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Abstract

Willow (*Salix* L.) species have good qualities for becoming a biomass crop for production of biofuels. They grow on marginal soils, produce high yields of wood in a short period of time, and are easily propagated. *Salix* is one of the few genera that may be cryopreserved as dormant winter buds (DBs) instead of using tissue culture techniques. The objective of our study was to evaluate selected factors that affect cryotolerance of nine *Salix* germplasm accessions in the USDA-ARS National Plant Germplasm System collection. One-year-old branches harvested in January over 3 yr were cut into either 6- to 7- or 10-cm segments containing at least two DBs. Segments were treated with a slow-cooling procedure and stored in vapor-phase liquid N (LNV). Control segments were stored at -5°C until planting. The LNV-exposed and the -5°C -stored nodal segments were warmed and directly planted in a cold greenhouse. Six weeks later, the material was evaluated for shoot and root development. Segments that developed a shoot were considered viable. Average viability varied among years (0–35.1%) even for the same accession, species (4.2–51.4%), and segment length. Dormant buds on 10-cm segments had higher average viability (82.2%) than did DBs on 6- to 7-cm segments (43.9%), suggesting higher suitability for cryopreservation. In the material studied, *Salix* DB viability was correlated with branch diameter, DB density, and the ability to develop shoots and roots prior to cryopreservation.

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ORIGINAL RESEARCH ARTICLE

Plant Genetic Resources

Salix dormant bud cryotolerance varies by taxon, harvest year, and stem-segment length

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Abstract

Willow (*Salix* L.) species have good qualities for becoming a biomass crop for production of biofuels. They grow on marginal soils, produce high yields of wood in a short period of time, and are easily propagated. *Salix* is one of the few genera that may be cryopreserved as dormant winter buds (DBs) instead of using tissue culture techniques. The objective of our study was to evaluate selected factors that affect cryotolerance of nine *Salix* germplasm accessions in the USDA-ARS National Plant Germplasm System collection. One-year-old branches harvested in January over 3 yr were cut into either 6- to 7- or 10-cm segments containing at least two DBs. Segments were treated with a slow-cooling procedure and stored in vapor-phase liquid N (LNV). Control segments were stored at -5°C until planting. The LNV-exposed and the -5°C -stored nodal segments were warmed and directly planted in a cold greenhouse. Six weeks later, the material was evaluated for shoot and root development. Segments that developed a shoot were considered viable. Average viability varied among years (0–35.1%) even for the same accession, species (4.2–51.4%), and segment length. Dormant buds on 10-cm segments had higher average viability (82.2%) than did DBs on 6- to 7-cm segments (43.9%), suggesting higher suitability for cryopreservation. In the material studied, *Salix* DB viability was correlated with branch diameter, DB density, and the ability to develop shoots and roots prior to cryopreservation.

1 | INTRODUCTION

Willows (*Salix* L., Salicaceae) are widespread deciduous trees and shrubs found through temperate and boreal plant communities. It is believed that the first primitive *Salix* species came to the Americas from an eastern region of Asia when the African and the American continents were adjacent (Dorn, 1976). In the Northern Hemisphere, willows are often pioneer

species in moist soils of the temperate regions. Willows are used in landscape arrangements, basketry, phytoremediation and biofiltration, traditional medicine, wildlife habitat, preventing soil erosion, and establishing riparian buffers (Keoleian & Volk, 2005). Willows may also be used as a feedstock for production of biofuels and therefore can be viewed as an energy crop (Krzyżaniak et al., 2014; Shield, Macalpine, Hanley, & Karp, 2015; Volk et al., 2006, 2018). *Salix* trees and shrubs make a good woody cropping system model because they produce a high yield of biomass in the span of a few years, they propagate easily, they are genetically diverse, they resprout after several harvests, and the energy from their biomass could be produced without adding CO₂ to the

Abbreviations: DB, dormant bud; LNV, vapor-phase liquid nitrogen; NCRPIS, North Central Regional Plant Introduction Station; NLGRP, National Laboratory for Genetic Resources Preservation; NPGS, National Plant Germplasm System.

environment (Volk, Ballard, Robinson, & Abrahamson, 2004). They can be grown on nutrient-poor soils and their wood is suitable for ethanol production, as in New Zealand (Herrera, 2006), and for generation of small-scale electricity, as reported by Goglio and Owende (2009). For over 30 yr, European researchers have studied the mechanization and efficient harvesting of willow biomass (Smart & Cameron, 2008) grown in a short-rotation coppice (Aylott, Casella, Tubby, Street, & Taylor, 2008; Labrecque & Teodorescu, 2005; Rosso, Faccioto, Bergente, Vietto, & Nervo, 2013; Simaliukas, Noreika, & Karalius, 2007). Still considered to be an emerging lignocellulosic energy crop, intense breeding efforts aimed at improving biomass materials and reducing production costs were undertaken in several countries (Ray, Brereton, Shield, Karp, & Murphy, 2012; Serapiglia et al., 2015).

Salix trees produce short-lived seeds that easily lose viability (Maroder, Prego, Facciuto, & Maldonado, 2000); cultivars, genetic lines, or hybrids are maintained as clones and are often propagated by rooting branch segments. Willow accessions can be conserved as field plantings, in vitro or in long-term liquid N storage. Cryopreservation (storage in liquid N or its vapor phase) can be more cost effective and a safer method for preservation of genetic resources than are field plantings or in vitro plant culture, if reliable cryopreservation procedures are available (Keller, Kaczmarczyk, & Senula, 2008; Reed, 2001). In cryogenic storage, a range of clonal propagules may be used: meristem shoot tips, dormant buds (DBs), and somatic embryos (Reed, 2001, 2002). The use of dormant winter buds has advantages over tissue culture because DBs do not require aseptic conditions; a few hundreds of accessions may be processed in one season, and the post-cryopreservation establishment of a plant is simpler and faster than growing a tree or shrub from an in vitro propagule. Cryotolerance of DBs is reported for several tree species, but their use in routine germplasm cryopreservation is limited to a relatively small group of plant genera (Jenderek, Forsline, Postman, Stover, & Ellis, 2011; Sakai, 1995; Stushnoff, 1991; Towill & Forsline, 1999; Towill, Forsline, Walters, Waddell, & Lauffman, 2004; Volk, Bonnard, Waddell, & Widrlechner, 2009).

The USDA-ARS National Plant Germplasm System (NPGS) maintains 52 willow accessions representing 29 species and hybrids at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA. Towill and Widrlechner (2004) proposed a DB slow-cooling method to cryopreserve *Salix* accessions. At the National Laboratory for Genetic Resources Preservation (NLGRP), Fort Collins, CO, this method was applied to 29 diverse accessions. Considerable variability in post-cryopreservation viability (evaluated as an ability to develop a shoot) was observed (0–95.8%). Since several of the accessions did not meet the NLGRP standard of $\geq 40\%$ viability for clonally cryopreserved germplasm, we

examined selected factors that may improve the survival of dormant willow buds in liquid N.

The objective of the study was to investigate the influence of branch harvest year, taxon, and length of branch segment with the DBs on post-cryopreservation viability of willow (*Salix* spp.) dormant winter buds.

2 | MATERIALS AND METHODS

2.1 | Plant material

One-year-old branches with dormant winter buds of eight accessions from four willow species, maintained at the NCRPIS in Ames, IA, were harvested between 14 and 17 January, in three consecutive years (2007–2009, Table 1); five accessions of *S. purpurea* L. and one accession from other three species (*S. × calodendron* Wimm., *S. × dasyclados* Wimm., and *S. × rubra* Huds.). In 2009, one additional accession of *S. exiqua* Nutt. subsp. *exiqua* var. *columbiana* Dorn was also included. The accessions were selected due to the large number of available branches. The branches, wrapped in plastic bags, were shipped overnight to the NLGRP. Upon arrival, on 10 randomly selected branches per accession, the top and bottom diameter was measured, and the number of vegetative buds per branch was counted to establish the bud density. The twigs were stored at $-5\text{ }^{\circ}\text{C}$ until processed for cryostorage. When material arrived, branches appeared dormant with the exception in 2008 where PI 487628 (*S. purpurea*) had already swollen buds on some branches and PI 505952 (*S. × calodendron*) had developed catkins on a few twigs. Material remained in the cold storage an average of 18 d before processing.

2.2 | Cryoprocessing

The protocol followed one of the slow-cooling experimental regimens described by Towill and Widrlechner (2004). Branches were cut with a band saw into 6- to 7-cm segments (denoted as 6 cm) with at least two nodes (DBs). During cutting, the branches were kept on ice in an ice chest and processed one twig at the time. In 2009, the protocol was enlarged by adding 10-cm segments with two to four nodes for *S. × dasyclados* and *S. exiqua* susp. *exiqua* var. *columbiana* and the five accessions of *S. purpurea* beside testing the 6-cm segments for all four species (*S. × calodendron*, *S. × dasyclados*, *S. × rubra*, and the five accessions of *S. purpurea*). On both segment lengths, the upper DB was 5–8 mm from the segment top. The nodal segments were sealed in polyolefin tubes (12 segments per tube in 2007, 10 segments per tube in 2008 and 2009) and cooled from -5 to $-35\text{ }^{\circ}\text{C}$ at a rate of $-5\text{ }^{\circ}\text{C d}^{-1}$ for 6 d (ScienTemp Chest Freezer). The

TABLE 1 *Salix* taxa used for cryopreservation study in 2007–2009 winter seasons

Species	Accession	Common name ^a	Year received in NPGS ^b
<i>S. purpurea</i> L.	PI 434309	Purple willow	1979
<i>S. purpurea</i> L.	PI 487628	Purple willow	1984
<i>S. purpurea</i> L.	PI 487629	Purple willow	1984
<i>S. purpurea</i> L.	PI 505943	Purple willow	1986
<i>S. purpurea</i> L.	PI 505949	Purple willow	1986
<i>S. × calodendron</i> Wimm.	PI 505952	Holme willow	1986
<i>S. × dasyclados</i> Wimm.; syn. <i>S. daphnoides</i> Vill.	PI 505953	Violet willow	1986
<i>S. × rubra</i> Huds.	PI 487627	Green-leaf willow	1984
In 2009 only			
<i>S. exigua</i> Nutt. subsp. <i>exigua</i> var. <i>columbiana</i> Dorn	PI 508553	Coyote willow, cv. Multnomah	1987

^aAccording to Germplasm Resources Information Network (GRIN) nomenclature: <https://npgsweb.ars-grin.gov/gringlobal/taxonomybrowse.aspx> (type in *Salix*).

^bNPGS, National Plant Germplasm System.

material was held at -35 °C for 24 h and then placed directly into vapor-phase liquid N (LNV; -180 to -196 °C ; also termed as cryostorage or cryopreservation, or LNV-exposed) in a cryotank (MVE 1842P150, Chart Industries) for 6–10 d. Control nodal segments were cut at the same time, sealed in polyolefin tubes, and placed back in the -5 °C storage until shipped to the NCRPIS for viability testing (two replications of 10 segments each for the control and LNV exposed material, in each testing year [2007–2009] and each DB segment length [6 and 10 cm]).

2.3 | Viability testing

Viability was evaluated by the segments' ability to develop shoot(s). The LNV-exposed nodal segments were transferred to 4 °C storage to warm for 1 d prior to overnight shipment in cold packs to NCRPIS for testing. A cold greenhouse under 10-h photoperiod, 12.8 °C soil temperature (root zone), and 4.4 °C air temperature (shoot zone) was used for testing. The bottom ends of the nodal segments of the control and the LNV-exposed material were dipped for ~ 5 s in a Dip 'N' Grow solution diluted 1:20 (final concentration of IBA 500 mg L^{-1} [indole-3-butyric acid] and NAA 250 mg L^{-1} [1-naphthalene acetic acid]), planted in a sterile substrate (peat moss, perlite, sand and vermiculate, 1:1:1:1), and saturated with sterile water 1–2 d before planting in 26- by 32-cm plastic containers (crispers) with 3–4 cm of the substrate. Viability testing was done in two replications (10 DB per accession per replication); sporadically, a segment was rotten or lost during planting. Nodal segments were considered viable when they developed at least one shoot with or without any roots. The ability to develop shoots was the primary factor in considering a propagule viable because shoots might be cut off and rooted or placed in tissue culture, whereas segments with roots will most likely be unable to develop and shoot. Evaluation was done 6 wk after planting. During 2007–2009, data

were recorded for the number of nodal segments with shoots and roots, and in 2009 also for the number of primary roots.

2.4 | Statistical analyses

A completely randomized experimental design was used, and data were evaluated by ANOVA. Means comparison was done using the Tukey–Kramer honestly significant difference test, and the interaction effects were tested by a fit model analysis. Correlations between the factors evaluated (year, nodal segment length, species, accession, viability of control, and LNV exposure) were tested using a multivariate method (pairwise correlations). The *F* statistics were considered significant when the reported *P* values were $< .05$. All statistical analyses were conducted with JMP 9 software (SAS Institute).

3 | RESULTS AND DISCUSSION

3.1 | Shoot development

The average 3-yr DB viability on 6-cm nodal segments exposed to LNV, evaluated as the ability to develop shoots, was significantly higher for *S. × dasyclados* (51.4%) than for the other willow species studied and varied significantly between the five tested accessions of *S. purpurea*: highest in PI 487628 and lowest in PI 50549 (30.6 and 2.8%, respectively; Table 2). The viability of the control nodal segments (not exposed to LNV) differed significantly only between *S. × rubra* (86.1%) and the *S. purpurea* average viability (67.5%). Most likely, the low average percentage of segments with shoots exposed to LNV was due to zero viability in 2008 (Table 3); this was probably related to the fact that an accession of *S. purpurea* had already swollen buds on some branches, and *S. × calodendron* had catkins on a few twigs. These were visible signs of past dormancy and

TABLE 2 Three-year (2007–2009) average viability of dormant buds cryopreserved on 6-cm segments of eight *Salix* taxa

Species	Accession	Segments with shoots		Rooted segments	
		Control	LNV ^a	Control	LNV
%					
<i>S. purpurea</i>	PI 434309	54.6c ^b	15.8b	79.6b	46.2ab
<i>S. purpurea</i>	PI 487628	75.0a	30.6a	86.1a	43.1b
<i>S. purpurea</i>	PI 487629	65.3b	8.3c	58.3b	18.1c
<i>S. purpurea</i>	PI 505943	75.0a	18.1b	87.5a	58.3a
<i>S. purpurea</i>	PI 505949	43.1c	2.8c	100a	19.5c
<i>P</i>		<.0001	<.0001	<.0001	<.0001
Avg. of <i>S. purpurea</i>	(5 accessions)	67.5b	18.2b	77.9b	41.4
<i>S. × calodendron</i>	PI 505952	54.2bc	4.6b	55.6c	26.4
<i>S. × dasyclados</i>	PI 505953	63.9abc	51.4a	70.6bc	50.0
<i>S. × rubra</i>	PI 487627	86.1a	4.2b	79.2abc	25.0
<i>P</i>		<.0001	<.0001	.0002	ns ^c

^aLNV, liquid nitrogen vapor.^bMeans in the same column followed by a common letter are not significantly different at $P < .05$.^cns, not significant.**TABLE 3** Viability of *Salix* dormant buds cryopreserved on 6-cm segments for each year during 2007–2009

Species	Accession	2007		2008				2009					
		With shoots		Rooted		With shoots		Rooted		With shoots		Rooted	
		Control	LNV ^a	Control	LNV	Control	LNV	Control	LNV	Control	LNV	Control	LNV
%													
<i>S. purpurea</i>	PI 434309	52.0	4.2	72.0	58.4a ^b	12.5	0	66.7b	16.7b	100	43.2b	100	63.6ab
<i>S. purpurea</i>	PI 487628	95.8	4.2	66.7	16.7b	29.2	0	95.9a	29.2a	100	87.5a	100	83.4ab
<i>S. purpurea</i>	PI 487629	95.9	12.5	62.5	25.0b	0.0	0	50.0b	0.0c	100	12.5c	100	29.2b
<i>S. purpurea</i>	PI 505943	100	8.4	25.0	79.2a	25.0	0	95.9a	0.0c	100	45.9b	100	95.9a
<i>S. purpurea</i>	PI 505949	41.7	8.4	100	50.0a	4.2	0	100a	0.0c	83.4	0.0c	100	8.4b
<i>P</i>		ns ^c	ns	ns	.002	ns		.0009	0.0014	ns	.003	ns	.003
Avg. of <i>S. purpurea</i>	(5 accessions)	85.9b	7.3b	56.6b	44.8	16.7b	0	77.1	11.5	100a	47.3	100a	68.0ab
<i>S. × calodendron</i>	PI 505952	100a	4.2b	29.2a	8.4	0.0b	0	70.9	33.4	62.5b	12.5	66.7b	37.5ab
<i>S. × dasyclados</i>	PI 505953	100a	83.4a	20.0a	37.5	16.7b	0	91.7	16.7	75.0ab	70.8	100a	95.9a
<i>S. × rubra</i>	PI 487627	100a	4.2b	58.3ab	37.5	62.5a	0	79.2	4.2	100a	43.2	100a	63.6ab
<i>P</i>		.0207	<.0001	.003	ns	.0029		ns	ns	.009	ns	<.0001	.0271

^aLNV, liquid nitrogen vapor.^bMeans in the same column followed by a common letter are not significantly different at $P < .05$.^cns, not significant.

a beginning of growth where perhaps DBs without visible signs already started growth and hence were not dormant any longer. In the same year, the control segments also showed relatively low shoot viability, except for *S. × rubra* (62.5%). Based on our observations of other plant genera, the post-LNV shoot development ability of the nodal segments relates to the initial (non-LNV exposed) shoot development capacity (unpublished data). In this study, an association of control sample viability to post-LNV storage viability was noticeable in PI 505953 (2007 and 2009), and in 2009 in three accessions of *S. purpurea* (PI 434309, PI 487628, and

PI 505943), *S. × dasyclados*, and *S. × rubra*. However, the relationship was not observed in *S. × calodendron*, *S. × rubra* (in 2007), and the five accessions of *S. purpurea* (Table 3). In the 2008 season, none of the LNV-exposed willow segments developed shoots; the percentage of developed shoots in the control segments was also significantly lower in that year. Although it is assumed that the 2008 processed branches were already past their dormancy and therefore were not viable after LNV exposure, the cause of the low shoot development on the control branches is unknown and difficult to speculate. Inclusion of the 2008 data was intended to show differences

TABLE 4 Average yearly viability of *Salix* dormant buds for 6-cm segments

Year	With shoots		Rooted	
	Control	LNV	Control	LNV
	%			
2007	85.7a ^b	16.1b	54.2c	39.1b
2008	18.8b	0.0c	81.3b	12.5c
2009	89.6a	35.1a	95.8a	55.9a
<i>P</i>	<.0001	.0012	<.0001	.0017

^aLNV, liquid nitrogen vapor.

^bMeans in the same column followed by a common letter are not significantly different at *P* < .05.

TABLE 5 Three-year (2007–2009) average viability for control and cryopreserved *Salix* dormant buds on 6-cm nodal segments

Treatment	With shoots	Rooted
	%	
Control	64.7a ^a	77.1a
LNV ^b	17.1b	35.8b
<i>P</i>	<.0001	<.001

^aMeans in the same column followed by a common letter are not significantly different at *P* < .05.

^bLNV, liquid nitrogen vapor.

TABLE 6 Average viability of *Salix* dormant buds on 6- and 10-cm nodal segments cryopreserved in 2009

Segment length cm	With shoots	Rooted	Avg. no. of roots per segment
	%		
6	43.9b ^a	62.4b	1.5b
10	82.2a	90.4a	3.1a
<i>P</i>	<.0001	<.0001	<.0001

^aMeans in the same column followed by a common letter are not significantly different at *P* < .05.

in viability outcomes between years even when the processing procedure was similar over the years (Table 4).

The 3-yr average percentage of segments that developed shoots was significantly higher in the control segments (64.7%) than in the cryopreserved ones (17.1%, Table 5). Shoot development observed for cryopreserved 10-cm nodal segments was significantly higher (82.2%) than for 6-cm segments (43.9%, Table 6). However significantly not different, viability of the 10-cm segments was between 80.6 and 100%; viability of the 6-cm segments varied from 37.8–70.8% for species. Viability for the five accessions of *S. purpurea* differed significantly and fell into three groups of 100% (PI 487628, PI 487629, and PI 505943), 87.5% (PI 434309), and 19.1% (PI 505949) for 10-cm segments,

and into four groups for the 6-cm segments, with the highest viability in PI 487628 (87.5%) and 0% in PI 505949 (Table 7).

3.2 | Rooting

The 3-yr average proportion of segments that developed roots after cryopreservation was not significantly different between the tested *Salix* species and was in the range of 25.0–50.0% but varied among some of the five accessions of *S. purpurea* and was significantly different only between PI 487629 and PI 505949 (18.1 and 19.5%, respectively) and PI 505943 (58.3%) (Table 2). The rooting ability of the control segments was significantly different only between the average rooting percentage of *S. purpurea* (77.9%) and *S. × calodendron* (55.6%). Rooting in the five *S. purpurea* accessions was significantly higher in PI 487628, PI 505943, and PI 505949 (86.1, 87.5, and 100%, respectively) than in the other two accessions (PI 434309 and PI 487629; 79.6 and 58.3%, respectively). In 2007, the percentage of LNV-stored segments with roots was not significantly different among species but was significantly higher in PI 434309, PI 505943, and PI 505949 of *S. purpurea* (58.4, 79.2, and 50.0% respectively) than in the two other *S. purpurea* accessions (Table 3). In the 2008 season, where no shoot development was observed on the LNV-stored (cryopreserved) segments, rooting was observed in all species (up to 33.4% in *S. × calodendron*) but was not significantly different between the species. The rooting percentage was significantly different for the accessions of *S. purpurea*; however, three accessions did not develop any roots (Table 3). In 2009, rooting of LNV-exposed DBs was not significantly different between species but varied significantly between PI 505943 and two other accessions (PI 487629 and PI 505949) of *S. purpurea* (95.9, 29.2, and 8.4%, respectively; Table 3). Overall, the lowest proportion of root development was seen in 2008, and the highest was seen in 2009 (12.5 and 55.9%, respectively) on the LNV-stored 6-cm nodal segments. Rooting in controls also varied significantly between the years (54.2, 81.3, and 95.8% for 2007, 2008, and 2009, respectively) (Table 4). The ability to develop roots in the LNV-stored material was approximately half that of the controls (35.8 and 77.1%, respectively) (Table 5). The 6-cm segments rooted to a significantly lower degree than the 10-cm-long segments (62.4 and 90.4%, respectively) (Table 6). The rooting percentage for the 6-cm segments was significantly higher in PI 487628 and PI 505943 (83.4 and 95.9%, respectively) than in the three other accessions of *S. purpurea* but was not different for the three species (Table 7). For the 10-cm-long segments, no significant differences were observed between *S. × dasyclados* and the average percentage of *S. purpurea* (100 and 94.0%, respectively), but *S. exiqua* subsp. *exiqua* var. *columbiana* DB segments rooted significantly lower (68.5%);

there were no significant differences between the five accessions of *S. purpurea* (Table 7). The average number of primary roots per 6-cm segment was not significantly different for species but was significant between PI 487629 and PI 505949 (0.4 and 0.1 roots per segment, respectively) and the other three accessions of *S. purpurea*. For the 10-cm segments, the number of roots per segment was significantly lower in PI 508553 (*S. exigua* subsp. *exigua* var. *columbiana*) (1.3 roots per segment) than in *S. × dasyclados* and the average of *S. purpurea* (4.3 and 3.3 roots per segment, respectively) and was significantly higher for PI 487628 and PI 505943 (4.9 and 4.5 roots per segment, respectively) than for the other three accessions of *S. purpurea* (Table 7).

3.3 | Correlations

The top diameter of the twigs used in this study ranged from 4.2–5.6 mm and was not significantly different among taxa; the average bottom diameter varied from 5.9–8.1 mm and was significantly different only between PI 434309 (*S. purpurea*) and PI 505953 (*S. × dasyclados*) (5.9 and 8.1 mm, respectively; Table 8). The average density of DBs on 10 randomly selected branches was only significantly different between PI 505953 (*S. × dasyclados*) and PI 487629 (*S. purpurea*) (3.3 and 2.3 cm, respectively). These measurements were taken to characterize branches and evaluate possible correlations between these characteristics and the viability of DBs after storage in LNV. The average post-cryopreservation viability, measured as the DB segments' ability to develop shoots, was

positively correlated at the $P \leq .001$ level with the rooting ability of the LNV-exposed segments and with shoot development of the control segments; at the $P \leq .05$ level with the top and bottom branch diameters (both negatively; the smaller the twig diameter, the higher was the percentage of viable DBs), rooting in the control, and the nodal spacing on the branches (Table 9). The rooting ability of the LNV-stored nodal segments was negatively correlated with the top and bottom branch diameters and positively correlated with the DB density, and with the root development of the control segments ($P \leq .05$). The correlations between the characteristics greatly aid in future twig harvest and suggest that branches with smaller diameter survive better a storage in LNV.

Differences in viability of the LNV-exposed and the control nodal segments were observed among the tested taxa, as well as among sampling years. The shoot development of LNV-stored nodal segments was significantly lower than that of the control nodal segments, which was expected, since not all DBs survive the ultralow temperature of LNV. The ability of the control segments to develop shoots seems to be indicative (sets the level) of potential viability after cryopreservation; practically, this would require testing the shooting potential of the controls prior to DB processing. Usually the number of available nodal segments for processing is limited; therefore, duplicate testing (before processing and a second time together with segments exposed to LNV) is often impractical but would be worthwhile. Shortage of quality material also limits the number of replications available for testing. Dormant buds have the highest cold resistance in their deep dormancy (endodormancy) phase (Cooke, Erikson, &

TABLE 7 Viability of dormant buds cryopreserved on 6- and 10-cm nodal segments for seven *Salix* taxa in 2009

Species	Accession	6-cm nodal segments			10-cm nodal segments		
		With shoots	Rooted	Avg. no. of roots per segment	With shoots	Rooted	Avg. no. of roots per segment
		%			%		
<i>S. exigua</i> subsp. <i>exigua</i> var. <i>columbiana</i>	PI 508553	47.4	60.6ab	1.3	80.6	68.5b	1.3b
<i>S. × dasyclados</i>	PI 505953	70.8	95.9a	2.3	100	100a	4.3a
Avg. of <i>S. purpurea</i>	(5 accessions)	37.8	56.1ab	1.4	81.3	94.0a	3.3a
<i>P</i>		ns ^b	.0295	ns	ns	.0005	.0001
<i>S. purpurea</i>	PI 434309	43.2b	63.6b	1.0ab	87.5b	100	3.8b
<i>S. purpurea</i>	PI 487628	87.5a	83.4a	2.7a	100a	100	4.9a
<i>S. purpurea</i>	PI 487629	12.5c	29.2c	0.4c	100a	93.8	2.4c
<i>S. purpurea</i>	PI 505943	45.9b	95.9a	2.7a	100a	100	4.5a
<i>S. purpurea</i>	PI 505949	0.0d	8.4d	0.1c	19.1c	76.2	0.9d
<i>P</i>		.0373	.0011	.004	<.0001	ns	.0017

^aMeans in the same column followed by a common letter are not significantly different at $P < .05$.

^bns, not significant.

TABLE 8 Average diameter and density of dormant buds on ten randomly selected branches used for cryopreservation of *Salix* accessions in 2007–2009

Accession	Species	Diameter		Density of nodes on branches
		Top	Bottom	
		mm		cm
PI 434309	<i>S. purpurea</i>	4.6	5.9b ^a	3.1ab
PI 487627	<i>S. × rubra</i>	4.7	7.0ab	2.5ab
PI 487628	<i>S. purpurea</i>	4.8	6.4ab	2.8ab
PI 487629	<i>S. purpurea</i>	5.4	7.1ab	2.3b
PI 505943	<i>S. purpurea</i>	5.6	7.1ab	2.7ab
PI 505949	<i>S. purpurea</i>	4.2	6.0ab	2.8ab
PI 505952	<i>S. × calodendron</i>	5.3	7.7ab	3.0ab
PI 505953	<i>S. × dasyclados</i>	5.4	8.1a	3.3a
<i>P</i>		ns ^b	.0232	.044

^aMeans in the same column followed by a common letter are not significantly different at $P < .05$.

^bns, not significant.

TABLE 9 Correlation between selected characteristics of branches with dormant buds and viability of 6-cm segments for eight *Salix* taxa cryopreserved during 2007–2009

Characteristic	Diameter top	Diameter bottom	Density of nodes on branches (cm)	Rooted control (%)	Rooted LNV ^c (%)	With shoots control (%)
Diameter bottom	***					
Density of nodes on branches (cm)	ns ^b	ns				
Rooted control (%)	ns	ns	ns			
Rooted LNV (%)	*	*	*	*		
With shoots control (%)	*	ns	ns	*	*	
With shoots LNV (%)	*	*	*	*	***	***

^a*Significant at the .05 probability level.

^b***Significant at the .001 probability level.

^cLNV, liquid nitrogen vapor.

^dns, not significant.

Junttila, 2012; Stushnoff, 1991). In each year of the study, the branches were harvested between 14 and 17 January. Studies on cryopreservation of *Vaccinium* L. DBs showed a strong influence of average air temperature 10 d before the branch harvest on cryopreservation viability (Jenderek et al., 2017); thus, the most favorable time for harvest for cryopreservation may vary from year to year, and weather forecast should be considered in setting twig harvest time for cryopreservation of *Salix* genetic resources also. It is assumed that in the 2008 season, the dormancy of the buds was already broken when the twigs were harvested, and their viability would have been enhanced with earlier harvest that was not known at that time.

Variation in post-cryopreservation viability among *Salix* taxa was also reported by Towill and Widrechner (2004).

In their study, shoot formation in LNV-exposed segments for three accessions, also used in this study (PI 434309 *S. purpurea*, PI 505949 *S. purpurea* [previously classified as *S. triandra*], and PI 487627 *S. × rubra*) was higher (19, 63, and 55%, respectively), but equally as variable as the 3-yr average viability of DBs reported in this study. The data from our study highlighted the importance of multiyear studies for cryopreservation. For example, in 2009, both PI 487627 (*S. × rubra*) and PI 434309 (*S. purpurea*) developed shoots on 43.2% of the LNV-treated segments, but neither of these accessions developed shoots in 2008. Towill et al. (2004) also reported an influence of year and cultivar on the cryopreservation outcomes of eight accessions of *Malus* spp. We also observed the influence of year in cryopreservation studies of selected *Prunus* species (data not shown).

The LNV-treated DBs on 10-cm segments had significantly higher post-cryopreservation viability than DBs on 6-cm segments, based on shoot and root formation, as well as the number of primary roots per segment. The exception was PI 508553 (*S. exiqua* subsp. *exiqua* var. *columbiana*), where the number of roots per segment was the same for both 10- and 6-cm segments. The number of primary roots might be less important than the ability to develop any roots at all. Some accessions might not be disposed to cryopreservation procedures and might require a different approach to cryopreservation. Our results strongly suggest that 10 cm nodal segments were more suitable for cryopreservation of *Salix* than 6-cm segments; the segment length may vary in cryopreservation protocols for other woody plant collections. However, the comparison of segment length on the viability outcome was conducted for only 1 yr, in 2011; the post-cryopreservation DB viability for six of eight *Salix* accessions cryopreserved on 10-cm segments was $\geq 75\%$ and for the other accessions was $> 40\%$ (data not shown). The viability data confirmed the importance of DB segment length in cryopreservation. The 10-cm segments may not be ideal for cryopreserving all willow accessions, but additional length and bud numbers likely contributed significantly to enhanced post-cryopreservation DB survival of the eight taxa tested. Processing 10-cm DB segments will require a larger number of twigs than processing shorter segments that might be a challenge. Some twig characteristics, such as top and bottom diameter, and density of DBs on twigs were significantly correlated with the ability to develop shoots and roots on cryopreserved and control segments and are relevant for selection of willow branches for cryoprocessing. However, the bud density on twigs is accession dependent, likely directed by genetic factors modified by environmental conditions, and thus not easily manipulated. By refining the cryopreservation method, our study supported development of a protocol for applied clonal cryopreservation of *Salix* genetic resources; however, any procedure might be further improved. Studies on defining an optimal branch diameter for cryopreservation of *Salix* DB and suitability of using cryoprotectants prior to LNV exposure might contribute to improvement of the cryoprotocol. With increasing cultivation of willow for biomass production, safeguarding of the genus germplasm will be similarly important as long-term preservation of many other agricultural crops.

4 | CONCLUSION

In this study, the survival and subsequent shoot and root development of *Salix* DBs following a slow-cooling method and a storage in LNV was dependent on the species, accession, year of harvest, length of branch segment, density of DB, and branch diameter. These results were used to develop a revised slow-cooling method for cryopreservation of the NPGS *Salix*

germplasm collection. The protocol involves the use of 10-cm branch segments and cryopreservation of accessions with low DB viability for a minimum of two consecutive years to ensure reliable viability after cryostorage of *Salix* DBs. Development of a dependable cryopreservation protocol is of importance as the willow collection enlarges, via addition of native accessions and cultivars developed for biomass production. Protocols such as this will support the ability of the NPGS to ensure preservation of the current and future *Salix* collection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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