Variation in Freezing Resistance During Different Phenological Stages in Some *Populus* and *Salix* Clones: Implications for Clonal Selection

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Abstract

Nineteen (19) clones of *Salix* and twenty-one (21) clones of *Populus* were examined for their variability in freezing resistance. A series of laboratory freezing tests were conducted, using visual assessment and electrolyte leakage to detect freezing injury and survival. Clones were tested at predetermined levels of freezing stress and during seven (7) phenological stages: dormant (D), early spring (ES), spring (S), flushing of terminal buds (FTB), new axillary bud growth (NAG), growing (G) and early-fall (EF) stages. Significant clonal variation in freezing resistance was detected at four (4) stages: S, FTB, NAG, and EF. At the D and ES stages, when freezing resistance was greatest, no significant differences in clonal survival were detected. Similarly, at the G stage in which clones exhibited the highest susceptibility to freezing stress, clonal variation for the estimated index of injury was negligible. At the G stage, significant clonal differences were detected only at relatively mild stress (–3 °C) in *Salix,* which accounted for 10% of the total variation. At the EF stage, clonal differences were highly significant for the index of injury and accounted for 34% of the variation in *Salix*, and 32% in *Populus*. For a subset of 14 *Populus* clones, all estimated SPEARMAN's rank correlation coefficients between stages were significant, except in the case between the S and EF stages. Implications of the results for clonal selection with respect to freezing resistance in *Populus* and *Salix* are briefly discussed.

Key words: clonal variation, freezing resistance, phenological stage, *Populus, Salix.*

Introduction

The increasing interest in *Populus* and *Salix* plantings for wood fiber and energy, has raised concerns about their vitality and optimal growth rates. Frost injury is one of the most significant economic obstacles in short rotation energy plantations for countries where freezing temperatures occur during the winter and, more importantly, during the growing season (CHRISTERSSON et al., 1983; LARSSON, 1998). VERWIJST et al. (1996) estimated that a single night with frost during the early growing season can cause losses of up to 60% of the annual yield in *Salix* plantations in Sweden (4 June 1993; Långaveka, 56°51'N, 12°35E'). To minimize such losses, and to allow expansion of *Populus* and *Salix* plantings in northern countries, selection for freezing resistant clones can be considered as an important option. However, before freezing resistance can be used as a trait for clonal selection, its clonal variability must be known.

The genetic effect of some traits in selected clones and inter-intraspecific hybrids of *Populus* and *Salix* has been found to be significant (ZSUFFA, 1982; MOSSESLER et al., 1988; KENNEY, 1990; RÖNNBERG-WÄSTLJUNG et al., 1994; RIEMENSCHNEIDER et al., 1996). However, knowledge of clonal variation in freezing resistance is relatively scanty, because screening requires expensive and time-consuming field trials. Recent indoor freezing tests in *Salix* (VON FIRCKS, 1994; ÖGREN, 1999; TSAROUHAS, et al., 2000) suggest that genetic variation in freezing resistance could be more systematically and quickly assessed. For conifers, such freezing tests have repeatedly demonstrated large genetic variation in freezing resistance that can be present among species (SUTINEN et al., 1992), families (*Pinus sylvestris*, NILSSON and ANDERSSON, 1987; *Pinus contorta,* REHFELDT, 1989; *Pseudotsuga menziesii* var. *menziesii,* AITKEN and ADAMS, 1996), clones (*Pinus silvestris,* NILSSON and WALFRIDSSON, 1995; *Picea sitchensis,* NICOL, et al., 1995) and provenances (*Pinus sylvestris,* NILSSON and ERIKSSON, 1986; *Picea glauca,* SIMPSON, 1993) which, in several instances, corresponded well with the survival of the same genetic entries in the field tests (NILSSON and ERIKSSON, 1986; NILSSON and ANDERSSON, 1987).

The current study was undertaken to examine the feasibility of using clonal selection to improve freezing resistance for *Populus* and *Salix* clones suitable for short rotation intensive culture (SRIC) systems. The objectives were to: (1) assess variation in freezing resistance among several clones of *Populus* and *Salix* after exposure to a series of predetermined freezing temperatures; and (2) study the effect of plant phenological stage on clonal variation in freezing resistance.

Material and Methods

Plant material

Twenty-one clones of *Populus* spp. and nineteen clones of *Salix* spp. were chosen as the plant material for the study (*Table 1*). The selection was based on clonal feasibility for SRIC systems. Because no prior testing for the freezing resistance of these clones has been conducted, this selection can be considered random with respect to this trait. First year shoot cuttings of *Salix* were collected in mid-January from stool nurseries at Maple and Orono, Ontario. At the end of December and January of the following year, dormant stem cuttings of *Populus* were collected from experimental trials in Ontario (Thunder Bay, Malancthon, and Maple), Minnesota and Iowa. All the cuttings were stored wrapped in plastic bags for 4 to 6 weeks at 3 °C (± 0.2 °C) until planting.

Cultural practices prior to freezing treatments

Since freezing temperature and duration in relation to freezing injury are very important factors, preliminary experiments were conducted to establish the freezing-thawing rate, the value as well as the length of the minimum testing temperature. Generally, a rapid rate of freezing may cause direct intracellular freezing (rapid killing) or supercooling effects in plants (LEVITT, 1980; SAKAI and LARCHER, 1987). To avoid these complications and because these phenomena rarely occur in nature (LEVITT, 1980), the preliminary testing was focused on slow

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Table 1. – List of clones used in this study.

N	Clonal codes	Species	Origin	Collection Site	Phenological stages tested
1	W724	*Salix nigra*	Missouri, USA	MO	*G,EF*
2	W762	*Salix nigra*	Adams City, Iowa	MO	*G,EF*
3	W918	*Salix nigra*	Butter City, Iowa	MO	*G,EF*
4	W78183	*Salix viminalis*	Sweden	MO	*G,EF,D*
5	W78101	*Salix viminalis*	Sweden	MO	*G,EF,D*
6	W78021	*Salix viminalis*	Sweden	MO	*G,EF,D*
7	W77683	*Salix viminalis*	Sweden	MO	*G,EF,D*
8	W77699	*Salix viminalis*	Sweden	MO	*G,EF,D*
9	W557	*Salix viminalis*	Sweden	MO	*ES,S,NAG,D*
10	W559	*Salix viminalis*	Sweden	MO	*ES,S,NAG,D*
11	W690	*Salix eriocephala*	Randolph City, Illinois	MO	*G,EF*
12	W721	*Salix eriocephala*	Bonne City, Missouri	MO	*G,EF*
13	W756	*Salix eriocephala*	Holt City, Missouri	MO	*G,EF*
14	W905	*Salix eriocephala*	Keva Poha City	MO	*G,EF*
15	W939	*Salix eriocephala*	Hennepin City, Minnes.	MO	*G,EF*
16	W1004	*Salix eriocephala*	Dane City, Wisconsin	MO	*G,EF*
17	W1045	*Salix eriocephala*	Pierce City, N. Dakota	MO	*G,EF*
18	W1055	*Salix eriocephala*	South Dakota	MO	*G,EF*
19	ERIO-24	*Salix eriocephala*	Pickering, Ontario	MO	*G,EF*
20	D102	*P. deltoides*	Minnesota	MIN	*ES,S,FTB,NAG,EF,D*
21	D108	*P. deltoides*	Minnesota	MIN	*ES,S,FTB,NAG,EF,D*
22	D120	*P. deltoides*	Minnesota	MIN	*ES,S,FTB,EF,D*
23	D190	*P. deltoides* cv. Brooks #4	Alberta	THB	*all (7)*
24	D191	*P. deltoides* cv. Brooks #6	Alberta	THB	*all (7)*
25	D207	*P. deltoides* cv. Brooks#1	Alberta	THB	*all (7)*
26	D208	*P. cv angulata x deltoides*	NEFES[1]	THB	*all (7)*
27	DTAC20	*P. cv angulata x trichocarpa*	NEFES	THB	*all (7)*
28	DTAC21	*P. cv angulata x trichocarpa*	NEFES	THB	*all (7)*
29	DTAC22	*P. cv angulata x trichocarpa*	NEFES	THB	*all (7)*
30	DTAC29	*P. cv angulata x trichocarpa*	Belgium	MLO	*all (7)*
31	DTACN1	*P. candicans x berolinensis*	Quebec	THB	*all (7)*
32	JACK31	*P. jackii*	Manitoba	THB	*all (7)*
33	POP856	*P. cv. angulata x balsamifera*	Maple, Ontario	THB	*all (7)*
34	TACN1	*P. x berolinensis/(P. laurifolia x nigra)*	Indian Head Nurs, Sask.	THB	*all (7)*
35	IW6424303	*P. deltoides*	Illinois	IWA	*NAG,EF*
36	IW7330500	*P. deltoides*	Illinois	IWA	*NAG,EF*
37	MWH12	*P. deltoides x maximowiczii*	Illinois	IWA	*NAG,G,EF*
38	ST66	*P. deltoides*	Stoneville, Mississippi	IWA	*NAG,G,EF*
39	IW6413503	*P. deltoides*	Illinois	IWA	*NAG,EF*
40	IW6410104	*P. deltoides*	Illinois	IWA	*NAG,EF*

MO = Maple, Ontario (43°N); THB = Thunder Bay, Ontario (48°N); MLO = Melancthon, Ontario, (46°N); MIN = Minnesota, (45°N); IWA = Illinois city, Iowa (40°N).
[1]) NEFES: North Eastern Forest Experimental Station, USDA Forest Service. ES: early spring; S: spring; FTB: flushing of terminal buds; NAG: new axillary bud growth; G: growing; EF: early fall; D: dormant.

freezing and thawing rates. Clonal material (one plant per clone) was subjected to several freezing temperatures, 1 °C to 3 °C apart. Temperatures with severe injury effects on plants or no effects were rejected. On average, the selected temperatures were expected to result in intermediate (30% to 70%) damage. At stages where plant material had developed a high degree of tolerance, no single temperature yielded intermediate damage. The applied freezing temperatures either slightly damaged (0% to 30%) or heavily injured (70% to 100%) the plants (data not shown). In these cases the lowest temperature resulting in slight (30%) damage was chosen for the main experiment. The plant material used to determine these temperatures was grown one to four weeks earlier than the plant material for the main experiment.

Laboratory testing for freezing resistance

Seven experiments were conducted to detect clonal variation in freezing resistance, each for a different phenological stage. The stages were: early spring stage (ES); spring stage (S); flushing of terminal buds stage (FTB); new axillary growth stage (NAG); growing stage (G); early fall stage (EF); and dormant (D) stage (*Table 2a and b*).

Table 2a. – Summary of experimental profile: ES, S, FTB, and NAG stages.

Phenological stage	Days at warm conditions after storage and until freeze-testing	Plant status at the freeze-testing	Starting freezing temperature	Cooling rates	Min freezing temperature/ duration	Thawing rates	Post-freezing conditions	Type of freezing injury assessment
ES	3	No sign of bud swelling or rooting	3.2°C (one night)	2-5°C /h	-15°C, 2h	2-5°C/h	one night at 2 °C and 15 days at SG conditions[2]	visual assessment: bud viability test scale: 0=flushing, 1=no flushing
S	10	Noticeable swelling of buds	3.2°C (one night)	2-5°C/h	-15°C, 2h	2-5°C/h	one night in the dark at 2 °C and 10 days at the SG conditions	visual assessment: bud viability test scale: 0=flushing, 1=no flushing
FTB	15-25 [1]	Bud scales fall off and first foliage *(well expanded)* appears	3.2° C (one night)	1-2°C/h	-3°C, 1h -5°C, 1h	1-2°C/h	one night in the dark at 2 °C and 10 days at SG conditions	visual assessment: RLI[3] scale: 0= RLI<50% (alive) 1= RLI>50% (dead)
NAG	18-28	Elongation of new stem (5-7cm) arising from the axillary buds	3.2° C (one night)	1-2°C/h	-3°C, 1h -5°C, 1h	1-2°C/h	one night in the dark at 2 °C and 10 days at the SG conditions	visual assessment: RLI scale: 0= RLI<50% (alive) 1= RLI>50% (dead)

ES: early spring stage; S: spring stage; FTB: flushing of terminal bud stage; NAG: new axillary growth stage; G: growing stage; EA: early autumn stage; D: dormant stage.

[1]) *Salix* clones were not included.
[2]) SG (Standard Growing conditions) = 25 °C/18 °C day/night air temperature, 18 h photoperiod and 40% to 60% relative humidity
[3]) RLI = the number of dead leaves of the total number of leaves.

ES, S, FTB and NAG stages

Stem cuttings 12 cm to 15 cm long were placed into tap water under standard growing conditions (SG): 25 °C/18 °C day/night, 16 h day, 40% to 60% humidity, and left to grow. When plants reached the desired phenological stage (*Table 2a*) they were planted in Pro-Mix soil medium (Pro-Mix, Premier Brand Inc. Red. Hill, PA.) inside of polypropylene trays (80 cm x 38 cm x 11 cm) and kept at SG conditions for three days before they transferred to a programmable freezer. Freezing tests were carried out in darkness by gradual cooling the air of the freezer at a rate of 1°C/h to 5 °C/h (*Table 2a*). To initiate extracellular freezing, plant material was sprayed with top water at –1 °C. At the ES, S and FTB stages, apical shoots containing one terminal bud or more (in some cases two to three joined terminal buds formed from small apical branches) were tested. Only the cuttings with axillary buds were included at the NAG stage. At all stages one set of plants (five cuttings per clone) was not subjected to freezing stress, but it was otherwise treated in the same way (control). Freezing injury was assessed visually 10 to 15 days after the freezing test (*Table 2a*). Only two clones of *Salix* were tested at the ES, S, and NAG stages (*Table 1*) while all *Salix* clones were excluded from the FTB stage experiment due to the inadequate number of terminal buds.

G and EF stages

Dormant stem cuttings, 10 cm long, were soaked in tap water until roots emerged and then they were planted in plastic Rootrainers (Spencer-Lemaire Industries, Edmonton, Alberta) using Pro-Mix soil medium. After eight weeks of growth in SG conditions, one randomized set of plants (ten cuttings per clone) was transferred for an artificial freezing treatment (G stage plants) while an other set (ten cuttings per clone) was exposed to an artificial hardening regime to induce growth cessation and to initiate the onset of dormancy (EF stage plants). The hardening conditions were: 17 °C/10 °C, 15 °C/4 °C day/night temperature for *Salix* and *Populus* respectively, 60% to 75% humidity and 9 hours photoperiod. The artificial hardening lasted 21 days for *Salix* and 32 days for *Populus*. At this point, apical stem growth was remarkably reduced in both *Salix* and *Populus* clones, but shoot tip abscission was pronounced only in *Populus*.

Freezing tests for G and EF stages were conducted in a programmable cooling chamber (Coldsream-Conviron). Following 1h exposure at selected temperatures (*Table 2b*), five leaves (three middle and two top) of each plant were removed and placed in test tubes containing 5.5 ml of distilled water, prefrozen in the same chamber with the plants. Test tubes were then transferred carefully in large insulated boxes, to other freezers for thawing to 2 °C. The freezing test was conducted on intact seedlings with roots insulated by Styrofoam on the sides and dry peat moss on the top of the soil. Root temperatures were never less than 2 °C. During the whole experiment, temperatures were monitored by copper-constantan thermocouples in contact with the plants. Clones of *Salix* and *Populus* were tested at different times.

Table 2b. – Summary of experimental profile: G, EF and D stages.

Phenological stage	Days at warm conditions after storage and until freeze-testing	Days of cold acclimation	Plant status at the freeze-testing	Starting freezing temperature	Cooling rates	Min. freezing temperature/duration	Thawing rates	Post-freezing conditions	Type of freezing injury assessment
G	56	0	actively growing plants (40-60 cm high)	3.2 °C (one night)	1°C/h	–3°C, 1h –4°C[1], 1h –5°C[2], 1h	1-2°C/h	12 hours in the dark at 2 °C, plus 24 hours in light and 5°C	electrolyte leakage; Index of Injury (IDX_t)
EF	56	21 (*Salix*) 32 (*Populus*)	cold acclimated plants	2.0 °C (one night)	1°C/h	–3°C[1], 1h –5°C[1], 1h –4°C[2], 1h –6°C[2], 1h –8°C[2], 1h	1-2°C/h	12 hours in the dark at 2 °C, plus 24 hours in light and 5°C	electrolyte leakage; Index of Injury (IDX_t)
D	0	28 (–3.2 ± 0.2 °C)	dormant shoots	–3.2	1.8-5 °C/h	–43 °C, 2h	4-5 °C/h	36 hours at 3.2 °C, 16 hours gradually thawing to 25 °C and 4 weeks at SG conditions until the evaluation	visual assessment Bud viability test: 0=flushing (alive) 1=no flushing (dead)

G: growing stage; EF: early fall stage; D: dormant stage.
[1]) Only *Salix* clones were tested.
[2]) Only *Populus* clones were tested.
[3]) SG (Standard Growing conditions)=25 °C/18 °C day/night air temperature, 18 h photoperiod and 40% to 60% relative humidity.

Electrolyte leakage of leaf tissue was used to assess the freezing injury (TSAROUHAS et al., 2000). Sampling of five leaf disks of 5 mm diameter, one disk per collected leaf, replicated three times for each plant was used for the electrolyte leakage procedure. Electrolyte leakage following freezing to temperature t was expressed as the index of injury (IDX_t) according to FLINT et al. (1967):

[1] $IDX_t = 100(RC_t\text{-}RC_o)/(1\text{-}RC_o)$ respectively,

where:

RC_t = Fractional release (c_t/c_{tk}) of electrolytes from freeze-treated samples

c_t = Specific conductance of leachate from sample frozen at temperature t.

c_{tk} = Specific conductance of leachate from sample frozen at temperature t and then heat-killed.

RC_o = Fractional release (c_o/c_{ok}) of electrolytes from unfrozen samples.

c_o = Specific conductance of leachate from unfrozen samples.

c_{ok} = Specific conductance of leachate from unfrozen, and then heat killed samples.

D stage

Fifteen dormant stem cuttings (5 cm to 7 cm long) for each clone were put into radiation sterilised polypropylene test tubes (30 mm x 115 mm), surrounded by ice chips to induce early ice crystal formation and thereby exclude supercooling effects (SAKAI and LARCHER, 1987). The test tubes were sealed with polypropylene caps and placed randomly in two insulated boxes with thermocouples inserted in selected tubes to monitor temperature. The insulated boxes were transferred to a programmable freezer. To ensure the hardening of our material, prior to freezing test all samples were subjected to artificial hardening (–3.2 °C ± 0.2 °C) for four weeks (SAKAI, 1965). The freezing test was conducted in the dark, at rates of 5 °C /h until the temperature of –20 °C and 1.8 °C/h to 2 °C/h until the minimum temperature of –43 °C (*Table 2b*). After freezing and complete thawing to 2 °C the samples were planted in Rootrainers containing Pro-Mix soil medium and placed under SG conditions (*Table 2b*). Evaluation of bud viability was conducted four weeks after the freezing treatment.

Analytical Methods

Because ES, S, FTB, NAG, and D stages consisted exclusively of categorical data, contingency tables ($p<0.05$) were used to test for independence of survival of treated and untreated plant samples. When significant differences in survival compared to the control were observed, clonal differences were tested using the G-test of independence (SOKAL and ROHLF, 1995). At the G and EF stages, two-way analysis of variance (ANOVA) for IDX_t was conducted with the clone and freezing temperature being the two factors. When necessary, data were normalized by square-root or logarithmic transformation prior to ANOVA. In the results and discussion, means are reported for non-transformed data, while all other estimates are based on analysis of transformed data. The following linear model was used in the analysis of variance (ANOVA) and in the calculations of variance components at the G and EF stages experiments:

[Model 1] $Y_{(ij)k} = \mu + T_i + C_j + CT_{ij} + e_{(ij)k}$ (two way ANOVA)

where:

$Y_{(ij)k}$ = the response of the k^{th} experimental unit of the i^{th} freezing temperature and j^{th} clone;

μ = overall mean

T_i = effect of the i^{th} freezing temperature

C_j = effect of the j^{th} clone

TC_{ij} = effect of the interaction of the i^{th} freezing temperature with the j^{th} clone

$e_{(ij)k}$ = experimental error of the k^{th} experimental unit associated with the i^{th} freezing temperature and j^{th} clone.

All the effects, except μ and T_i, are considered random and *F*-test values were based on type III estimation of sums of squares. The error mean squares was used as the denominator only for the clone x freezing temperature interaction effect (*C x T*). When the effects of freezing temperature (*T*) and clone (*C*) were tested the mean square of the interaction *C x T* was used as the denominator. The statistical analysis in G and EF stages were conducted separately for *Populus* and *Salix* clones since the two genera were not treated at the same time or with the same level of freezing stress. In the cases where the Model 1 was insignificant for the clonal effect, the within temperature clonal variation was tested by the following model:

[Model 2] $Y_{(j)k} = \mu + Cj + e_{(j)k}$ (one way ANOVA)

where:

$Y_{(j)k}$ = the response of the k^{th} experimental unit of the j^{th} clone

μ = overall mean

C_j = effect of the j^{th} clone

$e_{(j)k}$ = experimental error of the k^{th} experimental unit associated with the j^{th} clone that contains all effects not included in C.

All the statistical analysis was performed using the Statistical Analysis System (SAS, 1988).

Clonal ranking at the stages with significant variation among clones in freezing resistance was conducted by the PROC RANK procedure (SAS, 1988). In cases of tied scores, the smallest of the corresponding rank was used. SPEARMAN's rank correlation coefficients r_s between phenological stages were estimated. The set of clones included in the ranking procedure consisted of 14 clones of *Populus*. Clones absent from at least one of the stages in which significant clonal variation in freezing resistance was detected, were excluded from the ranking procedure.

Results and Discussion

Variation among clones at the different phenological stages

In the ES stage, no significant clonal differences in bud survival between the controls and plants treated at –15 °C were observed (*Table 3*). This suggests that a frost event of –15 °C during that stage is unlikely to cause significant bud injury in the clones studied here. However, after several days of deacclimation when swelling of the terminal buds was pronounced (S stage), a freezing stress of –15 °C did resulted in significant bud injury (*Table 3*). This phenological stage is seldom considered as a high-risk period for frost damage in *Populus* and *Salix,* possibly because the plants are considered to be dormant. However, this study shows that just before bud break, significant reduction in bud survival can occur. Reports from VON FIRCKS (1994) and SENNERBY-FORSSE (1986), showed new phloem cell activity in *Salix* before bud break. This in turn indicates that plants before bud break are no longer dormant, as well as suggests that low freezing temperatures after a warm spell at the end of winter or early in the spring, can be just as damaging as a late spring frost. The bud survival among clones in S stage varied significally (*Table 3*). This indicates that there is a potential for clonal selection to improve frost resistance prior to bud flushing.

Table 3. – Survival and *G* values at the early spring (ES), spring (S), flushing of terminal buds (FTB), new axillary growth (NAG) and dormant (D) stages. Denote *, **, *** are significant differences at p<0.05, 0.01 and 0.001, respectively.

	Phenological stages				
	ES	**S**	**FTB**	**NAG**	**D**
	(n=440)	(n=442)	(n=285)	(n=380)	(n=330)
	[2, 15][1]	[2, 15]	[0, 15]	[2,20]	[7,15]
Survival	0.988 (control)	0.938 (control)	0.968 (control)	0.971 (control)	0.817 (control)
	0.952 (-15°C)	0.547**[2] (-15°C)	0.746* (-3°C)	0.673** (-3°C)	0.786 (-43°C)
			0.524** (-5°C)	0.461** (-5°C)	
survival x clone test of independence	*df*	*G* value	*df*	*G* value	*df*	*G* value
	16	57.5*** (-15°C)	14	44.6*** (-3°C)	21	39.28** (-3°C)
				37.8*** (-5°C)		33.43* (-5°C)

Stage FTB tested only for *Populus clones.*

[1]) number of clones tested (*Salix, Populus*)

[2]) Significantly different compared to control as determined by the 2x2 test of independence (SOKAL and ROHLF 1995).

In the FTB and NAG stages, freezing temperatures of –3 °C and –5 °C had significant effect on the leaf injury of the plants (*Table 3*). Freezing resistance is very low at this stage, presumably due to high levels of physiological activity within the newly initiated leaf and shoot tissues. The significant clonal differences found in survival at both levels of stress (–3 °C and –5 °C) may be an important attribute in terms of clonal selection. It is important to note that all clones were tested at a standard stage in the development of flushing. In nature, clones will reach this stage at different time intervals. The time of bud break may contribute significantly toward the freezing resistance of a clone, since damage by spring frost events can be prevented by a late bud flushing. Furthermore, the reader is cautioned that the observed variation at the S and NAG stages, should be considered preliminary for *Salix* clones since only two clones were used.

At the G stage, freezing stress significantly (p 0.05) induced electrolyte leakage rates in both genera (*Table 4*). Even a 1 or 2-degree (Celsius) drop on the freezing temperature was enough to significantly increase electrolyte leakage in leaves, indicating a serious effect on cell membrane permeability (PALTA *et al.,* 1978; LEVITT, 1980). CHRISTERSSON *et al.* (1983), indicated that *Salix* clones grown in SRIC systems are extremely frost susceptible during the growing stage when freezing temperatures between –2 °C to –4 °C are usually lethal. The clonal variation between temperatures was insignificant for the estimated IDX_t and accounted for 2 % and 3 % of the variation in *Salix,* and *Populus* respectively (*Table 5*). The small effect of clones on the total variance for IDX_t suggests low heritability resulting in a poor response through clonal selection for freezing resistance at the G stage. However, the within temperature analysis of variance in *Salix* (Model 2) (*Table 5*) showed a significant ($p<0.05$) clonal variation at the –3 °C stress which might explain the obtained from Model 1 significant ($p<0.05$) temperature x clone interaction. As discussed above there is a sharp gradient in sensitivity to freezing stress between the 1 °C interval (*Table 4*), suggesting that most of the clones possibly be severely damaged in –4 °C. Mild freezing stress i.e. –3 °C, may be a valuable screening level for resistance to frost during the G stage in *Salix.*

Table 4. – Electrolyte leakage rates as estimated by the IDX_t (Index of Injury) after the freezing stress at the G and EF stage. Values are means ± SE.

	G	EF
Salix		
	10.70 ± 1.2*a* (control)	9.86 ± 0.7*a* (control)
	32.60 ± 3.7*b* (-3°C)	16.94 ± 2.8*b* (-3°C)
	57.52 ± 4.3*c* (-4°C)	59.21 ± 6.1*c* (-5°C)
Populus		
	11.02 ± 2.3*a* (control)	8.73 ± 1.21*a* (control)
	13.71 ± 3.2*ab* (-3°C)	18.90 ± 3.09*b* (-6°C)
	67.55 ± 8.6*c* (-5°C)	39.44 ± 4.30*c* (-8°C)

DUNCN's test was performed for treatments within each genus. Column values followed by different letters are significantly different (p 0.05).

In the EF stage, clones in both genera differed ($P<0.0001$) with respect to IDX_t (*Table 6*). Clonal variation in freezing resistance during the fall, has been reported earlier for *Populus* and *Salix* (FARMER et al., 1991; LIN et al., 1998; ÖGREN, 1999). It is important to note the high proportion of variation in IDX_t attributable to clones in both genera at this stage (*Table 6*). To date, several frost hardiness studies in perennial and woody plants suggest a number of biochemical and physiological changes during cold acclimation. Cellular and metabolic changes that occur during cold acclimation include increased levels of sugars, soluble proteins, proline, and organic acids (SAKAI and LARCHER, 1987; SAUTER et al., 1996; HUGHES and DUNN, 1996) as well as the appearance of new isoforms of proteins and altered lipid membrane composition (HUGHES and DUNN, 1990). The significant increase in the proportion of variation in IDX_t attributed to clones in EF stage, as compared to the G stage, suggests that these pathways are under strong genetic control in *Populus* and *Salix.* These differences appear to be regulated through changes in gene expression, and several low-temperature-responsive (LTR) genes have been isolated from a range of plants (HUGHES and DUNN, 1996; THOMASHOW, 1998). In our study, the proportion of the total variation in IDX_t attributable to clones was 32 % and 34 %, for *Populus* and *Salix* respectively. Similar patterns of variation with high prospects for improvement through clonal selection have been detected earlier for some quantitative traits in *Populus* and *Salix.* For instance, WILCOX and FARMER (1967) reported that in the first and second year of *Populus* growth, 25% and 31% respectively of the total variation in height, and 20% and 21% respectively of the total variation in diameter growth, were due to clones. RÖNNBERG-WÄSTLJUNG et al. (1994) have reported variance components, attributed to clones of 13% to 19% and 10% to 15% for height and diameter respectively, in forty families of *Salix viminalis* growing in different environments. This might indicate that freezing resistance at the EF stage can be improved through clonal selection, as can other quantitative traits. The significant variation in freezing resistance found at that stage has to some extent resulted from differences in timing of hardening among clones. While phenological characters related to autumn frost resistance i.e. bud set or growth cessation were not examined in this study, they are important contributors to optimal freezing resistance and should always be considered in the selection process for frost resistant *Salix* and *Populus* clones. Species and provenance variation within each genus were not significant ($p>0.05$) with respect to IDX_t at the G or EF stages (data of this analysis are not presented).

The analysis of variance indicated a significant temperature effect ($p<0.0001$) for the estimated IDX_t for both G and EF stages (*Table 5, 6*). Freeze-hardening is the positive effect of exposure to stress of the plant on its subsequent resistance to freezing (LEVITT, 1980). However, the proper time, temperature, and photoperiod will result in a maximum hardening rate (SAKAI and LARCHER, 1987). In the present study the *Populus* clones had developed a higher level of freezing resistance, compared to the *Salix* clones, at the EF stage. This could be attributed to the longer and cooler pre-hardening conditions that were applied. Temperatures 10 °C to 15 °C and short days i.e. 9 h, have been reported to trigger the acclimation process in *Salix* (JUNTTILA and KAURIN, 1990). However, the clones of *Salix* in our study are clearly in an earlier hardening stage than the *Populus*, eliminating any opportunities to compare the genera statistically. Perhaps a hardening period longer than three weeks was needed for our *Salix* material to reach a more remarkable hardening level.

In the D stage, the observed bud injury was not significantly different that of the control (*Table 3*). Both genera survived freezing stress as low as –43 °C after prefreezing to –3 °C for four weeks. This finding supports the hypothesis that the winter frost damage in fast growing tree species, i.e. *Salix,*

Table 5. – Results of the analysis of variance (ANOVA) for the index of injury (IDX_t) at the growing stage (G).

I) *Salix*

Source		df	Expected mean square (EMS)	Mean square	F	Component[1]
Model 1						
T_i		1	$\sigma_e^2 + 85\sigma_t^2 + 5\sigma_{ct}^2$	99.899	171.08***	—
C_j		16	$\sigma_e^2 + 10\sigma_c^2 + 5\sigma_{ct}^2$	0.636	1.09	3
TC_{ij}		16	$\sigma_e^2 + 5\sigma_{ct}^2$	0.588	1.92*	9
$e_{(ij)k}$		136	σ_e^2	0.307		88
Model 2						
C_j	*(-3°C)*	16	$\sigma_e^2 + 5\sigma_c^2$	0.324	1.78*	10
$e_{(j)k}$		85	σ_e^2	0.182		90
C_j	*(-4°C)*	16	$\sigma_e^2 + 5\sigma_c^2$	0.487	1.41ns	1
$e_{(j)k}$		85	σ_e^2	0.344		99

II) *Populus*

Source		df	Expected mean square (EMS)	Mean square	F	Component
Model 1						
T_i		1	$\sigma_e^2 + 70\sigma_t^2 + 5\sigma_{ct}^2$	84.957	344.43***	—
C_j		13	$\sigma_e^2 + 10\sigma_c^2 + 5\sigma_{ct}^2$	0.352	1.41ns	2
TC_{ij}		13	$\sigma_e^2 + 5\sigma_{ct}^2$	0.246	0.71ns	0
$e_{(ij)k}$		112	σ_e^2	0.350		98
Model 2						
C_j	*(-3°C)*	13	$\sigma_e^2 + 5\sigma_c^2$	0.291	0.82ns	1
$e_{(j)k}$		70	σ_e^2	0.352		99
C_j	*(-5°C)*	13	$\sigma_e^2 + 5\sigma_c^2$	0.355	0.95ns	0
$e_{(j)k}$		70	σ_e^2	0.374		100

T_i = effect of freezing temperature treatment, C_j = effect of the clone, TC_{ij} = effect of the interaction of clone with the treatment, $e_{(ij)k}$, $e_{(j)k}$ = error for Model 1 and 2, respectively. ***) Significant at $p<0.0001$ **) significant at $p<0.01$, *) significant at $p<0.05$; ns: non-significant at $p>0.05$.

[1]) Component of variation expressed as percent of total variance.

results from the incomplete winter acclimation and rather than from the inherent inability to develop adequate tolerance to winter temperature stress (VON FIRCKS, 1992). The high tolerance level and the non-significant clonal variation in survival observed in that study suggest that there is perhaps no need to consider winter resistance in *Populus* and *Salix* as a trait for selection. Reports from AITKEN and ADAMS (1996) indicate also a high tolerance level in extreme winter temperatures of coastal Douglas fir and a considerably lower genetic control in midwinter than that in fall. The preconditioning of tempera-

Table 6. – Results of the analysis of variance (ANOVA) for the index of injury (IDX_t) at the early fall stage (EF).

I) *Salix*

Source	df	Expected mean square (EMS)	Mean square	F	Component [1]
T_i	1	$\sigma_e^2 + 85\sigma_t^2 + 5\sigma_{ct}^2$	79.741	177.56 ***	—
C_j	16	$\sigma_e^2 + 10\sigma_c^2 + 5\sigma_{ct}^2$	2.267	5.78 ***	34
TC_{ij}	16	$\sigma_e^2 + 5\sigma_{ct}^2$	0.429	1.15 ns	3
$e_{ij(k)}$	136	σ_e^2	0.372		63

II) *Populus*

Source	df	Expected mean square (EMS)	Mean square	F	Component
T_i	1	$\sigma_e^2 + 105\sigma_t^2 + 5\sigma_{ct}^2$	73.696	272.98 ***	—
C_j	20	$\sigma_e^2 + 10\sigma_c^2 + 5\sigma_{ct}^2$	2.340	8.67 ***	32
TC_{ij}	20	$\sigma_e^2 + 5\sigma_{ct}^2$	0.269	0.60 ns	0
$e_{(ij)k}$	168	σ_e^2	0.446		68

T_i = effect of freezing temperature treatment, C_j = effect of the clone, TC_{ij} = effect of the interaction of clone with the freezing treatment, $e_{(ij)k}$ = error. ***) Statistical significance at $p < 0.0001$, ns: non-significant at $p > 0.05$.

[1])Component of variation expressed as percent of total variance.

tures below 0 °C is a prerequisite for enhancing maximum freezing resistance in woody plants. Winter temperature at about –3 °C is a required stage for hardening in *Salix* as has been reported by WEISER (1970), SAKAI (1972) and VON FIRCKS (1994). Hardening of willows at lower temperatures of –10 °C has been found less effective than –3 °C (SAKAI, 1965). In the current study, the winter hardening of –3 °C for four weeks assured the hardening for both *Populus* and *Salix* clones to withstand the –43 °C freezing stress.

Clonal ranking among phenological stages

The estimated rank correlation coefficients r_s indicate that superiority of a clone with respect to freezing resistance in one phenological stage may not occur in another stage. For instance the correlation between the clonal ranking in the S stage and the clonal ranking in the EF stage was found to be non-significant (*Figure 1*). A practical implication of this non-significant correlation is that clonal selection for early spring freezing resistance may ultimately have low or no impact on the fall freezing resistance. Therefore, freezing resistance prior to bud flushing and during the early fall should be considered as separate traits. For the *Populus* clones included in the ranking procedure, the flushing stages (FTB and NAG) appear to be the most effective stages in which to select for freezing resistance, since clonal rankings at these stages were highly correlated with all the other rankings in the remaining stages. However, this finding must be taken with caution since, during their flushing, all of the clones included in our study were tested at the same stage of development. In nature, by contrast, clones reach this standard point at different time intervals. Consequently, the time of bud break can contribute significantly towards the spring freezing resistance of a clone, since damage by frost events can be prevented by late bud flushing. Time of bud break and/or cessation of growth are necessary patterns for the optimal freezing resistance and more attention needs to be directed toward the synchronization between clonal phenology and local seasonality.

The WILCOXON's two-sample test for pairs of clones (*Figure 1*) confirms the significant clonal variation at the S, FTB, NAG and EF stages for the set of 14 clones of *Populus*. One of the principal difficulties in the development of clonal forestry programs for large-scale plantings is how to identify superior clones (ZOBEL and TALBERT 1984). With respect to freezing resistance and as clonal ranking shows, the superiority of a poplar clone may vary among the phenological stages. For instance TACN1, D207, D190 clones are present among the top five clones only at specific phenological stages (*Figure 1*). However, the clones DTACN1, POP856 and D208 are among the top five across all stages including the two stages (S, EF)

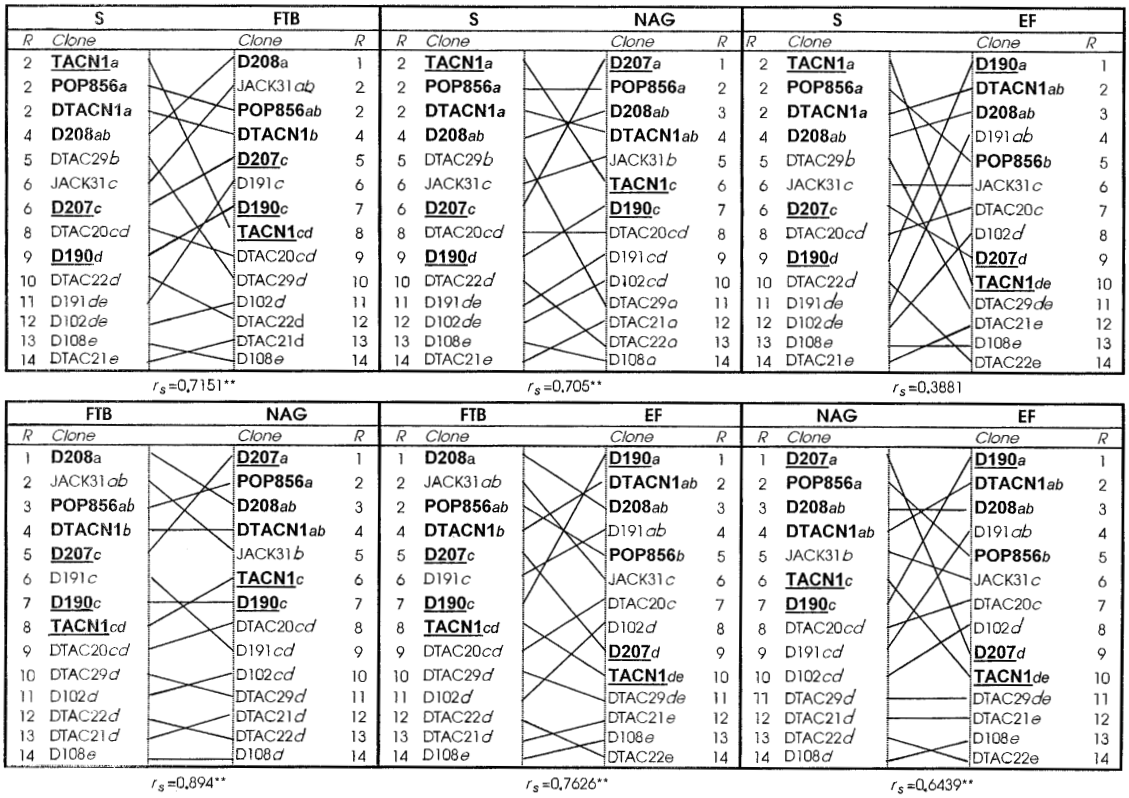

Figure 1. – Clonal ranking based on freezing injury (from the lowest to the highest injured clone) and SPEARMAN's rank correlation coefficients between rankings at different phenological stages.
r_s = SPEARMAN's rank correlation coefficient, ** = (< 0.01). Clones indicated by different letters within a column are significantly different (p 0.05) using the WILCOXON's nonpapametric rank test for pairs of clones (SAS, 1988).
Clones that appear to maintain their superiority in freezing resistance among phenological stages (always on the top five) are bolded. Clones with high freezing resistance at specific phenological stage (within the top five) are shadowed.

where the between stages clonal rank correlation r_s was very poor. This indicates the presence of another category of clones in our study i.e. clones that have maintained their superiority among stages. This may suggests that some, so-called plastic, *Populus* clones could be well adapted to a broad range of cold environments in which frost events could occur at different times of the year. Other clones could be more specific, and thus perform well only in a restricted range of environments, and with specific times of frost occurrence. Distinguishing plastic from season or site specific clones may imply three options for deployment in SRIC systems: (i) the exclusive planting of plastic clones, regardless of site differences with respect to the season in which frost occurs or; (ii) the development of specific populations of clones already adapted to different frost types or; (iii) a combination of (i) and (ii). The exclusive planting of plastic clones on heterogeneous, with respect to freezing temperature, sites may be advantageous. Operational considerations favor also the deployment of clones that are adapted to a wide variety of frost sites. However, a deployment scheme that exclusively favors plastic clones may inhibit optimal productivity (LUNDKVIST, 1988). Therefore, the previous three options need to be further studied in order to determine an optimum economic strategy that incorporates risk assessment.

Conclusions

Based on the present study some conclusions concerning clonal selection strategies for plantings in cold climates and breeding for freezing resistance in *Populus* and *Salix* can be suggested.

Significant levels of genetic variation in freezing resistance are present among *Populus* and *Salix* clones important for biomass production. However, the phenological stage of the plant subjected to freezing stress is an important factor that has to be considered. There is evidence from the present study that clonal variation in freezing resistance may not necessarily occur in all phenological stages. Thus, selection and breeding for resistance to frost might be feasible only at specific phenological stages (i.e. S, FTB, NAG and EF stages). The results of this study showed clonal selection for freezing resistance during the D or ES stage to be ineffective, since clones of both *Populus* and *Salix* are extremely frost resistance at these stages. Correspondingly, the negligible proportion of the total variation in index of injury (IDX_t) at the G stage attributable to clones, indicates that selection for resistance to frost at this stage may be doubtful. However, the significant within treatment clonal variation found in *Salix* indicates that some genetic variation at the G stage could be utilized towards a mild stress level. The relatively high proportion of the total variation attributable to clones obtained during the EF stage in both genera may prove to be valid information for clonal selection and improvement with respect to early autumn frost.

The non-significant correlation found in *Populus* clonal rankings between the S and EF stages implies that freezing resistance prior to bud flushing and during the early fall should be considered as separate traits. Furthermore, it suggests that the time of bud break and/or growth cessation are important aspects for the optimal freezing resistance. While the clonal phenology was not investigated here, it is an important contributor to optimal freezing resistance and should always be considered in

the selection process for frost resistant *Salix* and *Populus* clones.

The results obtained from the clonal rankings elucidate two categories of *Populus* clones with respect to their superiority in freezing resistance among phenological stages: (a) those that maintained their superiority among their phenological stages; and (b) those that exhibited high freezing resistance in specific stages. Therefore two types of selection with respect to freezing resistance can be suggested: (a) selection for superior clones which maintain their superiority among phenological stages; and (b) selection for specific set of clones highly resistant to a specific season frost.

Finally, there is evidence that controlled-freezing tests, as used in the present study, may provide a means of screening large numbers of *Populus* and *Salix* genotypes for resistance to frost. While this study provides evidence of clonal variation in freezing resistance, future field-testing is needed to evaluate such variation in light with interaction of other stresses occurring in nature (drought, salinity, flooding, pathogens, soil quality, etc.).

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