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Evidence of cell deterioration in winterfat seeds during refrigerated storage

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Abstract

Effective storage of wildland seeds helps alleviate supply shortages and mitigates variable production associated with annual weather patterns. The storage environment is critical for seeds like winterfat [*Eurotia lanata* (Pursh) Moq.) that rapidly lose viability under ambient conditions. Defining seed response to storage conditions is basic to effective seed storage programs. We used electron micrographs of freshly collected, and of stored winterfat seeds, with vigor tests to compare seedling vigor and to relate seed performance to seed cell biology as influence by; (a) seed age under known storage conditions, and (b) imbibition temperatures. We found that imbibition temperatures had little influence on the vigor of fresh seeds but significantly influenced aged seeds. Mitochondrial deterioration was evident in winterfat seeds stored 5-6 years at 5°C, and in fresh, but incompletely hydrated seeds held at 20°C. We recommend seeds be held at –18°C or colder for long-term storage and that field seedings be done during the cold season to reduce the chance that incompletely hydrated seeds will be exposed to warm temperatures.

Key Words: Seed aging, mitochondria, vigor, imbibition, *Eurotia, Ceratoides, Krascheninnikovia*

Resumen

El almacenamiento efectivo de semillas silvestres ayuda aliviar la falta de suministro y mitigar la producción variable asociada con los patrones anuales de clima. El ambiente de almacenamiento es critico para semillas como las de "winter fat [*Eurotia lanata* (Pursh) Moq.]que rápidamente pierden viabilidad bajo las condiciones ambientales. El definir la respuesta de la semilla a las condiciones de almacenaje es básico para implementar programas efectivos de almacenamiento de semilla. Utilizamos micrográficas del microscopio electrónico de semillas recién cosechadas y semillas de "winter fat" almacenadas. Se utilizaron las micrográficas con pruebas de vigor para comparar el vigor de la plántula y relacionar el comportamiento de la semilla con la biología celular influenciado por: (a) la edad de la semilla bajo condiciones de almacenamiento conocidas y (b) las temperaturas de imbibición. Encontramos que la temperatura de imbibición tiene poca influencia en el vigor de semillas recién cosechadas, pero influencio significativamente el de las semillas envejecidas. La deterioro mitocondrial fue evidente en semillas de "winter fat" almacenadas durante 5–6 años a 5°C y en semillas frescas pero hidratadas incompletamente a 20°C. Para el almacenamiento por largos periodos, recomendamos que las semillas deben ser conservadas a –18°C o temperaturas mas frías y que las siembras de campo sean hechas durante la época fría para reducir la probabilidad de que semillas hidratadas incompletamente sean expuestas a temperaturas calientes.

Only in laboratory studies has it been possible precisely to separate effects on germination from effects on subsequent survival.—John L. Harper (1977) in his book, 'Population Biology of Plants'.

Winterfat [*Eurotia lanata* (Pursh) Moq.][1] and closely related species are important forage plants on the cold deserts of North America and Asia, but seeds of these species rapidly lose viability when stored at ambient conditions (Wilson 1931, Hilton 1941, Springfield 1968, 1974b). Springfield (1974b) recommended refrigeration for long-term storage and reported little germination from collections held at ambient temperatures for 8 years compared to 68% germination for seeds held at 5°C. Although 5°C storage extends seed viability, its effect on seedling vigor is unknown.

Our objective was to determine if seed aging under 5°C-storage reduced winterfat seedling vigor. Our hypothesis was developed from studies by Booth (1992), Booth and McDonald (1994), and Agustrina (1995). Booth (1992) reported seedling vigor was reduced by warm imbibition temperatures (Fig. 1). Booth and McDonald (1994) found that rapid

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[1] *Krascheninnikovia* Gueldenstaedt. is the current synonymic favorite, replacing *Ceratoides* J.T. Howell. To call attention to the need to stabilize scientific nomenclature except where new evidence clarifies the phylogeny, we have retained *Eurotia* (Adanson 1763) which was used by most publications between 1840 and 1971.

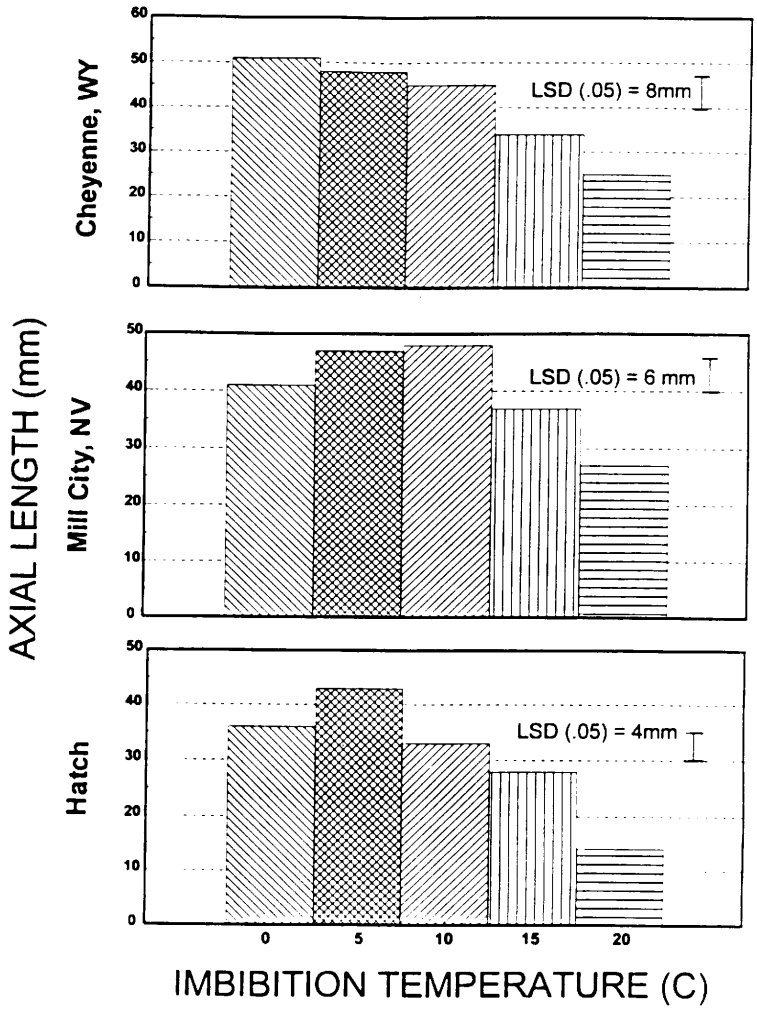

Figure 1. The effect of imbibition temperature on winterfat seedling vigor from seeds collected in Nevada and Wyoming, and from the released variety 'Hatch', as reported by Booth (1992).

imbibition of winterfat seeds at warm temperatures did not damage seed membranes. Agustrina (1995) found freshly harvested winterfat did not exhibit the imbibition-temperature response evident in the stored seeds used by Booth (1992). Because increases in respiratory activity during germination of other species have been associated with rapid development of mitochondrial ultrastructure, including rehydration of its membrane system (Nawa and Asahi 1973, Solomos et al. 1972), we hypothesized (1) that winterfat seeds stored more than 2 years at 5°C contained mitochondrial-ultrastructure changes that reduced seedling vigor and, (2) that imbibition temperatures affected winterfat seedling vigor by influencing mitochondrial hydration, metabolism and development in seeds stored more than 2 years at 5°C.

Methods and Materials

Plant Material

Winterfat diaspores (seed-containing dispersal units) were collected in October, 1986 (Booth 1992); and from the same stand on the High Plains Grassland Research Station, Cheyenne, Wyo., (elevation of 1,909 m) in 1993 (Agustrina 1995). The diaspores of both collections were stored at room temperature for approximately 2 months, then stored at 5°C (Springfield 1974a). Using the methods described by Booth and Griffith (1984), a portion of the diaspores were threshed and seeds stored at 5°C. The 1986 seeds were tested by Booth in 1989 and 1990. Seeds from diaspores collected in Cheyenne in 1993 were tested about 6 months after collection (Agustrina 1995).

Germination and Growth of 1986 and 1993 Seeds

Booth (1992) and Agustrina (1995) report tests for differences in seedling axial length (a measure of seedling vigor) due to imbibition temperature. We used these reports to compare seedling vigor from the 1986 and 1993 seeds by calculating the 95% confidence interval for mean axial lengths of seedlings from the 1986 seeds. We used the MSE from an ANOVA comparing seedling axial length by imbibition temperature for day 10 and for day 12 of incubation (n = 75) (Booth, unpublished 1992 data). Confidence intervals were not calculated for the 1993 data because interactions among the several treatments prevented pooling of experimental units (Agustrina 1995). In their procedures Booth (1992) and Agustrina (1995) divided seeds into groups of 20, weighed the groups to 0.01 g, and humidified the seeds for 3 days at 2°C prior to imbibition to reduce imbibitional injury (Vertucci and Leopold 1984, Vertucci 1989). The seeds were then incubated on slant boards (Jones and Cobb 1963). The slant boards containing humidified seeds were placed in plastic boxes with tight fitting lids; the boxes filled to a depth of 5 cm with distilled water, then placed in incubators for 4 days at treatment temperatures that included 5 and 20°C. All treatments were then incubated in the dark at 20°C. The axial lengths of germinants were measured after 5, 7, 10, and 12 days of incubation using a digitizing tablet (Booth and Griffith 1994).

Mitochondrial Ultrastructure Study

Winterfat diaspores of the 1986 and 1993 Cheyenne collections were used for an electron microscope study of mitochondrial structure. The diaspores were threshed, the seeds weighed, and placed in a humidity chamber at 5 or 20°C until the seed weight reached 1.2 or 1.5 times the dry weight. After humidification, 1 mm was cut from the tip of the embryonic radicle and fixed in 5% glutaraldehyde at room temperature for 4 to 5 hours. For comparison, dry seeds were submerged in 1% glutaraldehyde in 0.1 M sodium cacodylate (pH > 4) for 48 hours, then fixed in 5% glutaraldehyde. Standard methods were used for specimen dehydration and

mounting (Hayat 1986). The specimens were trimmed using an LKB ultratome (LKB Instruments, Inc., Rockville, Md.) For light microscopy, the 1.0 micron sections were cut using a Reichert Jung Ultracut (Reichert-Jung Optische Werke Ag, Hernalser Hauptstr, Wien, Austria), stained with 1% toluidine blue, and then observed under the light microscope. Using the light microscope we selected a representative area from the winterfat radicle tip for study by transmission electron microscope (TEM). A section approximately 79 nanometers thick was cut from the chosen area using the Reichert Jung Ultracut, stained with uranyl acetate and lead citrate, and then observed by TEM. A second group of representative cells were chosen from these TEM observations. Micrographs were obtained using an internally mounted camera on the TEM, the magnification was 50,700 x. Samples were cut from 3 different seeds in each treatment and 2–6 micrographs were made from each sample.

Ratio of Mitochondrial Area to Cell Surface Area (MA/CA)

Slides of TEM photos were used to determine the mitochondria number and a ratio of mitochondrial area to cell area (MA/CA). Relative area was measured by tracing projected images on tracing paper. Traced images were cut out, weighed, and the data used to calculate the ratio.

Experimental Design and Statistical Analysis

To compare MA/CA, mitochondrial number, and size of mitochondria we used a random design and the ANOVA procedure in SAS. Comparison of individual treatment means used the Least Significant Difference (LSD) for variables with a significant F-statistic (SAS 1985).

Results

Germination and Growth of 1986 and 1993 Seeds

Seedlings from the 1993 seeds had about twice the axial length of seedlings from the 1986 seeds (Table 1), and axial lengths from 1993 seeds did not fall within the axial-length confidence intervals of seedlings from 1986 seeds. These findings imply a significant difference in the seedling vigor between the 2 seedlots.

Table 1. Seedling axial-length comparison between Cheyenne seeds collected in 1986 and tested between 1989–1990 vs. Cheyenne seeds collected in 1993 and tested in 1994. Seeds were imbibed at the temperature indicated for 4 days, then incubated at 20°C. The 95% confidence interval is indicated for the 1986 seeds.

Temperature	Days Measured	Mean Length by Collection 1986	1993
--°C--		--------(mm)----------	
5	10	48 + 4.8	80
	12	48 + 4.7	78
20	10	25 + 4.8	77
	12	25 + 4.7	76

The seedling axial length means for 1986 seeds were obtained from earlier data (Booth, data on file). Means for 1993 are for seeds, at treatment temperature, without fungicide (Agustrina 1995).

Mitochondrial Ultrastructure

Dry Seeds. Radicle-tip cells of dry seeds from 1986 and 1993 collections contained mitochondria with few cristae and numerous translucent areas in the mitochondria matrix. Hydration reconstituted radicle mitochondria for both 1986 and 1993 seeds.

Hydration at 5°C. When hydrated at 5°C to the 20% moisture level, the mitochondria of 1993 seeds contained, (1) more distinct lipid bilayers of the outer membrane, (2) a greater number of cristae, and (3) a more uniformly dense matrix— typical for normal mitochondria of hydrated cells—than did cells from 1986 seeds (Fig. 2a versus 2b).

When hydrated at 5°C to 50% moisture content, mitochondrial ultrastructure of 1986 and 1993 seeds was similar (data not shown).

Hydration at 20°C. Hydration to 20% moisture at 20°C resulted in 1993 seeds with thinner mitochondrial cristae than were observed in seeds hydrated at 5°C (data not shown); however, the mitochondrial matrix was more uniformly distributed. Hydration to 50% moisture at 20°C produced 1993 seed with the most deteriorated mitochondrial structures seen among all treatments (Fig. 3). The outer membrane and cristae were completely degraded while in the 1986 seeds the outer membrane was partially degraded and the cristae structures could still be observed.

MA/CA

Neither imbibition temperature, collection year, moisture, nor their interactions significantly affected numbers of mitochondria. P values ranged from 0.14 for the temperature x year interaction (Table 2) to 0.84 for moisture x year. However, the MA/CA ratio was affected by an imbibition-temperature x collection-year interaction (P=0.03). The greatest MA/CA ratio was observed from 1993 seeds imbibed at 20°C (Table 2). This was significantly greater ($P < 0.05$) than for 1986 seeds imbibed at 20°C. The 2 collections did not differ when imbibed at 5°C.

Discussion

Mitochondrial Degradation

Early metabolic activity, which begins near 20% seed moisture, consists of membrane reorganization, glycolysis, repair of organelles, and activation of enzymes in preparation for an increasing respiration rate and subsequent radicle emergence (Bewley and Black 1985). Loss of seedling vigor due to seed aging has been associated with a marked decline in soluble carbohydrate (Bermal-Lugo and Leopold 1992), increases in chromosomal aberration (Dimitrov 1994, Guiterrez et al. 1993), changes in ribosomal protein content (Zalewski 1985, Hallam et al. 1973), and abnormal mitochondrial structures (Hallam et al. 1973, Abu-Shakra and Ching 1967). Aging produces an accumulation of a free fatty acid mixture in

Table 2. Data for Cheyenne seeds collected in 1986 and 1993 showing the effects of temperature and collection year on total surface area of mitochondria per surface area of the cell (MA/CA) and on the number of mitochondria per cell. The temperature x year interaction was significant for MA/CA (P=0.035).

Temperature	Year	Mean MA/CA	Mean number Mitochondria[2]
NT (dry)[1]	86	0.268a[2]	31.67
NT (dry)	93	0.310ab	33.67
5	86	0.305ab	40.50
5	93	0.299ab	37.34
20	86	0.274a	32.67
20	93	0.373b	50.17

[1]Dry and not treated

[2]Means with the same letter are not different as determined by $LSD_{0.05}$. There were no differences in mitochondria numbers among treatments (P ranged from 0.14 for temperature x year, to 0.85 for moisture x year).

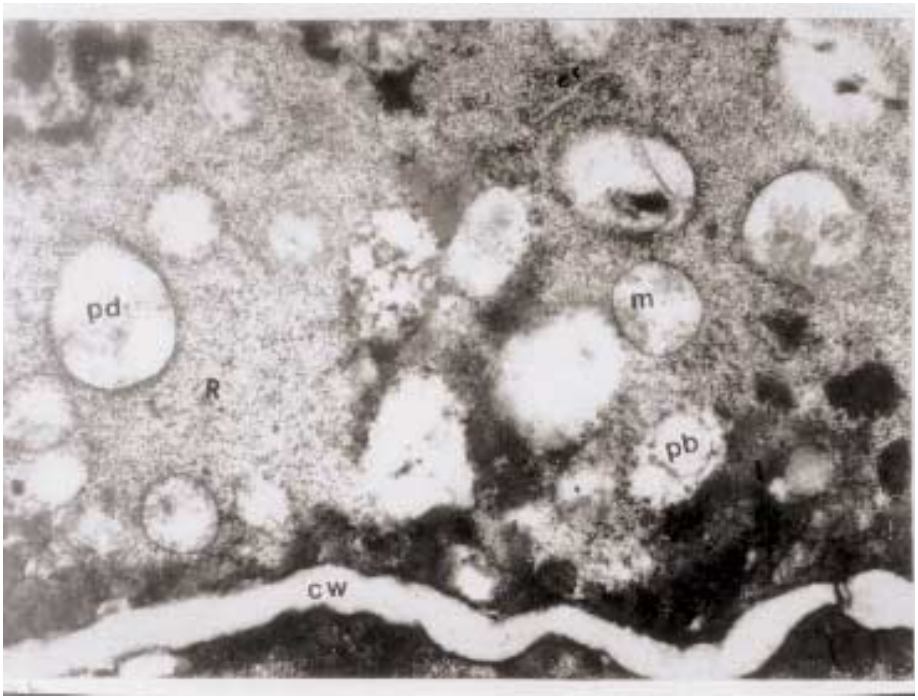

Fig. 2a

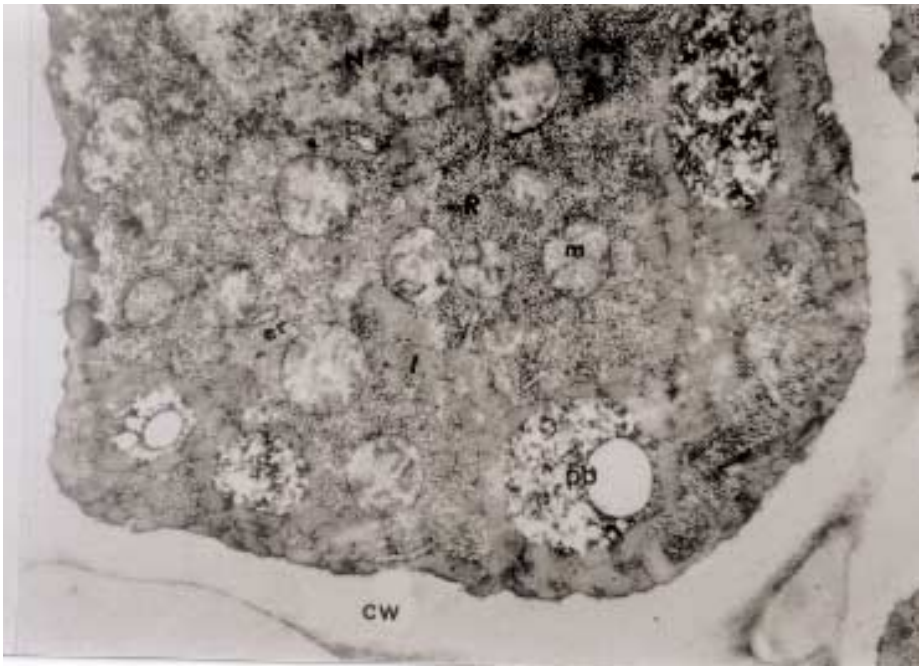

Fig. 2b

Fig. 2. The organelles of embryonic winterfat radicle cells (50,700 x) of 1986 (a) and 1993 (b) seeds, hydrated at 5°C to 20% moisture. The organelles are represented as follows: (m) mitochondria; (cw) cell wall; (l) lipid body; (N) nucleus; (pd) plastid; (pb) protein body,(R) ribosome, and (er) endoplasmic reticulum.

mitochondrial membranes as a result of activation of phospholipase A_2 (Luzikov et al. 1985; Nachbaur et al. 1972). This accumulation induces uncoupling of oxidative phosphorylation, reduces endogenous ATP levels, and leads to mitochondrial swelling (Luzikov et al. 1985). The characteristics of early mitochondrial degradation are dilution of the matrix, swelling and subsequent straightening of the folds formed by the inner membrane, rupture of the outer membrane, vacuolarization of the matrix, loss of the matrix content, and total disappearance of cristae (Luzikov et al. 1985); in short, a decrease in respiration capability and efficiency. Thus the lack of mitochondrial ultrastructure correlates with the low vigor of the 1986 seeds (Table 1).

Effects of Seed Moisture and Temperature

There were few differences between dry or hydrated seeds. This agrees with Baird et al. (1979) who also reported no appreciable differences between mitochondria in dry and imbibed radicle tissues.

Seed hydration at low temperature did produce differences in MA/CA between the 1986 and 1993 seeds as evident by the significant temperature x collection-year interaction. The greater MA/CA for 1993 seeds imbibed at 20°C, compared to that of 1986 seeds imbibed at 20°C, and the lack of difference when these seeds were imbibed at 5°C (Table 2), suggest that early metabolic activity at 5°C, maintained or repaired mitochondria in the 1986 seeds. Thus, these data provide a physiological explanation for Booth's (1992) finding that winterfat seedling vigor decreased with increasing imbibition tempertures (Fig. 1). The same explanation may apply to other chenopods shown to benefit from imbibition at temperatures suboptimal for germination (Haferkamp et al. 1990, Shaw et al. 1994).

Hydration at warm temperatures produced unexpected differences due to moisture content of the 1993 seeds. Why did these seeds show so much mitochondrial degradation at 50% moisture whereas the 1986 seed did not? Bain and Mercer (1966) reported that transportation of hydrolyzed food reserves from cotyledon cells of peas did not occur until the moisture level in the seeds reached more than 93%. This suggests that translocation of hydrolyzed food reserves from winterfat seed-storage tissue may not have occurred at 50% moisture.

Vartepetian et al. (1976) reported that ATP-regeneration was essential in maintaining the integrity of mitochondria in excised seedling tissues. Hodson et al. (1987) hypothesized that a delay in the development of mitochondrial structure may be related to the available energy supply in the seeds. The lack of activity

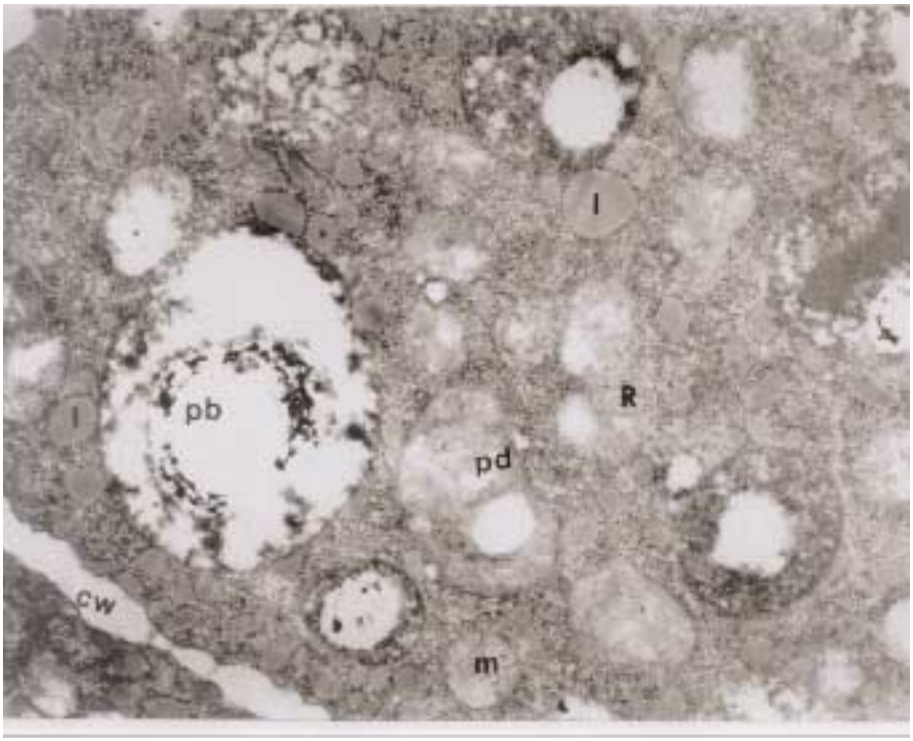

Fig. 3. The organelles of embryonic winterfat radicle cells (50,700x) in 1993 seed hydrated to 50% moisture at 20°C. See Fig. 2 for organelle labeling information.

in the mitochondria of pea seedlings after their peak activity, was correlated with shrinking of the cotyledons (Malhotra et al. 1970).

So lack of an energy supply may have caused mitochondrial degeneration in the 1993 seed at 50% moisture. Would not this also occur in the 1986 seed? Aging may have reduced active catabolic enzymes in the 1986 seeds. Das and Sen-Mandi (1992) reported a decline in amylase activity of aged seeds. Declines in enzyme activity or in the cellular ability to synthesize proteins could indicate reduced catabolic activity. Thus, the 1986 mitochondria could be more degraded than in the 1993 seed at 20% moisture, then be less degraded at 50% moisture.

Implication of Findings and Related Information

The evidence is that storage at 5°C did not protect winterfat seeds from age-related degradation. The seeds remained viable, but lost vigor. Other chenopod seeds stored at –12 to –23°C were reported to have little loss of vigor after 22 years (Pack and Owen 1950). Roos (1989) recommended seeds be at –18°C or lower for long term storage. Springfield (1968) compared winterfat seeds which had been stored in a warehouse (13 to 35°C) with refrigerated storage (3 to 6°C), and with freezer storage (–20 to –32°C). He did not directly compare refrigerated and freezer storage but commented that "subfreezing temperatures appear slightly superior". However, he subsequently (Springfield 1974a) recommended refrigerated storage.

The severe degradation of mitochondria in 6-month-old seeds at 50% moisture and 20°C, suggests that fresh, partially hydrated seeds are at risk during periods of warm weather. We believe that the combination of warm soil temperatures (> 15°C) and incomplete seed hydration could account for the failure of late-spring and summer winterfat seedings.

Judging the Evidence

Micrographs. The micrographs of the 1986 seed were made 3–4 years after the vigor test was conducted, and thus represent a more advanced stage of mitochondrial degradation than was present when the seeds were tested. The fact that the micrographs are of seeds that are older than the vigor test is perhaps fortunate since we may not have detected these gradual structural changes in 2-year-old seeds.

Comparing Seedlots from Different Years. The Coefficients of Variation (Steel and Torrie 1980) for mean monthly temperature and precipitation for June through September, 1986 and 1993 at the High Plains Grasslands Research Station are smaller than the coefficients for the three 1993 seed collections (Cheyenne and Pine Bluffs, Wyo; and

Table 3. Climatological data and Coefficients of Variation for Cheyenne and Pine Bluffs, Wyo., and Sterling Colo.[1]

	Mean Temperature	Mean Precipitation
Cheyenne 1986	-------(°C)-------	--------(mm)--------
June	18.2	62
July	20.4	26
August	19.6	39
Sept	13.1	63
Cheyenne 1993		
June	15.0	84
July	NA	NA
August	18.4	56
Sept	12.5	80
Pine Bluffs 1993		
June	15.8	66
July	NA	NA
August	19.1	22
Sept	12.5	40
Sterling 1993		
June	19.9	52
July	22.8	41
August	22.5	50
Sept	15.4	30
Coefficients of Variation for:	Temperature	Precipitation
Cheyenne 1986 & 1993	17.7	32.6
Chey., P. B., & Sterl. 1993	20.9	37.7

[1]National Oceanic and Atmospheric Administration (1986, 1993). Data for Sterling, Colorado was courtesy of the High Plains Climate Center, University of Nebraska, Lincoln (personal communication).

Sterling, Colo.) tested by Agustrina (1995) (Table 3). Agustrina (1995) detected no significant differences in seedling axil length among the three 1993 seeds collections. This implies that differences in the seeds due to production year are small relative to aging differences, and are unlikely to confound our interpretation of the data.

Extrapolation from 1 Seed Source. We detected differences in basic cell biology that have been observed in seeds of other species. A basic process that occurs across species is not likely to change due to seed source within a species. Further, the 3 seed sources tested by Booth (1992) were all more than 2 years old and had been stored under similar conditions and gave similar responses to imbibition temperature. Finally, though Springfield (1968) did not grasp the importance of the differences between "refrigerated" and "freezer"-stored seeds, his observations add to the evidence that winterfat seed aging during 5°C-storage is an important consideration for all winterfat seeds.

Conclusion and Recommendations

We conclude that storage of winterfat seeds at 5°C resulted in age-related degradation of seed mitochondria and other organelles during storage, and in a significant loss of seedling vigor. Also, that age-related degradation was the physiological reason for the seedling-vigor response to imbibition temperature found by Booth (1992) for 3 separate winterfat seed sources. We now believe that warm imbibition temperatures do not significantly reduce vigor of healthy seeds. However, incomplete hydration (>20% and <90% seed moisture) at warm temperatures (near 20°C) will result in rapid mitochondria degradation and loss of vigor. Also, imbibition at warm temperatures will reduce the vigor of seeds needing mitochondrial repair. For these seeds, cold imbibition reduces inefficient respiration and allows repair to proceed.

We recommend winterfat seeds be held < –18°C to protect seed vigor during long term storage and, that the seeds be imbibed at 0 to 5°C as a standard laboratory practice. Cold imbibition does no damage to winterfat seeds and it does protect winterfat seed-vigor potential. For that reason we also recommend winterfat be seeded during the cold season.

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