Effect of Liquid Nitrogen Storage on Seed Germination of 51 Tree Species

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ABSTRACT

Two liquid nitrogen storage experiments were performed on 51 tree species. In experiment 1, seeds of 9 western tree species were placed in a liquid nitrogen tank for 3 time periods: 24 hours, 4 weeks, and 222 days. A corresponding control sample accompanied each treatment. For three species, *Calocedrus decurrens, Pinus jefferyi,* and *Pinus contorta*, the germination percent was not significantly different from the controls in any of the liquid nitrogen treatments. Exposure to 24 hours of liquid nitrogen did not affect the germination percent for any of the 9 species compared to their controls. Two species, *Abies x shastensis* and *Picea engelmannii,* exhibited a significant negative response to 4 weeks exposure to liquid nitrogen. Four species *Abies amabilis, Abies concolor, Pinus monticola,* and *Pseudotsuga menziesii,* exhibited a significant positive response to the 222-day exposure to liquid nitrogen when compared with Control D.

Experiment 2 examined the germination response to liquid nitrogen storage after a 24 hour exposure for 42 tree species. The germination percent for nine of the 42 species, *Acer rubra, Celtis occidentalis, Lonicera tartarica, Malus prunifolia, Physiocarpus opulifiolius, Pinus banksiana, Pinus clausa, Pinus nigra,* and *Pinus rigida*, was significantly affected by 24 hours exposure to liquid nitrogen. Liquid nitrogen exposure had a negative affect *on germination for 7 species and a positive effect for 2 species, Pinus nigra,* and *Pinus rigida.* Only 8 species had enough data to calculate the correlation coefficient between moisture content and germination after exposure to liquid nitrogen. Correlations were significant for 4 species. Two species, *Abies fraseri* and *Liriodendron tulipfera* had negative correlations; two species, *Pinus ponderosa* and *Pinus taeda* had positive correlations.

INTRODUCTION

Seeds are an inexpensive way of conserving populations of genes in a gene conservation program. Conventional long-term storage for orthodox tree seeds is usually conducted at –20 to –7 °C, but seed deterioration and viability loss can occur with increased time in storage. Changes in seed storage temperature resulting from long periods without electrical power or mechanical breakdowns of mechanical refrigeration systems can shorten the life of the seed (Stanwood and Bass 1981). Large seed samples must be maintained to allow for periodic germination testing and viability losses. Large storage facilities are needed to house these large seed samples which is costly. Losses in viability may also change the original gene content of the population as individuals are lost in the sample.

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Cryopreservation of seed is not dependent on electrical and mechanized refrigeration. Liquid nitrogen (–196 °C) is stored in self-contained tanks. The extreme cold temperature greatly reduces all sources of metabolic seed deterioration (Stanwood and Bass 1981). Cryo-storage of seed populations may provide the potential for the long-term maintenance of our valuable plant resources.

Before this method can be recommended as a viable option for gene conservation, the seed from the species needing to be stored must be tested. The duration which seed can be stored in liquid nitrogen without loss of viability is unknown (Stanwood and Bass 1981). Many agronomic crops' gene pools are being successfully preserved with cryopreservation at the USDA-ARS National Center for Resources Preservation in Fort Collins, Colorado. A similar procedure may apply to orthodox seeds of forest tree species; therefore, experiments were devised to test the possibility of cryopreservation of tree seed.

MATERIAL AND METHODS

Two separate experiments are presented. Both experiments examine the influence of liquid nitrogen storage on seeds of various tree species. A liquid nitrogen tank SC 33/32 with a MVE roller base, manufactured by Minnesota Valley Engineering Inc., was used for both experiments. The tank holds 30 liters of liquid nitrogen (–196 °C). The tank was kept in a walk-in cooler maintained at –7 °C during the experiment and only brought out into a room at ambient temperature to load and unload the seed samples. In experiment 1, seeds were placed in 1.8 and 2.0 ml cryogenic vials; the vials were snapped into aluminum canes designed to hold the vials. For experiment 2, nylon stockings were used to house the seeds. Both set of samples were placed into an aluminum tube, which was inserted into the tank so the samples did not come into contact with the liquid. Seeds were rewarmed at room temperature for about one hour before planting in germination dishes.

Experiment 1:

Seeds from nine western forest tree species were tested in the liquid nitrogen tank for 3 time periods: 24 hours, 4 weeks, and 222 days. Moisture content tests were performed before the study to insure the seed was sufficiently dry for long-term storage. Moisture content was calculated using the wet weight as the denominator (Table 1).

A 200 seed sample of each species was used for each liquid nitrogen treatment with a corresponding control sample (no liquid nitrogen treatment). Each seed sample was planted into 8 germination dishes of 25 seeds each. Kimpak® was used as media except with the true firs where metromix® was used. Approximately 80 ml of water was applied to each germinating dish before planting. All but 2 species received 21 days of prechilling before the germination test. Incense cedar, *Calocedrus decurrens* (Torr.) Florin seeds received 30 days of prechilling and western white pine, *Pinus monticola* Dougl. seeds received 3 months of prechilling before germination. *Pinus monticola* was the only seedlot that was not prechilled in the germination dish but in a cloth bag surrounded by peat moss in a small plastic container.

Table 1. Initial moisture tests for the seeds of species tested in experiment 1.

Common name	Species	Moisture Content % (Wet weight)
Incense cedar	*Calocedrus decurrens* (Torr.) Florin	4.44
Douglas-fir	*Pseudotsuga menziesii* (Mirb.)Franco	10.22
Engelmann Spruce	*Picea engelmannii* (Parry) Engelm.	5.44
Jeffrey Pine	*Pinus jeffreyi* Grev. & Balf.	5.60
Lodgepole Pine	*Pinus contorta* Dougl.	6.85
Pacific Silver Fir	*Abies amabilis* (Dougl.) Forbes	6.85
Shasta Red Fir	*Abies x shastensis* (Lemmon) Lemmon	6.51
White fir	*Abies concolor* (Gordon & Glend.) Lindl. ex Hildebr.	6.88
Western White Pine	*Pinus monticola* Dougl.	5.60

Table 2. Number of days of seed prechilling and temperature range before germination tests from experiment 1.

Species	Prechill	Temp (°C)
Calocedrus decurrens (Torr.) Florin	30 days	20–30
Pseudotsuga menziesii (Mirb.) Franco	21 days	20–30
Picea engelmannii (Parry) Engelm.	21 days	20–30
Pinus jeffreyi Grev. & Balf.	21 days	20–30
Pinus contorta Dougl.	21 days	20–30
Abies amabilis (Dougl.) Forbes	21 days	15–25
Abies x shastensis (Lemmon) Lemmon	21 days	20–30
Abies concolor (Gord & Glend.) Lindl.	21 days	20–30
Pinus monticola Dougl.	90 days	20–30

Abies amabilis (Dougl.) Forbes, Pacific silver fir, seeds were germinated at 15 °C in darkness for 16 hours and 25 °C with light for 8 hours; whereas, the other species' seed were germinated at 20 °C in darkness for 16 hours and 30 °C with light for 8 hours (Table 2). Germination counts were recorded weekly until seeds were fully germinated. Association of Official Seed Analysts (AOSA 2002) rules was followed for each species.

Experiment 2:

Forty-two tree species were selected for analysis. Each liquid nitrogen treatment was accompanied by a control sample (no liquid nitrogen treatment). Liquid nitrogen exposure for 24 hours was the only treatment. The germination test for the seeds each, while the germination test for the liquid nitrogen

treatment sample contained 200 seeds planted into 2 dishes of 100 seeds each. Kimpak® was the media used in the germination dishes unless the species' seed required a different media to maximize germination. Approximately 80 ml of water was applied to each small germination dish with the amount doubled for the large germination dishes. Various prechill time periods were used but not included in the analysis. AOSA (2002) rules were followed for each species to maximize germination.

Statistical Analysis:

SAS® software was used to analyze data in both experiments. SAS® statistical procedures were proc glm, proc corr, and proc ttest.

Specific comparisons in Experiment 1 were performed using contrast statements within proc glm. Control A was compared to the 24 hours liquid nitrogen treatment; Control B was compared to the 4 weeks liquid nitrogen treatment; and Control D was compared with the 222-day liquid nitrogen treatment. Control C and its corresponding treatment was eliminated due to a sampling error and replaced with the 222-day treatment. The controls were contrasted with each other, but the treatments were not contrasted with each other. Species were not compared with each other because treatment response was the hypothesis, not magnitude responses by species. There was no significant correlation between moisture content % and treatment so it was not reported in the results (Table 2).

A folded *F* test was calculated to test equality of variances for the *t* test in experiment 2. If variances were equal then the pooled standard deviations were used; whereas, with unequal variances the Satterthwaite method was used to calculate the *t* test. Germination counts were converted to percent and the angular (arcsine $\sqrt{Y}$) transformation was applied. Statistical analysis of the transformed data was almost identical to the percentages data, so the percentages were used in the analysis instead of the transformed data. There were only sufficient data for 8 species to run a correlation analysis of moisture content % with germination after the liquid nitrogen treatment. This was done as a check. If sufficient moisture is present in a seed it is possible such extreme cold could rupture cells causing loss of viability.

RESULTS

In experiment 1, seed from the 9 western tree species did not respond the same for every treatment. For three species, *Calocedrus decurrens, Pinus jefferyi* Grev. & Balf., and *Pinus contorta*, the germination was not significantly different from the controls in any of the liquid nitrogen treatments (Table 3). Exposure to 24 hours of liquid nitrogen did not affect the germination percent for any of the 9 species compared to their controls. Two species, *Abies* x *shastensis* (Lemmon) Lemmon, and *Picea engelmannii* (Parry) Engelm., exhibited a significant negative response to 4 weeks exposure to liquid nitrogen. Four species *Abies amabilis, Abies concolor* (Gordon & Glend.) Lindl. ex Hildebr., *Pinus monticola,* and *Pseudotsuga menziesii* (Mirb.) Franco, exhibited a significant positive response to the 222-day exposure to liquid nitrogen when compared with Control D.

Table 3. Seed germination percentages and tests of significance of liquid nitrogen treatments against controls from experiment 1.

Species	Control†			Liquid Nitrogen		
	A	B	D	24 hrs	4 wks	222 days
	%					
Calocedrus decurrens	11	10	0	14	8	3
Pseudotsuga menziesii	71	61	23	67	55	38‡
Picea engelmannii	96	85	89	97	70‡	85
Pinus jeffreyi	94	93	90	93	87	89
Pinus contorta	42	48	44	51	48	43
Abies amabilis	41	40	17	42	40	34§
Abies x shastensis	48	58	43	44	46‡	33
Abies concolor	45	38	13	37	34	39§
Pinus monticola	69	70	30	73	72	70§

† Control A for 24 hours treatment; Control B for 4 week treatment; Control C for 222 day treatment.

‡ Significant at $\alpha = 0.05$.

§ Significant at $\alpha = 0.01$ from its corresponding control.

Control D germination percentage of five species, *Abies amabilis, Abies concolor*, *Calocedrus decurrens, Pinus monticola,* and *Pseudotsuga menziesii,* declined when compared to Control A and B. It appears the seed may have degraded while in cold storage.

In experiment 2, the seed germination from 9 of the 42 species, *Acer rubra* L., *Celtis occidentalis* L., *Lonicera tartarica* L., *Malus prunifolia* (Willd.) Borkh., *Physiocarpus opulifiolius* (L.) Maxim, *Pinus banksiana* Lamb., *Pinus clausa* (Chapm. ex Engelm.) Vasey ex Sarg. , *Pinus nigra* J. F. Arnold subsp. *Nigra,* and *Pinus rigida* Mill., were significantly affected by 24 hours exposure to liquid nitrogen (Table 4). Liquid nitrogen exposure had a negative affect on germination for 7 species, *Acer rubra, Celtis occidentalis, Lonicera tartarica, Malus prunifolia, Physiocarpus opulifiolius, Pinus banksiana, Pinus clausa,* and a positive effect for 2 species, *Pinus nigra, and Pinus rigida.*

Only 8 species had enough data to calculate the correlation coefficient between moisture content and germination after exposure to liquid nitrogen. Correlations were significant for 4 species. Two species had negative correlations, *Abies fraseri* (Pursh.) Poir ($r = -0.79$, $n = 36$, $P < 0.001$) and *Liriodendron tulipfera* L. ($r = -0.996$, $n = 12$, $P < 0.001$). Two species, *Pinus ponderosa* C. Lawson ($r = 0.94$, $n = 12$, $P < 0.001$) and *Pinus taeda* L. ($r = 0.23$, $n = 96$, $P = 0.026$) had positive correlations.

DISCUSSION

Orthodox seed of the forest tree species reported in this study and by Stanwood (1985, 1984, 1981) were not, for the most part, adversely affected by

Table 4. Moisture content (MC) and tests of germination from experiment 4.

Species	MC (Wet weight basis)	Germination	
		Control	LN
	---------- % ----------		
Abies balsamea (L.) Mill.	—	57	50
Abies balsamea (L.) Mill. Var. *Canaan*	—	49	*52*
Abies fraseri (Pursh.) Poir.	7.9*[1]	24	22
Acer rubra L.	15.9	48	21**[2]
Celtis occidentalis L.	—	85	40**
Cercis canadensis L.	—	36	33
Fraxinus pennsylvanica Marshall	—	53	55
Ilex glabra (L.) A. Gray	—	0	1
Juniperus virginiana L.	—	8	6
Larix larcinia (DuRoi) K. Koch	—	51	48
Liquidambar styraciflua L.	9.6	86	74
Liriodendron tulipfera L.	10.9*	43	42
Lonicera tartarica L.	—	62	44*
Maclura pomifera (Raf.) C. K. Schneid.	—	39	36
Malus prunifolia (Willd.) Borkh.	12.7	74	49*
Morus rubra L.	—	67	62
Physiocarpus opulifolius (L.) Maxim	—	24	10*
Picea abies (L.) H. Karst.	—	53	52
Picea glauca (Moench) Voss	5.6	64	59
Picea pungens Engelm.	—	90	88
Pinus banksiana Lamb.	—	89	83*
Pinus clausa (Chapm. ex Engelm.) Vasey ex Sarg.	9.9	92	84*
Pinus contorta Douglas ex Loudon var. *latifolia*	5.7	91	81
Pinus echinata Mill.	9.1	64	61
Pinus elliottii Engelm.	7.4	92	92
Pinus michoacana	8.5	74	69
Pinus nigra J. F. Arnold subsp. *Nigra*	—	11	25*
Pinus oocarpa Schiede ex Schltdl.	8.2	63	59
Pinus palustris Mill.	7.8	66	63
Pinus ponderosa C. Lawson	8.9*	63	60
Pinus resinosa Aiton	—	89	89
Pinus rigida Mill.	—	86	95*
Pinus serotina Michx.	—	96	97
Pinus strobis L.	6.3	74	73
Pinus sylvestris L.	—	85	80
Pinus taeda L.	7.2*	89	89
Pinus virginiana Mill.	9.5	90	88
Platanus occidentalis L.	10.8	48	43
Pseudotsuga menziesii (Mirb.) Franco	4.5	72	69
Taxodium distichum (L.) Rich. Var. *distichum*	—	13	15
Thuja occidentalis L.	—	3	3
Vitex agnus-castus L.	—	86	90

[1] moisture content correlated with liquid nitrogen (LN) treatment.

[2] *Significant at $\alpha = 0.05$.

**Significant at $\alpha = 0.01$.

liquid nitrogen storage over the time periods in which they were tested. Stanwood (1985) exposed seed from 10 trees species, *Abies concolor, Abies procera* Rehder, *Picea sp.* Brewer, *Pinus lambertiana* Douglas, *Pinus ponderosa, Pseudostuga menziesii, Pyrus malus* L., *Thuja plicata* Donn ex. D. Don, *Tsuga heterophylla* (Raf.) Sarg., *Ulmus americana* L., to 1 to 3 years of liquid nitrogen storage without significant harm to the seed.

Stanwood's seed samples and the samples in this study were all at acceptable moisture content percentages (below 10 %) for conventional and liquid nitrogen seed storage. At these moisture contents there was little water to freeze in the cells, so ice crystal formation and damage by dehydration appeared to be insignificant (Stanwood 1981). The moisture limit of each species' seed needs to be discovered before cryopreservation becomes routine practice.

This is not to say that much longer liquid nitrogen exposure would not have created significant loss of viability. The assumption that seed surviving 24 hours exposure to liquid nitrogen would survive 100 years exposure without adverse effect on seed germination cannot be made, because extrapolation beyond the scope of the experiment is not valid. To know if the species' seed would survive for 50 to 100 years of liquid nitrogen storage, the seed would have to be tested for that time period.

In experiment 2, the seed coats of some species stored in nylon stockings cracked when they were removed from liquid nitrogen and exposed to ambient temperatures. Seed stored in cryovials in experiment 1 did not experience seed coat cracking. This problem can be overcome by storing seed in liquid nitrogen tight containers or in vapor phase storage (–150 °C) (Stanwood 1981).

Much of the germination in experiment 1 and 2 was not significantly high enough for gene conservation. The germination should be as high as possible to insure against possible loss of genetic integrity. It is not economically or biologically reasonable to store dead or degraded seed for long time periods. The seed quality in experiment 1 should have been upgraded before the experiment began. Any loss in viability would have been due to the liquid nitrogen treatment and not to the degraded condition of the seed.

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