *Salix triandra* showing bud growth.

Cooling[1]	Warming[2]	% Sections showing bud growth[3]
-30ºC	+ 2ºC	94 (8)a
-30ºC, PSN	-3ºC methanol	60 (28)ab
-30ºC, LN vapor	-3ºC methanol	93 (9)a
-30ºC, PSN	+ 2ºC	53 (25)ab
-30ºC, LN vapor	+ 2ºC	40 (16)b
-35ºC	+ 2ºC	100 (0)a
-35ºC, LN vapor	+ 40ºC, water	0c
-35ºC, LN vapor	- 3ºC ice	38 (11)b
-35ºC, LN vapor	+ 2ºC	63 (25)b
-35ºC, LN vapor	styrofoam box	0c

[1] All samples were cooled at 0.21ºC/hr within tubes. The –30ºC sections were removed from the tube and transferred to PSN. The –35ºC samples were cooled to LNV in tubes.
[2] For the –30ºC samples in LNV, bare twigs were transferred to –3ºC methanol; tubes were transferred to +2ºC air. For the 35ºC samples in LNV, tubes were transferred to warming conditions except for +40ºC water, where sections were transferred to plastic bags which were exposed to +40ºC water. The Styrofoam box was equilibrated at –175ºC, four tubes were inserted, and the box was closed and placed to room temperature.
[3] Means (standard error) based on four replicates of four sections. Within a transfer temperature, different letters signify statistical difference (P=0.05).

Since warming in +40ºC water gave an intermediate rate (734-453ºC/min), but a higher final temperature (25ºC), we hypothesize that the 0% survival resulted from too rapid uptake of melt water from the apoplast into the freeze-desiccated cells. In partial support of this hypothesis was the high levels of shoot formation that were obtained with sections warmed more rapidly, but to a temperature below 0ºC. Although not tested with *Salix* in this experiment, in further tests scions of *S. triandra*, pear and apple cooled at 0.21ºC/hr to –30ºC were damaged during warming in a +40ºC water bath (Towill, unpublished). Sakai (9) observed damage by rapid warming from –25ºC and –30ºC for winter twigs of *S. sachalinensis* and *Betula plataphylla*, two very cold hardy species. Rapid thawing of winter pear sections from LNV to above 0ºC also is damaging (11).

Sections did not form shoots after very slow warming from LNV. A possible explanation for this is that the slow rate allowed either devitrification or ice recrystallization in the cells. In these samples, the bark's green color was retained for about 2-3 weeks before visibly darkening whereas the rapidly warmed samples (+40ºC water) quickly developed a brown cambium (1-2 days). At 6 weeks some buds were sectioned and were still green and firm, but had not expanded. Injury due to ice recrystallization or devitrification within cambial or apical cells should be evident very quickly, so damage in another portion of the section,

perhaps in the xylem ray parenchyma, may have ultimately led to cambium deterioration and retarded bud growth. Xylem ray parenchyma viability was not assessed here. Plasmolysis and vital staining tests are needed to define sites of injury within the section and to determine differences among *Salix* species.

Overall applicability of the cryo-procedure was tested on 8 species and 2 interspecific hybrids in addition to *S. triandra* (Table 3).

Table 3. Percent of sections showing bud growth for 11 Salix taxa after cooling at 0.21ºC/hr to –35ºC and transfer to the vapor phase over liquid nitrogen.

Species	-3.5ºC	-35ºC[2]	-35ºC, LNV[3]
S. alba Ames 7658	78 (21)[4] a	89 (16) a	83 (13) a
S. eriocephala PI 505945	58 (7) a	72 (16) a	60 (24) a
S. integra Ames 13709	46 (26) a	45 (12) a	5 (8) b
S. lemmonii PI 573104	11 (8) a	50 (14 a	0 a
S. purpurea PI 434309	67 (29) a	36 (10) ab	19 (7) b
S. sitchensis PI 508558	79 (8) a	51 (7) ab	45 (21) b
S. viminalis PI 502256	89 (8) a	100 (0) a	95 (7) a
S. sp. 'Korso' PI 502255	100 (0) a	75 (0) a	75 (20) a
S. triandra PI 505949	100 (0) a	100 (0) a	63 (25) b
S. x rubens PI 502250	100 (0) a	100 (0) a	81 (18) a
S. x rubra PI 487627	95 (7) a	95 (7) a	55 (10) b

[1]Samples held at –3.5ºC and warmed at +2ºC.
[2]Samples cooled at 0.21ºC/hr to –35º, held 24 hrs then warmed at +2ºC.
[3]Samples cooled at 0.21ºC/hr to –35º, held 24 hrs then transferred to LNV; samples warmed at +2ºC.
[4]Percentage of sections showing bud growth (standard error) based on 3 replicates of 6 sections. Within a row, different letters represent significance with a chi-square test (P=0.05).

The initial viability (-3ºC treatment) of sections was maintained upon exposure to –35ºC for all accessions. In five of these, the shoot formation percentages were statistically the same among the -3ºC, –35ºC and the –35ºC, LNV samples, suggesting little injury to the bud. Eight of these eleven had shoot formation percentages after LN-treatment of 45% or greater. Three species showed reduced percentages of shoot development after cryo-exposure. These values are adequate for gene banking (6).

The lower sprouting percentages of non-cooled sections (-3ºC) from *S. integra, S. purpurea, S. lemmonii* and *S. eriocephala* were indicative of the variability in the material available. Variability in growth responses of *S. lemmonii* sections precluded assessment of the established cryoprocedure. Survival in these experiments exhibited considerable variability among replicates, which may have been related to non-uniformity of the scions. Although scion diameter was not measured and sections were randomly assigned to samples, we noticed that small diameter (1-2 mm) sections did not sprout or root as well as larger (2-3 mm) ones. Some buds also contained catkin primordia (as contrasted to vegetative meristems).

Salix is a large genus and the application of this cryopreservation protocol would be applicable to species that are cold hardy. For several species recovery of the plant is probably feasible using the methodology described. However reasons for differences in shoot and root formation among species need to be further explored to improve the efficiency of the procedure.

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