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Longevity of cryogenically stored seeds☆

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Abstract

Though cryogenic storage is presumed to provide nearly infinite longevity to cells, the actual shelf life achieved under ultra-cold temperatures has not been addressed theoretically or empirically. Here, we report measurable changes in germination of dried seeds stored under liquid nitrogen conditions for >10 years. There was considerable variability in the extent of deterioration among species and accessions within a species. Aging time courses for lettuce seeds stored at temperatures between 50 and −196 °C were fit to a form of the Avrami equation to determine rate coefficients and predict half-life of accessions. A reduction in the temperature dependency on aging rate, determined as a break in the Arrhenius plot, occurred at about −15 °C, and this resulted in faster deterioration than anticipated from extrapolation of kinetics measured at higher temperatures. The break in Arrhenius behavior occurred at temperatures in between the glass transition temperature (28 °C) and the Kauzmann temperature (−42 °C) and also coincided with a major triacylglycerol phase change (−40 to −7 °C). In spite of the faster than anticipated deterioration, cryogenic storage clearly prolonged shelf life of lettuce seeds with half-lives projected as ~500 and ~3400 years for fresh lettuce seeds stored in the vapor and liquid phases of liquid nitrogen, respectively. The benefit of low temperature storage (−18 or −135 °C) on seed longevity was progressively lost if seeds were first stored at 5 °C. Collectively, these results demonstrate that lowering storage temperature progressively increases longevity of seeds. However, cryogenic temperatures were not sufficient to stop deterioration, especially if initial stages of aging were allowed to progress at higher storage temperatures. This work contributes to reliable assessments of the potential benefit and cost of different genebanking strategies.
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Keywords: Aqueous glass; Cryopreservation; Desiccation; Genebank; *Lactuca sativa*; Longevity; Kauzmann temperature; Seeds; Storage; Water content

Cryogenic storage is favored for preservation of DNA, tissue, and germplasm because extremely low temperatures are believed to stop most biological activity. The actual time scale for biological change at liquid nitrogen temperatures (−120 to −196 °C) is of both practical and fundamental interest because it relates to questions of the most efficient way to bank biological materials as well as the nature and kinetics of reactions that occur under ultra-cold conditions. Measurements of change in materials that are cryogenically stored are sparse because the time scales are large,

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experiments are few (routine use of cryogenic storage began just 30 years ago), and quantitative assessments are often difficult to make.

Existing evidence clearly shows that degradation is slower and shelf-life enhanced when cells are stored at cryogenic rather than refrigerator temperatures. Pathogens, cell cultures, spermatozoa, and embryos remain viable in cryogenic storage for 10–40 years (e.g. [11,12,14,16,22,25,27–29,32,33]). However, since quantitative assessments of change are difficult in these systems, the above studies are limited to conclusions about longevities for the cells that survived and do not document losses of cell viability. Unchanged viability of mouse embryos exposed to a dose of about 2000 years of background radiation while stored in liquid nitrogen for 5 months [18] may imply that cells surviving the initial stress of cryogenic exposure will survive at least 2000 years. The validity of this supposition rests on the hypothesis that cryogenic temperatures virtually stop thermally regulated biological reactions, an hypothesis that will not be tested for many years to come. The little existing evidence that demonstrates degradation during cryogenic storage is usually attributed to temperature fluctuations that lead to devitrification of aqueous glasses and the growth of damaging ice crystals, rather than to aging reactions that occur at the storage temperature (e.g. [30,39,40]).

Problems of devitrification are mostly avoided in dry seeds where glass transition temperatures (T_g) are greater than the melting temperature of water (T_m) (e.g. [4,24]). In spite of the obvious advantage of avoiding ice crystals, the beneficial effects of ultra-cold storage in seeds are still not clear [15,13,35]. Currently deterioration kinetics in seeds stored at temperatures less than 0 °C are extrapolated from experiments conducted at much higher temperatures. These empirically derived models predict exponential increases in seed lifespans (e.g., Harrington's Thumb Rules: Q10≈2 [21]) to minor increases or potential reductions in seed lifespans as temperatures fall below about −60 °C (e.g. [13,23]). Assuming a limited benefit of low temperature, "ultra-dry technology" sought to achieve comparable seed lifespans without the high cost of refrigeration or liquid nitrogen [15,37,43].

Deterioration rates of seeds correlate to intracellular viscosity [8,9,38], suggesting models for viscoelastic or cooperative reactions may be appropriate to describe deterioration kinetics. The Avrami function [2] is a classic model of cooperative kinetics giving the familiar sigmoidal form typically observed for seed deterioration time courses. Used to model the rate of ice nucleation and crystal growth (i.e. [17]), forms of the Avrami equation have also been used to describe the kinetics of hemolysis in erythrocytes exposed to low temperature [31] or osmotic stress [41]. Here, we use the Johnson–Mehl–Avrami form of the Avrami equation to calculate time coefficients (ϕ) for lettuce (*Lactuca sativa*) seeds stored at temperatures between 50 and −196 °C

$$\ln\left(\frac{N_0}{N}\right) = \left(\frac{t}{\phi}\right)^n, \tag{1}$$

where t is storage time, N_0/N is interpreted as the reciprocal of percentage germination, and n is the Avrami coefficient that reflects how rapidly change occurs once it has been initiated. The time coefficient, ϕ, can be treated as an Arrhenius function of temperature, enabling us to evaluate temperature dependencies of seed aging rates [41].

In this paper, we report quantitative changes in viability of seeds stored for >10 years in liquid nitrogen (−196 °C) or above liquid nitrogen (vapor phase temperature ≈−135 °C). Aging rates predicted for lettuce seeds stored in vapor or liquid phases of liquid nitrogen are compared with rates measured at higher storage temperatures and extrapolations based on empirical models. We relate the temperature dependency of lost viability in stored lettuce seeds to the temperature dependency of molecular motion in seeds [7–9,38].

Methods and materials

The USDA National Center for Genetic Resources Preservation (NCGRP) (formerly National Seed Storage Laboratory (NSSL)) serves as a geneBank to conserve the genetic diversity of economically important plant and animal species. The plant collection was initiated in the late 1950s

Table 1
Summary of the experiments used in this paper to report time and temperature-dependent changes in seed viability

Storage temperature (°C)	Species	Seed source	No. of accessions	Harvest year	Storage initiated	Analyses	Data reported
−196 (constant)	42, including Lettuce	Commercial	1–21 (9 for Lettuce)	1975–1976	1977	Linear trend of pooled data	Table 3, column 1
						Paired t test of initial and final percentage germination among accessions	Table 3, column 1 (sig. indicated by *)
						95% CI of initial and final percentage germination, averaged among accessions	Table 3, columns 3, 4 and 5, 6
						Percentage germination averaged among accessions and time; linear trend and Avrami curve-fit (lettuce)	Fig. 1 and Fig. 2D, Table 4 (right side)
						Avrami curve-fit of individual accessions	Table 4 (left side)
−135 (constant)	Lettuce	NCGRP collection	70	1978–1990	$\leqslant$1 year post-harvest	Percentage germination averaged among accessions and time; linear trend and Avrami curve-fit	Fig. 1 and 2C, Table 4 (right side)
						Avrami curve-fit of individual accessions	Table 4 (left side)
						95% CI of percentage germination averaged among accessions, time and monitoring frequency	Table 2
−135 after 5 and −18 °C	Lettuce	NCGRP collection	256	1967–1976	1984 (half samples at −18 °C	Percentage germination averaged among accessions and time; Avrami curve-fit	Fig. 8
−18 (constant)	Lettuce	NCGRP collection	43	1976–1982	$\leqslant$1 year post-harvest	Percentage germination averaged among accessions and time; linear trend and Avrami curve-fit	Fig. 1 and 2B, Table 4 (right side)
						Avrami curve-fit of individual accessions	Table 4 (left side)
−18 after 5C	Lettuce	NCGRP collection	256	1967–1976	1977	Percentage germination averaged among accessions and time; Avrami curve-fit (lettuce)	Fig. 7
5 (constant)	Lettuce	NCGRP collection	61	1954–1969	After harvest	Percentage germination averaged among accessions and time; linear trend and Avrami curve-fit	Fig. 1 and Fig. 2A, Table 4 (right side)
						Avrami curve-fit of individual accessions	Table 4 (left side)
−12, −1, 10, 21, 33 (constant)	Lettuce	Commercial	1 (cv. 'Great Lakes')	1959	After harvest	Avrami curve-fit at each temperature	Fig. 3, Table 4 (lower portion)

when dried seeds were stored at 5 °C. The temperature for conventional storage of dried seeds was reduced to −18 °C in 1977, though processing and short-term storage remain at 5 °C. Studies of the feasibility of liquid nitrogen storage were introduced to NCGRP in 1977, when 1–21 commercially available varieties of 42 species of seeds were placed in liquid nitrogen (−196 °C). Seeds for this initial feasibility study were donated by commercial growers to NSSL and were selected on the criteria of recent harvest, high viability, and sufficient seed numbers for viability monitoring (Table 1). Beginning in 1984, many species within the NCGRP collection were routinely stored above liquid nitrogen (vapor phase of liquid nitrogen $\approx -135 \pm 15$ °C). The data presented in this paper rely mostly on accessions contained in the USDA NCGRP collection (except for samples obtained from commercial growers as indicated in Table 1), that were harvested at official sites and processed and stored at NCGRP under various storage regimens (Table 1), depending on the date they were received and the quantity of seed available. Data about accessions were retrieved from the NCGRP database through a genetic resources information network (GRIN) query with criteria of high initial viability, constant storage conditions, and $\geqslant 3$ (5 or −18 °C storage) or 2 (vapor storage) viability assessments.

For −196 °C storage, aliquots of accessions were separately sealed in cans and submerged in liquid nitrogen. All cans remained submerged with only the top one from each stack removed when it was time to test germination. [Please note, that storage of aluminum cans under liquid nitrogen presents a danger of explosion. Cans were deemed too unsafe and this experiment had to be terminated in 1999.] Seeds stored in cryovats above liquid nitrogen (∼−135 °C), were sealed in large plastic straws, and the entire accession was removed and warmed to room temperature for approximately 2 days each time viability was assessed. An analysis of seed viability for lettuce seed that had been removed once or twice from storage (during 13 years of storage) and two or three times (during 21 years of storage) revealed no detectable differences with monitoring frequency (Table 2). Samples stored at 5 and −18 °C were packaged in foil-laminate bags and were also warmed to room temperature during viability assessments. Storage temperatures for all regimens have been continuously monitored since the early 1960s and no anomalies were reported until 1999, when the −196 °C experiment was terminated. In the vapor phase storage, the temperature of the cryovat fluctuated between −120 °C and −150 °C over the 20 years of the experiment. Modern temperature reporting devices and automatic filling keep current temperatures at a more constant −150 °C; but for the sake of these analyses an intermediate storage temperature of −135 °C is assigned for vapor phase storage.

Germination of stored seeds was monitored, usually at 5 year intervals, providing longevity data and regeneration alerts when viability declines below an acceptable level. Germination assays were/are conducted by certified seed analysts using AOSA Rules [1]. Data for lettuce seeds were collected since the early 1960s by Dr. Louis Bass or his protégé, Ms. Patricia Conine.

Detailed longevity studies reported here used data for lettuce (*L. sativa*) seeds since this species serves as a good model for seed deterioration studies [3,19–21,23], and NCGRP has a large number of accessions of lettuce in cryogenic storage, giving the opportunity to study the long-term

Table 2
Effect of sampling frequency on percent germination of lettuce seed stored in the vapor phase above liquid nitrogen (−135 °C)

# times germination assayed during storage	Years that germination was assayed	Storage time for reported germination results (years)	Percent germination (95% confidence interval)	# samples
2	1, 13	13	97.5–95.2	32
3	1, 7, 13	13	97.2–94.8	64
3	1,7, 21	21	96.8–87.4	3
4	1, 7, 13 or 17, 21	21	96.4–92.4	3

Germination data were transformed to calculate mean germination and 95% confidence interval.

effects of various storage regimens (Table 1). For constant temperature studies, 61, 43, 70, and 9 accessions of lettuce seeds were placed almost immediately (<1 year of processing time) into storage at 5, −18, −135, and −196 °C and monitored for about 30, 20, 15, and 14 years, respectively. Lettuce seeds (cv. 'Great Lakes' Ferry Morse Seed, Mountain View, CA), that were sealed in cans in 1959 by Dr. Louis Bass and stored at 32, 21, 10, −1, and −12 °C ([3,21]), were re-assayed and used as an additional data set. Initial germination percentages were high for each group of accessions, ranging from $95 \pm 5\%$ (experiment at constant 5 °C) to $99 \pm 2\%$ (experiment at constant −135 °C). In another experiment, 256 accessions of lettuce were stored for 2–10 years at 5 °C (harvest year ranged from 1967 to 1975) and then about 6 years at −18 °C and finally split in 1984 and stored at both −18 and −135 °C for an additional 18 years (Table 1). Accessions used for this experiment were selected based on high percentage germination ($94 \pm 5\%$) at the onset of cryogenic storage in 1984. Water contents of stored lettuce seed ranged from 0.05 to 0.08 g H_2O/g dry weight (g/g) and averaged 0.065 ± 0.007 g/g for all storage temperatures. Depending on accession, germination percentage was monitored 2–4 times during liquid nitrogen storage and 3–7 times during −18 or 5 °C storage.

Statistical analyses (trend, averages and tests of differences) used angular transformed percent germination data and SigmaStat software. Significant change in viability during storage was tested in liquid phase storage by linear regression of all germination percentage versus time data points for the species (Table 3, column 1), regression of a single time course created from transformed data averaged among storage time and accession (results for lettuce given in Fig. 1), and a paired t test of initial and final percent germination among accessions (Table 1, significance indicated by a * in column 1). The 95% confidence interval for initial and final germination percentages was calculated by multiplying the standard error of the mean germination (retransformed) by 1.9.

Longevity parameters for each storage treatment were calculated by solving for the coefficients in Eq. (1) using least squares linear fit of $\ln[\ln(N_0/N)]$ and ln(time) and a standard spreadsheet [Thus, the regression equation is $\ln(\ln(N_0/N)) = n \cdot \ln(\text{time}) + y_0$, where y_0 is the y intercept. The variables N and time are known and the coefficients n and y_0 are calculated]. The value of N was assigned as the number of seeds germinating normally ÷ number of seeds planted. As with many viability models, the coefficient representing initial viability (N_0 in Eq. (1)) strongly influences the kinetics. Here, N_0 was assigned a value that was 0.5 greater than the maximum value of the time course (usually the initial percentage germination). The coefficient ϕ and the exponential factor n in Eq. (1) were calculated from the coefficients of the regression line ($\phi = e^{(-y_0/n)}$ and $n =$ slope). The time for germination to drop to 50% (P50) was calculated by interpolating (storage at temperatures $\geqslant -1$ °C) or extrapolating (storage at $\leqslant -18$ °C) Eq. (1) using the coefficients calculated from fitted time courses. Germination time courses were analyzed in two ways: as a representative time course created by averaging transformed germination among accessions for each storage time and as individual accessions if germination was assayed three or more times. When individual accessions were fit to the Avrimi function, it was necessary to constrain the slope of the regression to values $\geqslant 0.1$ so that modeled germination time courses always decreased even if detectable changes within the accession were not apparent within the experimental time frame. When this was done, values of P50 of the order of 10^4 years were calculated. Similar calculations of Avrami coefficients and P50s were made using published viability time courses of lettuce seeds containing 0.06–0.07 g H_2O/g dw and stored at temperatures between −12 and 50 °C [19–21]. For the work considered here, uncertainty of P50 was approximated from coefficients ± standard error of coefficients calculated from regression analyses. Future work will analyze variability of P50 among accessions.

Calorimetric parameters were used to determine phase transitions in lettuce seeds that might influence temperature dependency of deterioration kinetics. The water contents of seeds were adjusted by storing seeds over different relative humidities or by adding known amounts of water to weighed

Table 3
Seed germination following storage in liquid nitrogen (−196 °C) for 10–20 years

Species	# Accessions tested	Percentage germination				Storage time (years)
		Initial (95% CI)		Final (95% CI)		
Significant trend ($P < 0.05$)						
Abies procera	4	56.3	41.6	53.1	16.5	11.7
*Allium cepa**	21	95.6	90.3	90.6	79.6	10.4
*Beta vulgaris**	11	94.5	88.9	79.2	53.8	13.7
Eragrostis curvula	3	96.7	93.9	82.6	53.6	14.2
Helianthus annuus	3	95.1	91.5	92.9	74.7	20.4
*Lactuca sativa**	9	98.6	97.7	97.3	93.8	12.1
Medicago sativa	4	93.0	84.6	87.1	77.5	13.4
Papaver somniferum	3	99.4	88.9	94.6	51.6	14.2
Petroselium crispum	2	85.0	76.9	74.8	60.0	21.2
Ulmus americana	1	89[a]		77[a]		21.6
*Vicia spp.**	3	95.6	92.4	88.6	84.8	14.2
*Zinnia violacea**	4	91.2	88.3	87.8	78.3	13.7
No significant trend ($P > 0.05$)						
Apium graveolens	2	95.4	80.6	94.6	89.2	20.9
Astragalus spp.	3	89.7	85.6	92.5	76.4	21
Brassica oleracea	18	96.7	94.1	97.3	95.0	11.7
Capsicum annuum	3	98.9	97.1	98.2	96.8	20.5
Citrullus lanatus	5	99.1	95.5	99.9	85.6	20.9
Crambe abyssinica	4	84.5	68.1	86.7	75.3	21.0
Cucumis melo	7	97.2	92.9	95.8	91.8	21.3
Cucumis sativa	7	98.6	96.5	99.6	96.3	21.6
Daucus carota	19	89.2	82.4	87.6	81.2	14.4
Elymus condensatus	1	14[a]		11[a]		20.7
Festuca rubra	6	93.5	83.1	97.3	85.3	21.1
Glycine max	1	64[a]		57[a]		14.1
Hordeum vulgare	3	98.6	88.4	98.9	85.1	15.7
Lespedeza stipulacea	1	88[a]		89[a]		20.7
Lotus corniculatus	3	81.0	57.6	84.3	61.6	21.2
Lycopersicum escluentum	6	90.9	82.1	90.6	85.2	10.6
Nicotiana tabacum	4	97.8	90.3	99.4	85.6	14.2
Onobrychis viciifolia	3	85.6	65.3	86.4	81.6	21.2
Oryza sativa	2	97.4	93.4	95.0	92.5	20.8
Pennisetum glaucum	1	86[a]		75[a]		20.6
Petunia spp.	6	94.5	84.7	93.9	85.0	14.1
Phaseolus vulgaris	3	97.7	88.7	99.4	92.7	21.2
Poa pratensis	3	87.7	85.6	94.6	89.2	13.9
Raphanus sativus	8	98.8	97.8	99.3	96.7	19.9
Sorghum bicolor	7	97.9	95.2	97.6	94.2	14.0
Spinacia oleracea	5	97.7	92.5	96.7	90.8	21.6
Trifolium pratense	3	92.9	71.8	84.6	74.6	13.4
Trifolium repens	3	91.9	87.4	97.7	92.2	13.4
Triticum aestivum	3	96.8	81.5	99.7	79.4	21.2
Zea mays	1	98[a]		77[a]		20.5

Species are divided into categories that do and do not show significant trends ($P \leqslant 0.05$) towards reduced germination over the study period, based on a linear regression model of storage time (5–7 sampling times) versus angular transformed percent germination data. As an additional test of significant change, transformed germination data from initial and final assays were compared among accessions within a species in a paired t test, and significant differences ($P \leqslant 0.05$) are indicated by an * in the first column. Germination data, transformed, and averaged among accessions were used to calculate 95% confidence intervals for initial and final germination for each species.

[a] Only 1 accession of this species was included in the experiment, precluding calculation of 95% confidence intervals for initial and final germination assays.

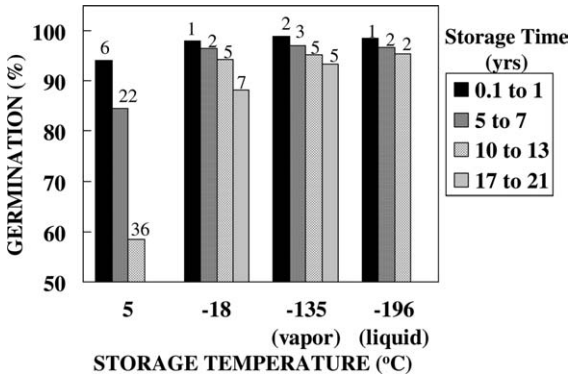

Fig. 1. Average percentage germination of lettuce seed accessions containing 0.06–0.07 g H_2O/g dry weight and stored at the indicated temperatures for up to 21 years. "Liquid" and "vapor" refer to seeds stored in cryovats submerged in or above liquid nitrogen, respectively. Averages and standard deviations (numbers above bars) were calculated from angular transformed data from 61, 43, 70, and 9 accessions stored at 5, −18, −135, and −196 °C for the indicated time and temperature ranges. Germination of seeds stored at 5 °C for 17–21 years was $7 \pm 13\%$. Data are unavailable for seeds stored at −196 °C for the same interval. Linear regressions of time courses calculated from averaged data and SigmaStat software were significant ($P \leqslant 0.04$) for all storage regimens, though slopes for −196, −135, and −18 were not significantly different from each other based on the standard errors of the slopes. The 95% confidence intervals for germination percentage following 20 years of storage were 3–10% (5 °C), 86–90% (−18 °C), and 92–95% (−135 °C).

seed samples. Transitions were detected using a differential scanning calorimeter (DSC) (Perkin–Elmer DSC7) during warming at 10 °C/min between −150 °C and +110 °C. Triacylglycerol melting transitions (lettuce seeds contain about 35% lipid) were assigned using scans of almost completely dried seeds (<0.01 g H_2O/g dw) and water melting transitions were distinguished in wetter seeds from a difference in temperature or energy. Aqueous glass transitions were assigned to seeds with water contents between 0.02 and 0.12 g H_2O/g dw from step-wise discontinuities in the power baseline; T_g was calculated as the temperature at half-height of the discontinuity.

Results

Reduced germination was measured in seeds from 12 of the 42 species that were stored at −196 °C for 10–20 years (Table 3) ($P \leqslant 0.05$ in regression model of time versus percentage germination by species). When viability declined during storage, it was progressive with time (e.g., $P \leqslant 0.04$ in regression models of averaged time courses for lettuce (Fig. 1)) and faster at higher temperatures (e.g., the 95% confidence intervals for germination of lettuce following 20 years of storage at 5, −18, and −135 °C do not overlap (Fig. 1)). Extent of deterioration varied among accessions, which is demonstrated by the increased breadth of the 95% confidence interval of final germination percentages for species showing deterioration (Table 3), the range of percentage germination from 0 to 99% among 61 lettuce accessions that were stored at 5 °C for about 14 years (Fig. 2A), and the range of P50s calculated among accessions in Table 4. In spite of the large variation in viability observed among lettuce accessions after 14 years storage at 5 °C, there was little variation after 25 years as most seeds in most accessions had died.

Deterioration time courses, measured using germination percentage assays, exhibit a sigmoidal shape in which an initial period giving minor change in germination percentage is followed by a period of rapid decline and then one of lingering low viability [21] (Figs. 2 and 3). The sigmoidal shape of deterioration curves is most easily visualized when single accessions are plotted (Fig. 3), but is also apparent when percentage germination among accessions is averaged for a given storage period (Fig. 2A). Deterioration time courses, measured for 20–40 years for either individual accessions (assayed at least three times) or for percentage germination averaged among accessions, were fit to a version of the Avrami equation (Eq. (1)) allowing calculation of the coefficients ϕ and n (Table 4). These parameters were also calculated for deterioration time courses of lettuce reported in the literature [19,20]. The value of P50 (the time for germination to decrease to 50%), calculated from the coefficients of the Avrami equation, increased from about 13 years when seeds were stored at 5 °C to a few thousand years when seeds were stored in liquid nitrogen (Table 4, Fig. 4). The wide variability in projected longevity among accessions is evident from the range of minimum and maximum longevities calculated

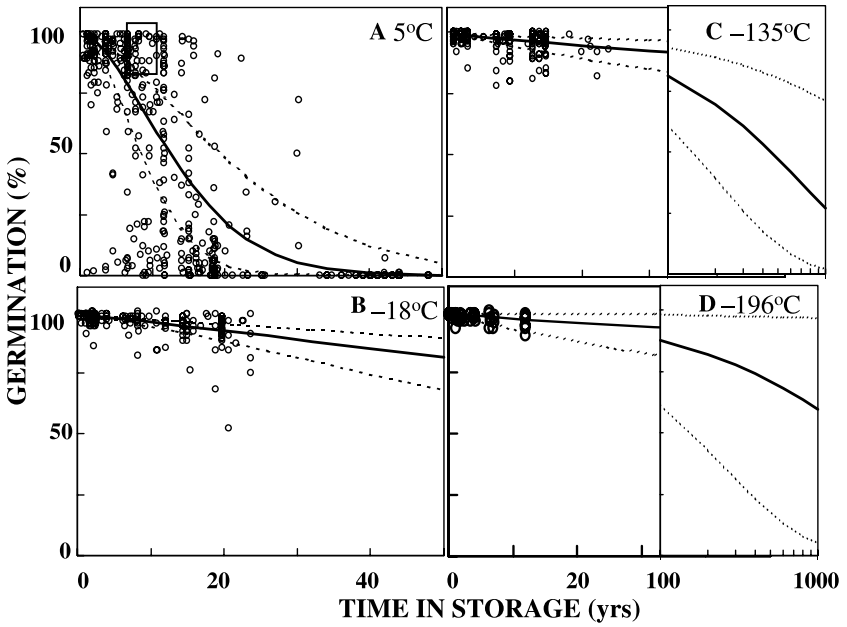

Fig. 2. Percentage germination of lettuce seed as a function of storage time at different temperatures. Each point represents percentage normal seedlings developing from an accession at a time-temperature combination and most accessions were assayed 3–5 times during the storage period. These data were transformed and used to calculate average percentage germination given in Fig. 1, parameters in Eq. (1) (Table 4) and P50 values in Fig. 4. Solid curves are the Avrami function using coefficients given in Table 4 and dashed curves are the Avrami function using these coefficients plus and minus their calculated standard errors. The boxed accessions in A have characteristics similar to accessions used in experiments on effects of 8–10 years prestorage at 5 °C (Figs. 7C and D and 8C and D).

Table 4
Coefficients calculated from linear regressions of ln(ln (1/percentage germination)) versus ln(storage time) of lettuce seeds containing 0.065 g H_2O/g dw and stored at the indicated temperatures

Temperature (°C)	Accessions considered individually					Averaged among accessions			
	Slope	Intercept	P50 (years)			Slope	Intercept	ϕ	P50 (years)
			Min	Max	Median				
5	2.1 (0.7)	−5.4 (2.4)	2	42	12.2	2.0 (0.1)	−5.4 (0.4)	15.5	13 (9–19)
−18	1.0 (0.5)	−5.3 (0.7)	29	982	169	1.2 (0.1)	−6.2 (0.2)	205	150 (88–280)
−135	0.8 (0.6)	−5.8 (0.6)	95	3244	942	0.8 (0.1)	−5.4 (0.2)	828	524 (199–1282)
−196	0.5 (0.2)	−4.7 (0.3)	1805	2×10^4	5296	0.5 (0.1)	−4.8 (0.3)	6586	3377 (702–3×10^4)
	'Great Lakes' stored for 40 years at different temperatures								
32						2.1 (0.3)	−0.06 (0.3)	1	0.9 (0.8–1.0)
21						4.2 (0.9)	−7.2 (1.3)	6	5 (3–12)
10						2.8 (0.5)	−6.8 (1.1)	11	10 (5–27)
−1						2.3 (0.3)	−6.2 (0.9)	15	13 (7–29)
−12						1.3 (0.2)	−5.7 (0.8)	80	61 (21–264)

In the upper portion of the table, regression analyses are performed on individual aging time courses (left side) for 61, 43, 70, and 9 accessions of lettuce stored at 5, −18, −135, and −196 °C or on a single time course calculated from transformed germination percentages averaged for each storage time interval (right side) (data in Fig. 2). Values in parentheses represent standard deviations of calculated coefficients among accessions (left side) or standard error of coefficients from the single linear regression (right side). In the lower portion of the table, regression analyses are performed on a single accession of lettuce seed stored at the indicated temperatures (data in Fig. 3).

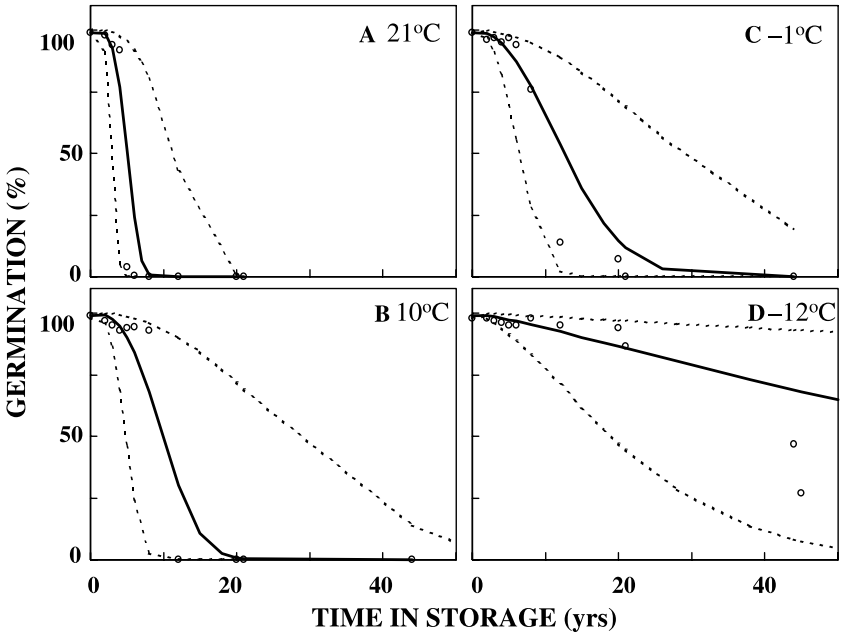

Fig. 3. Percentage germination of 'Great Lakes' lettuce seed harvested in 1959 by Ferry Morse Seed Company and stored at the indicated temperatures for 42 years. Germination data for the first 20 years of storage were reported by [3] and [21]. Coefficients for Eq. (1) were calculated from time courses (Table 4) and used to draw the curves (calculated coefficients (solid curves) ± standard error of coefficients (dashed curves)) and P50 values in Fig. 4.

from time course data for individual accessions (Table 4), with the minimum P50 equal to about 10–30% of the value of the median or pooled P50.

Despite variability among accessions, the median P50 values and P50 values calculated from averaged time courses correspond well to P50 values calculated from Viability equations [13,23] at storage temperatures $\geqslant -18\,^{\circ}C$ (Fig. 4). Estimates of P50 based on deterioration data of seeds stored at −135 and −196 °C were greater than expected from Viability equations containing a quadratic term for temperature, and less than expected from Viability equations when an exponential relationship was used to describe the effect of storage temperature on longevity.

Calorimetric measurements describe state changes occurring in lettuce seed components that may influence aging kinetics at the temperature range studied. Triacylglycerols melt at about −80 °C (α crystals), recrystallize at −55 °C, and show a major melting transition (β' crystals) between −45 and −7 °C in seeds at all water contents (Fig. 5). Water melting transitions occur between −30 and −10 °C, but are only detectable in seeds containing more than 0.15 g H_2O/g dw (Fig. 5 shows increased peak area in seeds with water contents $\geqslant$0.17 g/g). Two aqueous glass transitions were detected at temperatures $\geqslant 0\,^{\circ}C$ in seeds with $<$0.13 g H_2O/g dry weight (Fig. 5 inset shows seeds with 0.06 and 0.07 g/g). According to these data, T_g for seeds containing 0.065 g H_2O/g dry weight was assigned at 28 °C, which is consistent with other dry systems reported in the literature [4,7,24,42].

The temperature dependency of aging reactions can be described by the parameter ϕ (Eq. (1)) when graphed in an Arrhenius plot (Fig. 6). Here, we scaled temperature by T_g (28 °C for lettuce seeds containing 0.065 g H_2O/g dw (Fig. 5)) and ϕ by ϕ at 28 °C (ϕ_{T_g}, calculated from parameters determined from data sets used in Fig. 4) in order to incorporate potentially relevant data about molecular mobility (rotational motion [7–9] and glass relaxation [38]) on the same graph. A change in the temperature dependency of lettuce seed aging reactions occurred at −15 °C ($T_g/T = 1.17$), with a greater slope at the higher temperature

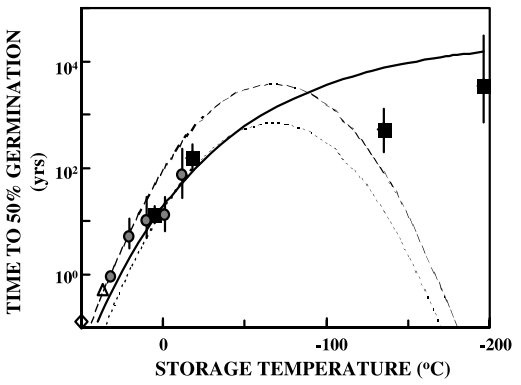

Fig. 4. The storage time calculated to reduce percentage germination to 50% (P50) for lettuce seed stored at temperatures between 50 and −196 °C. Values for P50 were calculated from time courses fit to Eq. (1) (i.e., the time giving 50% germination from solid curves in Figs. 2 and 3) and symbols represent various data sets used: squares (analyses from Fig. 2), circles (analyses from Fig. 3), triangle [19], and diamond [20]. Error bars represent the range of P50 values calculated when the Avrami function uses best-fit coefficients plus and minus their calculated standard errors (i.e., the time giving 50% germination from the dashed curves in Fig. 2). The curves represent P50 values calculated using viability equations and coefficients for lettuce seed: Eq. (4) in [13] (solid curve), Eq. (2) in [13] (dotted curve), and Eq. (1) in [23] (dashed curve). The parabolic shape is predicted because the temperature term is a quadratic equation.

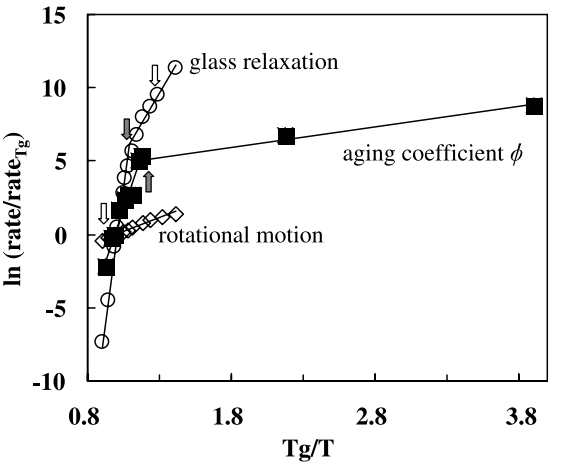

Fig. 6. Arrhenius plot, scaled by values at T_g, of aging rate in lettuce (ϕ in Eq. (1) as described in Table 4) (solid squares). Measures of molecular mobility are also given for comparison: open diamonds represent rotational motion (from [7]) and open circles represent glass relaxation in seeds (from [38]). Lines are drawn from linear regressions ($r^2 > 0.96$) that intersect at −15 °C (aging) and 2 °C (glass relaxation) as indicated by shaded arrows. Open arrows represent T_g (28 °C) and T_K (−40 °C). Slopes of lines drawn at high and low temperature ranges were 8.6 (SE = 0.5) and 0.4 (SE = 0.1), respectively, giving apparent activation energy (E_a) of 71 and 3.5 kJ/mol for lettuce seed aging above and below the break temperature.

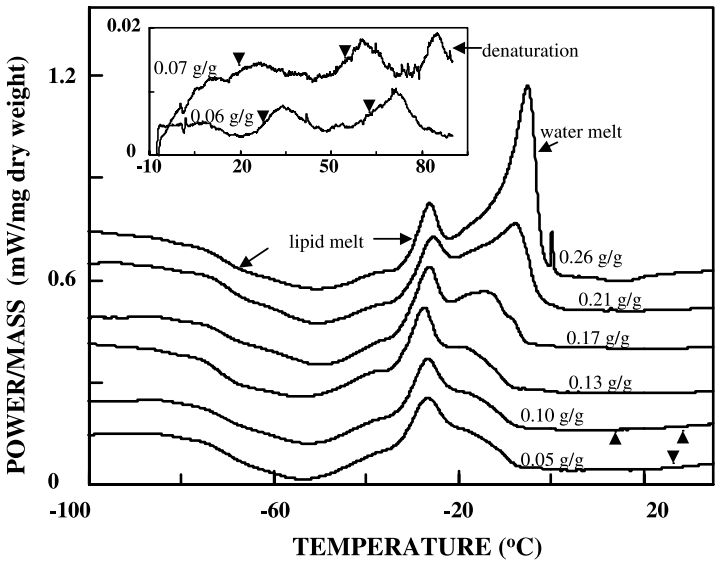

Fig. 5. Representative DSC scans of lettuce seeds containing indicated amounts of water. Various first order transitions are labeled. Aqueous glass transitions are indicated by arrows at temperatures above 0 °C.

range ($r^2 = 0.98$, $E_a = 71$ kJ/mol for temperatures $\geqslant -15$ °C; $r^2 = 0.97$, $E_a = 3.5$ kJ/mol for temperatures $\leqslant -15$ °C). A change in the temperature dependency was also measured for glass relaxation (break temperature = 2 °C and Arrhenius activation energy (E_a) = 178 and 41 kJ/mol at temperatures above and below 2 °C, respectively [38]), and rotational motion (break temperature = 28 °C, $E_a \approx 30$ and 9 kJ/mol at temperatures above and below 28 °C [7,9]).

Viability time courses described so far in this paper reflect the aging kinetics of seeds placed at the storage temperature within 1 year after harvest. In practice, conditions for seed storage vary during the storage period, as is illustrated in the following experiment where seeds were harvested between 1967 and 1975 and stored at 5 °C until 1977, when routine storage at −18 °C was implemented at NCGRP; then, in 1984 samples were split and stored at both conventional (−18 °C) and liquid nitrogen (−135 °C) temperatures. Viability time courses and calculated Avrami parameters for seeds stored for 2–10 years at 5 °C, then 7 years at −18 °C, and then an additional 18 years at either −18 or −135 °C are shown in Figs. 7 and 8, respectively. Avrami predictions of P50 in seeds stored at −18 and −135 °C decreased almost exponentially with prestorage time at 5 °C, approaching P50 = 13 years (Table 4) expected for uninterrupted storage at 5 °C (Fig. 9). Four years of 5 °C prestorage reduced P50 values at −18 and −135 °C to about half the values projected if prestorage was $\leqslant 1$ year (compare 64 and 267 years, respectively, with P50 values given in Table 4). In this experiment, only accessions with 1985 tests showing high percent germination were used, and so most of the loss of germination was observed when the seeds were stored at −18 or −135 °C. For example, the viability of seeds harvested in 1973 and stored for 4 years at 5 °C and 7 years at −18 °C was 96 ± 2% (retransformed mean ± standard deviation of 68 accessions), but

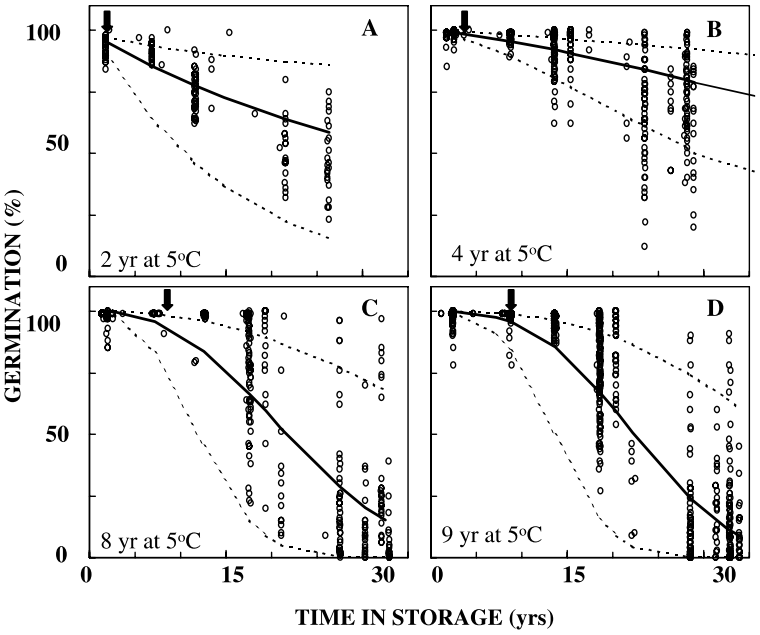

Fig. 7. Percentage germination of 256 accessions of lettuce seed as a function of storage time at 5 and −18 °C. Each point represents percentage normal seedlings developing from an accession at a time–temperature combination and most accessions were assayed 5–6 times during the storage period. Seeds were initially stored at 5 °C for 2 (A), 4 (B), 8 (C) or 9 (D) years and then placed permanently in conventional storage at −18 °C (solid arrows). Data points were transformed and averaged for a 1–3 year storage interval and the average time course was used to calculate parameters in Eq. (1) (solid curve) ± standard error of coefficients (dashed curves) and P50 values in Fig. 9. Data for accessions stored for 6 and 10 years at 5 °C before transfer to −18 °C are not given, but show similar trends.

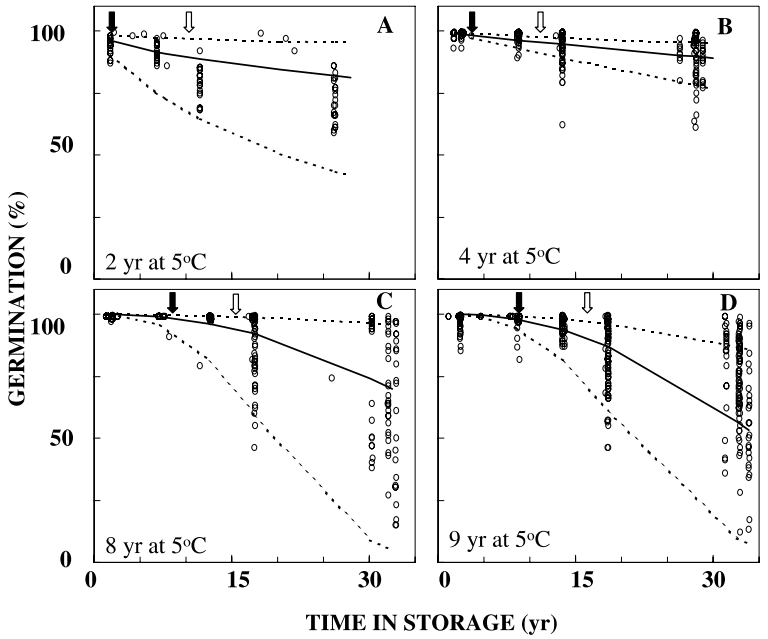

Fig. 8. Percentage germination of 256 accessions of lettuce seed as a function of storage time at 5, then −18, then −135 °C. Each point represents percentage normal seedlings developing from an accession at a time–temperature combination and most accessions were assayed 5–6 times during the storage period. Seeds were initially stored at 5 °C for 2 (A), 4 (B), 8 (C) or 9 (D) years and then placed in conventional storage at −18 °C (solid arrows) and then permanently placed in vapor above liquid nitrogen (open arrows). Initial data are identical to those presented in Fig. 7 until indicated by the open arrow which signifies the time that each accession was split into two storage regimens. Data for accessions stored for 6 and 10 years at 5 °C before transfer to −18 and −135 °C are not given, but show similar trends. Data are analyzed as described in Fig. 7 to give P50 values plotted in Fig. 9.

decreased to $72 \pm 17\%$ (Fig. 7B) and $91 \pm 6\%$ (Fig. 8B) after an additional 18 years of storage at −18 and −135 °C, respectively. Similarly in this experiment, $99 \pm 1\%$ of seeds germinated after 10 years of storage at 5 °C (retransformed mean ± standard deviation of 14 accessions), but germination decreased to about 75% in 1984 after 7 additional years of storage at −18 °C, and $7 \pm 7\%$ or $22 \pm 13\%$ after 18 additional years of storage at −18 or −135 °C, respectively (time course data for 10 years at 5 °C not shown but follow similar trends as displayed in Figs. 7 and 8: P50 for seeds stored at 5 °C for 10 years and then at −18 or −135 °C was 18 and 24 years, respectively (Fig. 9)).

Discussion

In this paper, we describe changes in viability of cryogenically stored seed and, for lettuce, we compare the kinetics of deterioration at extremely low temperatures with storage experiments conducted at higher temperatures. Despite previous predictions that viability can be maintained indefinitely in cryogenically stored materials, we show statistically significant, though slight, losses in germination percentage within the first 20 years of storage. Decreases in viability of cryogenically stored material have been attributed to inadvertent warming through T_g, causing acceleration of deteriorative reactions or formation of damaging intracellular ice [30,39,40]. Ice is unlikely to form in seeds at the water contents studied in this experiment (Fig. 5), and monitoring data for temperatures in cryovats and storage vaults indicate that seeds were never warmed to temperatures as high as T_g during storage. Except for the seeds submerged in liquid nitrogen, seeds were briefly (2–3 days) and periodically (1–4 times) exposed to room temperatures when accessions were retrieved

for viability testing, but water contents remained sufficiently low to preclude ice formation during re-cooling (monitoring data not shown).

The slight deterioration observed in cryogenically stored lettuce seeds allowed us to calculate coefficients for the Avrami equation (Eq. (1)) and predict the rate of future deterioration. Estimates of P50 using Avrami kinetics are consistent with reports from other studies for storage temperatures $\geqslant -18\,^{\circ}C$, and are intermediate to predictions made for storage at liquid nitrogen temperatures using different versions of the viability equations [13,23] (Fig. 4). To our knowledge, the data provided in this paper represent the first estimates of longevity under cryogenic conditions based on deterioration time courses measured at those conditions. Though there is a large degree of uncertainty because time courses are extrapolated beyond the realm of inference, our analyses suggest that 50% of lettuce seeds containing 0.065 g H_2O/g dw will survive 524 years at $-135\,^{\circ}C$ and 3377 years at $-196\,^{\circ}C$ (Table 2, Fig. 4).

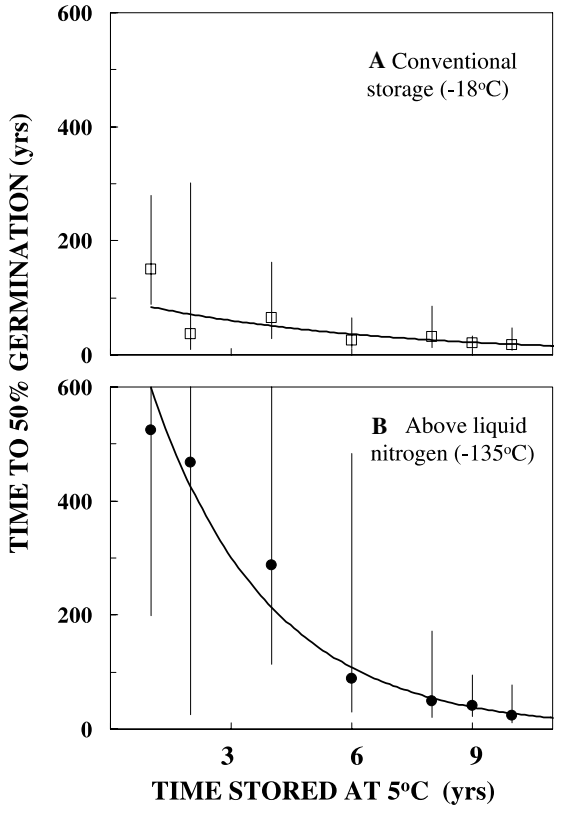

Fig. 9. The time required for percentage germination to decrease from ~95 to 50% (P50) for lettuce seed stored at 5 °C for 1–10 years and then transferred to −18 °C (A) or −135 °C (B). Values for P50 for 1 year prestorage were taken from Fig. 4 and for 2–10 years prestorage were calculated from data in Figs. 7 and 8 fit to Eq. (1). Error bars represent P50 values calculated from Eq. (1) coefficients ± standard error of those coefficients. The curves are exponential decays giving $r^2 = 0.76$ (A) and 0.98 (B).

While the projected longevity reported here may seem sufficient for genebanking, it raises larger questions about seeds that are particularly prone to deterioration and the relative benefits of liquid nitrogen storage versus alternatives. Our data show that there is considerable variability in aging kinetics within a seed species that is not accounted for by water content or temperature of storage. This variability is clearly demonstrated from the range in germination percentage for accessions stored under similar conditions (Figs. 2, 7, 8 and Table 4). Variability based on seed provenance may also be interpreted from the poorer longevity of accessions harvested in 1975 compared to those harvested in 1973 even though the latter accessions were stored for 2 additional years at 5 °C (Figs. 7A and B and 9A). Further, the accessions stored for 8–10 years at 5 °C before they were used in cryogenic studies (time courses described in Figs. 7C and D and 8C and D) display phenotypes with exceptional longevity (analogous to accessions in the box in Fig. 2A) compared to the wider cross-section of lettuce seeds where the average germination was $67 \pm 17\%$ (means ± SD of 104 accessions) after 8–10 years (Fig. 2A). The basis for this variability is under investigation and is surely affected by both genetic and environmental components. Before reasons for the extremes in longevity are known, genebank operators cannot assume that a particular accession will exhibit average deterioration kinetics.

An outstanding feature of average deterioration behavior is the low temperature dependency of aging under cryogenic conditions (Fig. 6). The Arrhenius activation energy ($E_a = 3.5$ kJ/mol) is lower than −40 kJ/mol expected for diffusion-

driven processes. Measurable rotation [6] and relaxation at temperatures below the Kauzmann temperature (T_K), the theoretical limit to molecular mobility in glasses ($T_K = -42\,°C$ for seeds at about 0.065 g H_2O/g dw; [38]), attest to the remaining molecular mobility in seeds at cryogenic temperatures, and this is apparently sufficient for aging reactions to proceed, albeit slowly. Further, our data suggest that the later stages of deterioration are particularly insensitive to low temperature: when aging was initiated by initial storage at 5 °C, it progressed when seeds were transferred to −18 or −135 °C (Figs. 7–9). In fact, transfer of these seeds to −18 or −135 °C after 9–10 years of storage at 5 °C did not increase P50 substantially beyond what was measured for seeds with exceptional longevities stored at a constant 5 °C (compare P50 = 35 years for seeds stored at 5 °C for 9 years then transferred to −135 °C (Fig. 8D) or P50 = 18 years for seeds stored at 5 °C for 10 years and transferred to −18 °C (Fig. 9) with 20–40 years measured for seeds exhibiting the same phenotype and stored continuously at 5 °C (Fig. 2A, Table 4).

The relative insensitivity to temperature observed in aging of lettuce seeds under cryogenic conditions is not observed when seeds are stored under ambient or higher temperatures (Fig. 6). "Breaks" in Arrhenius behavior of seed and pollen aging leading to decreasing E_a with decreasing temperatures have been attributed to a glass melting transition [5,9] or a shift in the temperature dependency of mobility within glassy matrices from Vogel–Tamman–Fulcher (VTF) to Arrhenius kinetics [38]. A combination of these factors would lead to a curvilinear Arrhenius plot with high E_a at $T > T_g$ (−160 kJ/mol reported for pollen by [5,8]; not considered here for analyses of lettuce), lower E_a near T_g (−60 kJ/mol reported for pollen for temperatures between 28 and 5 °C [5,8] and 71 kJ/mol reported here for lettuce for temperatures between 50 and −18 °C (Fig. 6)), and even lower E_a at temperatures near T_K (3.5 kJ/mol at temperatures less than the "break" temperature calculated at −15 °C, which is halfway between $T_g = 28\,°C$ and $T_K = -42\,°C$). Estimates of the temperature coefficients of molecular mobility do not completely correspond to each other or to E_a values measured for seed aging, with mobility coefficients that are larger (178 and 41 kJ/mol for molecular relaxation above and below a break at 2 °C (Fig. 6, Walters, 2004)) or smaller (30 and 9 kJ/mol for rotational motion above and below T_g (Fig. 6 [5,8])) than aging coefficients at similar temperature ranges.

Results reported here only consider the special case of lettuce seeds containing 0.065 g H_2O/g dw. However, aging rates and molecular mobility are affected by an interaction between water content and temperature. These interactions are demonstrated by numerous reports including hypothetical models that assume different moisture and temperature coefficients for the complex suite of reactions believed to be involved in aging [36], measures of rotational motion at different water content and temperatures [6], and optimum water contents for storage of seeds already in the glassy state that increase with decreasing temperature [5,34,36]. Consideration of these interactions leads directly to the conclusion that aging kinetics will follow non-Arrhenius behavior at water contents and temperatures well below T_g and that E_a will be small once temperature decreases to a point that water content becomes less than optimum [5,8,34,36]. Though the physical basis of optimum water contents is still conjectural, values for lettuce seeds, which contain 35% lipid, are expected to be intermediate between those reported for soybean (20% lipid) and peanut seeds (45% lipid) [34]. The break in Arrhenius behavior at −15 °C for lettuce seeds (Fig. 6) corresponds to a projected optimum water content of 0.07 g H_2O/g dw for this species based on a weighted average of soybean (0.08 g/g at −15 °C) and peanut (0.05 g/g at −15 °C). Hence, the water content of lettuce seeds used in these experiments and the reduced temperature dependency of aging reactions at and below −15 °C (Fig. 6) are consistent with earlier predictions of the limited benefits of both drying and temperature on longevity. These arguments are also applicable to desiccation-sensitive materials that are stored at higher water contents since critical water contents conferring desiccation damage appear to be temperature-dependent.

The above discussion focuses on temperature interactions in the aqueous domain that may contribute to changes in Arrhenius behavior. However, DSC scans (Fig. 5) show that the

temperature range for transitions of lipids is coincident with the break temperature observed in the Arrhenius plot for lettuce seed aging (Fig. 6). Many seeds contain triacylglycerols as storage reserves and those containing ~30% linoleic acid (such as lettuce seed) exhibit crystallization and melting transitions between −40 and −10 °C [10]. Triacyglycerols have long been linked to poor storage behavior (e.g. [26,36]) though the mechanism of their involvement remains unclear.

Conclusions

Time scales for biological change at cryogenic temperatures provide information on efficacy of preservation treatments and mechanisms of biological activity at extremely low temperatures. Here we report measurable changes in dried seeds within 20 years of storage at −196 °C, which allows us to predict time scales of the order of hundreds to thousands of years for seed survival under cryogenic temperatures. In the dried materials used here, damage by ice formation is considered improbable, and so storage at −18 °C is also an effective preservation strategy giving time scales of about 100–200 years. Quantitative assessments of the risk of deterioration with time will allow geneBank operators to evaluate storage protocols based on operating costs and benefits of longevity.

The degree to which low temperatures prolong the lifespan of seeds is dependent on intrinsic properties of the seed and how the seed is handled. Harvest year and prestorage at higher temperatures have dramatic effects on longevity under cryogenic conditions, and longevity can vary by more than 300% among samples with similar provenances. Our experiments refute the commonly held idea that all biological activity ceases at temperatures less than −130 °C ($-T_g$ of pure water): molecules remain sufficiently mobile at this low temperature to allow aging reactions to proceed.

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