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Evaluating seed longevity: use of RNA integrity to characterize variation within species of legume grains

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Abstract

Seed genebanks must maintain collections of healthy seeds and regenerate accessions before seed viability declines. Seed shelf life is often characterized at the species level; however, large, unexplained variation among genetic lines within a species can and does occur. This variation contributes to unreliable predictions of seed quality decline with storage time. To assess variation of seed longevity and aid in timing regeneration, ten varieties of pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medikus subsp. *culinaris*) from the Australian Grains Genebank were stored at moderate temperature (20°C) and moisture (7–11% water, relative humidity [RH] ~30%) and deterioration was assessed by yearly germination tests for 20 years. Decline in germination was fit to a sigmoidal model and the time corresponding to 50% germination (P50) was used to express seed longevity for each genetic line. The feasibility of using RNA fragmentation to assess changed seed health was measured using RNA integrity number (RIN) from RNA extracted from seeds that were stored for 13 and 20 years. Seed lots of legume grains that maintained high survival throughout the 20 years (i.e. they aged slower than other lines) had higher RIN than samples that degraded faster. RIN was lower in embryonic axes compared with cotyledons in the more deteriorated samples, perhaps indicating that axes exhibit symptoms of ageing sooner than cotyledons. Overall, RIN appears to be associated with longevity indicators of germination for these legumes and indicating that RIN decline can be used to assess ageing rate, which is needed to optimize viability monitoring.

Introduction

Seed longevity (i.e. the duration seeds survive during storage) is fundamental to seed companies and plant genebanking operations (Hay and Whitehouse, 2017). We typically think of seed longevity as the threshold between when seeds retain and lose the capacity to germinate (Rajjou and Debeaujon, 2008; Walters et al., 2010; Hay et al., 2022). This threshold of functionality is analogous to 'expiration dates' for other materials such as dried foods and pharmaceuticals, and can vary considerably across species and seed lots within a species (Walters et al., 2005, 2010; Nagel et al., 2009; Probert et al., 2009; Lee et al., 2013; Willis, 2017; Pritchard, 2020). Predicting expiration dates for seeds remains elusive because numerous interacting factors are involved and underlying mechanisms contributing to each factor are poorly understood.

Longevity is often characterized by a cut-off value marking changed functionality, such as P85 or P50, the time at which the sample is 85 or 50% viable, respectively. Plant genebanks use an 85% viability metric because this appears to minimize risk of genetic erosion as well as ensure successful stand establishment during regeneration (FAO, 2014). Because P85 cannot be predicted, genebanks rely on periodic testing of stored seeds, which provides 'snap-shots' of seed germination potential. This does not mitigate the issue of predicting longevity, because the frequency of testing heavily relies on crude estimates of seed longevity. Testing intervals of 10–20 years are recommended for genebanks storing seeds at −20°C. Thus, a sample that survives 100 years may be tested 5–10 times, consuming hundreds to thousands of valuable seeds. Too frequent or infrequent testing poses major risks of loss within genetic resource collections, either by depleting long-lived samples or samples dying in between monitor tests (Hay et al., 2013; Fu et al., 2015; Hay, 2021). Tools are needed to reliably predict longevity (the time that a seed remains viable) or to measure ageing rate (i.e. longevity^{-1}) at times before P85. Unfortunately, ageing in seeds is mostly characterized by the final effect of ageing – mortality – rather than its less catastrophic symptoms. Because there are no recognized symptoms of

ageing besides germination behaviour, it is a challenge to assess the onset of viability loss in a viable seed lot.

Seed longevity, and its reciprocal, seed ageing, are dependent on the storage conditions (moisture and temperature) and intrinsic properties of the seed collectively referred to as 'initial quality'. Moisture and temperature control the molecular mobility within the cytoplasm of seeds and are major contributors to ageing kinetics (Ellis, 2022; Nadarajan et al., 2023; Rao et al., 2023). These principles are increasingly understood in the context of material sciences related to glassy properties (Ballesteros and Walters, 2019). Given moisture and temperature conditions, seed longevity can be approximated from empirically based models (Ellis, 1988; SER, 2023) within boundaries of about 20–25% relative humidity (RH) and −30 to −40°C (Vertucci and Leopold, 1987; Ellis et al., 1988; Ellis and Hong, 2006).

The components of initial seed quality that contribute to seed longevity remain elusive, mostly because they are both hard to quantify and to control. Typically, species may be characterized as producing short-lived (e.g. onion) or long-lived (e.g. oat) seeds and numerous studies report relative longevity among species or survival times expected in warehouse or genebank storage (Nagel and Börner, 2010; Thirusendura Selvi and Saraswathy, 2018; Solberg et al., 2020). Importantly, genebanks report high, unexplained variation of longevity among accessions within a species despite similar provenances (Walters et al., 2005; Nagel et al., 2009, 2011; Solberg et al., 2020). Studies of ecotypes and QTLs regulating longevity of dry seeds strongly suggest genetic mechanisms (Kueneman, 1983; Bentsink et al., 2000, 2010; Clerkx et al., 2004; Schwember and Bradford, 2010; Nagel et al., 2011; Rehman Arif et al., 2012; Agacka-Mołdoch et al., 2015; Zhang et al., 2019). This is consistent with numerous studies of genetic control of desiccation tolerance in diverse organisms (Hesgrove et al., 2021; Nguyen et al., 2022; Packebush et al., 2022). Maturity at harvest and pre- and post-harvest treatments also influence seed survival during storage (Argerich and Bradford, 1989; Bradford et al., 1990; Grass, 1994; Hay and Probert, 1995; Pinheiro et al., 2021), clearly indicating that longevity is a complex trait influenced by genetic × environmental (G × E) interactions.

A correlation between initial germination percentage and performance during storage is often assumed (e.g., Ellis, 1988). Logically, progressive deviation from 100% viability places the seed lot in ever-closer proximity to the longevity threshold when seeds begin to die rapidly and this would indicate that ageing is occurring. However, tracking aging by subtle changes in germination requires large sample sizes and frequent monitoring. Also, some seed quality traits that affect initial germination are neutral to seed longevity, and some highly vigorous seed lots age rapidly (e.g. willow (Ballesteros and Pence, 2017)). An evolving concept of ageing-induced mortality uses 'the straw that breaks the camel's back' analogy, whereby a minor event can have a major effect. In this concept, germination and longevity are disassociated and accumulation of minor events (i.e. 'straws') will have mostly undetectable effects on viability. In other words, ageing is not detectable by viability loss until it is too late and the longevity threshold is reached without warning.

Stress tests can help unify various concepts of ageing mechanisms by addressing how much stress can be endured before a seed lot succumbs (Silva et al., 2024). This information may provide a better starting point than % germination for empirical models of longevity. One common approach named 'accelerated ageing' or 'controlled deterioration' places seeds in unfavourable conditions, usually a combination of high humidity (≥75% RH) and high temperature (>45°C). Seed lots that succumb first are expected to die soonest even under more favourable conditions, that is dry (10–25% RH) and cold temperatures (−18 to 5°C). These kinds of tests come under perennial criticism because they are hard to translate back to actual genebanking conditions. Genebank conditions approach, or extend beyond, boundary conditions of empirical models, with limited data to verify that seed lots identified as relatively short- and long-lived in a stress test perform as expected in the genebank.

An alternative approach is to seek assays that detect degradation within stored seeds *before* the longevity threshold – that is, an assay of non-lethal ageing rather than a viability assay. Linking the kinetics of presumed reactions of dried materials with longevity (or longevity^{-1}) involves waiting for the longevity signal; the waiting period could be decades to confirm that the assay is relevant to the dry and cold conditions of genebanks. One candidate assay involves assessing RNA fragmentation, which was studied in legacy collections that were 30 and 60 years old (Fleming et al., 2019). RNA molecules are labile and are rapidly degraded by RNases in actively metabolizing cells (Wurtmann and Wolin, 2009). However, in dry seeds, RNase activity is low and RNA molecules persist during storage. RNA molecules tend to fragment at random which can be quantified from total cellular RNA using RNA integrity number (RIN) (Fu et al., 2015; Fleming et al., 2017, 2018a, 2018b; Walters et al., 2020; Tetreault et al., 2022). Initial studies show that RIN decline occurs linearly and this decline is detected in all tested species before reaching the longevity threshold that marks rapid mortality (Fleming et al., 2019). This assay seems especially promising because the temperature dependency of RIN decline in soybean and other species was indistinguishable from temperature dependency for viability loss, which indicates that factors controlling molecular mobility affect both processes in similar ways (Fleming et al., 2019; Walters et al., 2020).

To be a valid indicator of ageing rate (longevity^{-1}), the rate of RIN decline should correlate with factors in addition to temperature that affect seed longevity, namely, moisture and seed quality. The work presented in this paper uses a 20-year-old legacy collection of different varieties of chickpea, pea and lentil with similar harvest and storage provenances (Redden and Partington, 2019). The original purpose of the collection was to demonstrate variation of seed longevity within a species that was independent of initial germination percentages (the standard method to quantify initial seed quality) (Redden and Partington, 2019). Here, we hypothesized that RIN values would be higher in longer living species and longer living cultivars within a species. Taking advantage of the large seed size of legumes, we extended the study to investigate RIN decline at the tissue level and hypothesized that RIN decline would be comparable in seed tissue from cotyledons and embryonic axes. Experiments comparing ageing of varieties used 20°C storage and a single moisture range. Additional samples were stored at 2°C and different moisture levels, allowing us to test correlations of RIN with storage moisture and confirm temperature effects reported previously in an independently designed experiment.

Materials and methods

Seed treatment design

Seeds from ten lines of chickpea (*Cicer arietnum* L.), pea (*Pisum sativum*) and lentil (*Lens culinaris* Medikus subsp. *culinaris*) were

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used in this study. All accessions were grown in 2002 from regeneration plots at the Australian Grains Genebank (AGG, Horsham, Victoria, Australia) as described previously (Redden and Partington, 2019). Water content of the seeds was adjusted as previously described (Redden and Partington, 2019), and the seeds were sealed in three-ply laminate pouches with an aluminium foil layer that was at least 15 µm. Each pouch represented a sampling time and pouches were placed at either 2 or 20°C (Table 1). Seeds stored at 2 and 20°C represent 'Part A' and 'Part B', respectively, of a larger experiment described in Redden and Partington (2019). In Part A, water content of two lines of chickpea, pea and lentil were adjusted to 9–12% (high), 7–10% (medium) and 5–8% (low). In Part B, water content of seeds from ten lines of each crop was adjusted to 7–10% (medium).

A subset of the seeds was sent to the National Laboratory for Genetic Resource Preservation (NLGRP, Fort Collins, Colorado) in 2016 for RIN analysis and in 2023 for RIN, water content and germination measurements. Seed germination and water content were measured upon receipt at NLGRP. Seeds were stored at −20°C until RIN measurements were completed.

An additional study of the effect of moisture on RIN decline was conducted at NLGRP using pea cv. 'Alaska' seeds stored from 2017 to 2023 at room temperature and different RHs, obtained using saturated solutions of LiCl (13% RH), $MgCl_2$ (33% RH), K_2CO_3 (43% RH) and $CaNO_3$ (52% RH). These represent the same lot used for the initial time point in Fleming et al. (2019) in which initial germination was 99% and the initial RIN values were reported as 7.8 ± 0.3 (axes) and 8.0 ± 0.7 (cotyledons).

Seed moisture

Water content of seeds was initially measured at AGG in 2003 using a Mettler LP16/PM400 seed moisture meter (Redden and Partington, 2019) and subsequently for this paper in 2016 and 2019 by the high-constant-temperature oven method (ISTA, 2003) (Table 1). Water content was additionally measured at NLGRP in 2023 by comparing fresh and dry mass of seeds, dry mass determined by placing seeds at 95°C for 3 d (Table 1). There was some variation in water content measurements among pouches of the same treatment which could indicate experimental uncertainty or water movement over time if pouches were not perfectly waterproof.

RH surrounding the seeds can be estimated from water content measurements using generic isotherms constructed for species (SER, 2023). RH was measured at room temperature in 2023 (NLGRP) by placing a wireless RH sensor (BlueMaestro, London, UK) into the foil pouches containing seeds immediately upon opening them. Measured (Table 1) and SID RH values were consistent. The 'low' moisture treatment corresponded to 14–27% RH for all species. There was greater variation of RH at 'medium' and 'high' treatments ranging from 25 to 47% RH (pea and lentil at medium), 38 to 51% RH (chickpea at medium), 38 to 58% RH (pea and lentil at high) and 55 to 67% RH (chickpea at high).

The water contents of pea seeds cv. Alaska in precisely controlled RH chambers were comparable to the measured RH as well as the SID isotherm models (Table 1, bottom).

Germination assays and determination of longevity

Change in seed quality over storage time was measured using an initial germination assay and subsequent assays were conducted almost yearly at first and then spread out at 4- to 6-year intervals. Methods used at AGG (before 2023 assay) followed the *International Seed Testing Handbook* (ISTA, 2003); 100–200 seeds were spread over a paper towel, placed at 20°C and normal seedling development was assessed at 5 and 7 d (Rao et al., 2006; Redden and Partington, 2019). Similar methods were used in the 2023 assay conducted at NLGRP except that 50 seeds were used for germination assays.

The longevity of seed lots was calculated by fitting time course germination data across the 20 years of storage to an Avrami equation (Avrami, 1941; Walters et al., 2005) to determine P50. The value of P50 (storage time to which germination declines to 50%) was interpolated or extrapolated (for long-lived samples in which germination was >50% at the time of publication). The ageing rate is expressed as $P50^{-1}$.

The expected deterioration of each species was also fitted to the Ellis and Roberts longevity model as posted on the Seed Information Database (SER, 2023, visited 20 August 2023) using initial viability = 99%; storage temperature = 20°C; water content = 7.7, 9.9 and 9.6% (chickpea, pea and lentil) and species constants (Ellis, 1988; Ellis et al., 1988; Whitehouse and Norton, 2022).

RNA degradation (RIN) assays

RNA degradation was quantified using RIN of 4–12 individual seeds per line separated into embryonic axes and cotyledons. RIN assays were measured in 2017 and 2023.

RNA extractions were used between 1 and 20 mg of dry tissue. Tissue was ground, in the presence of liquid nitrogen and 1–2 mg polyvinylpyrrolidone-40 (PVP-40; Fisher Scientific, Fair Lawn, NJ), in a 2 ml centrifuge tube using a nickel/lead steel shot bead (Ballistic, Inc., Hamel, MN) using the TissueLyser II (Qiagen, Hilden, Germany). RNA was extracted from ground tissue using the Qiagen Plant RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA yield was quantified using a DeNovix DS-11 FX+ Spectrophotometer (DeNovix, Wilmington, DE). Samples were diluted to 2 ng μl^{-1} in nuclease-free water.

The RIN of diluted RNA samples was quantified on an Agilent Bioanalyzer (Agilent, Waldbronn, Germany), using Agilent RNA 6000 Pico chips and the Plant RNA Pico assay (Agilent 2100 Expert software version B.0208.SI648 R3), following manufacturer protocols. Fragment sizes of diluted RNA were assessed by electrophoresis using Agilent RNA 6000 Pico chips followed by analysis with the Agilent 2100 Expert software where electropherograms are assessed for peak areas of different fragment sizes and assigned a RIN value using a proprietary formula that involves peak sizes for rRNA (Fleming et al., 2017; Tetreault et al., 2022).

Statistical and data analysis

Linear regressions of RIN and most recent germination proportion, P50 and $P50^{-1}$, were calculated using Excel 'linest' functions. Linear regressions were first performed using data from all three species combined to represent effects of species having different seed longevities. Subsequent linear regressions were performed for each species separately to examine the within-species variation of aging rates and RIN decline. To estimate the confidence of a P50 estimate for the 30 varieties, germination data were also modelled using a general linear model with binomial error distributions following the 'dose.p' function using the statistical environment R (R Core Team, 2019). Analyses of variance tests

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Table 1. Environmental treatments of seeds used in this study

			20°C Experiment			2°C Experiment		
	Cultivar #	Cultivar or salt treatment (NLGRP)	Water content 2016 (%, AGG)	Water content 2019 (%, AGG)	Water content 2023 (%, NLGRP)	Water content (%, high)	Water content (%, medium)	Water content (%, low)
Chickpea	1	Howzat	8.00	No data	No data	12.58 (60–67% RH)	9.98 (38–42% RH)	5.54 (15–24%)
	2	FLIP 94-079C	7.85	No data	No data	11.81 (55–65% RH)	10.78 (46–51% RH)	5.69 (15–23% RH)
	3	ICCV 96836	7.36	No data	No data			
	4	Kaniva	No data	No data	No data			
	5	FLIP 94-090C	No data	No data	No data			
	6	Tyson	7.74	8.20	7.34 (25–31% RH)			
	7	Paider-91	7.69	7.80	6.86 (22–30% RH)			
	8	WACPE 2075	7.73	7.60	5.51 (15–29% RH)			
	10	FLIP 94-508C	7.62	7.80	7.04 (22–31% RH)			
	Average		**7.71**	**7.85**	**6.69**	**12.20**	**10.38**	**5.62**
Pea	1	Snowpeak	10.32	No data	No data	10.77 (46–58% RH)	8.56 (30–33% RH)	6.1 (16–18% RH)
	2	Kaspa	9.82	9.9	9.82 (40–44% RH)	9.49 (38–57% RH)	7.73 (25–47% RH)	5.54 (14–27% RH)
	3	Excell	10.06	10.6	8.33 (30–49% RH)			
	4	Parafield	9.97	6.9	7.65 (25–41% RH)			
	5	Paravic	9.25	9.7	7.16 (22–40% RH)			
	6	90-158*8-1	9.96	10.5	8.13 (28–47% RH)			
	7	95-072*3	9.91	10.7	7.64 (25–43% RH)			
	8	90-131-*27-7	9.99	10.4	7.23 (22–38% RH)			
	9	89-036-*9-8	9.75	10.1	8.02 (28–44% RH)			
	10	90-166*30-5	9.89	10.7	9.89 (40% RH)			
	Average		**9.89**	**9.94**	**8.21**	**10.13**	**8.15**	**5.82**
Lentil	1	Cumra	9.47	9.7	9.47 (36–38% RH)	11.22 (48–54% RH)	8.52 (30–39% RH)	6.47 (18–20% RH)
	2	Cobber	10.12	10.7	10.12 (40–42% RH)	11.08 (48–57% RH)	9.14 (34–46% RH)	5.58 (14–21% RH)
	3	Northfield	8.98	8.7	7.75 (25–36% RH)			
	4	CIPAL 102	8.96	9.2	7.64 (25–38% RH)			
	5	Matilda	10.1	11.7	8.94 (32–36% RH)			
	6	Digger	10.1	5.4	8.92 (32–48% RH)			
	7	Aldinga	10.35	No data	No data			
	8	CIPAL 205	9.24	5.9	7.89 (26–36% RH)			
	9	CIPAL 206	9.2	9.7	7.84 (26–40% RH)			
	10	CIPAL 106	No data	11.8	9.08 (34–47% RH)			

	Average		9.61	9.20	8.63	11.15	8.83	6.03
Pea	Alaska	52% RH, RT			11.11			
		43% RH, RT			9.91			
		33% RH, RT			7.83			
		13% RH, RT			6.54			

Water contents (wc) were adjusted in a bulk sample of each genetic line and temp × wc treatment in 2003-harvested seeds. Seeds were then sealed in foil laminate bags and stored at the Australian Grains Genebank (AGG) at either 20 (9–10 lines) or 2°C (2 lines). Water content was resampled for this experiment in 2016 and 2019 (20°C, AGG) and 2023 (20 and 2°C, NLGRP), and was expressed here as a percentage (%) on a fresh weight basis. Cell entries of 'no data' indicate that water content was not measured to conserve seeds for germination testing. Empty cell entries indicate that this temp × wc treatment did not occur. To provide an additional assessment of moisture effects, an independent dataset of pea seeds stored for 7 years at various RHs at room temperature (20–22°C) is also provided. A range of RH is provided by direct measurement using a BlueMaestro wireless Tempo Disc™.

were performed within species to determine significance in the RIN values from seeds stored at 2°C with varying moisture contents; *post-hoc* Tukey's honestly significant difference (HSD) tests were conducted to assess the significant differences between RIN means. Analyses of variance tests were performed using JMP 12.2.0 (JMP Statistical Discovery LLC 2022).

Results

Changes in seed germination during storage

Initial seed germination was high for all varieties studied, being greater than 90% and mostly between 98 and 100% (Tables 2 and 3). A measurable effect of moisture environment on seed longevity was difficult to discern for seeds stored at 2°C even after 20 years of storage (Table 2). Germination remained relatively high for most treatments except the high moisture treatment of chickpea cv. 'Howzat' (56% germination) and the low moisture treatment of pea cv. 'Kaspa' (83% germination) (Table 2). The effect of moisture environment was apparent in a subsidiary experiment using pea seeds stored at room temperature (20–22°C) for 7 years. In this case, germination declined substantially in seeds stored at RH higher and lower than 33% RH. Maximum germination of 90% (for pea seeds stored at 33% RH) was slightly reduced from initial values (Fleming et al., 2019; Table 2). The lower germination of seeds stored at 13 compared with 33% RH was previously reported (Vertucci and Roos, 1990).

Germination declined in most of the AGG seeds that were stored at 20°C and 30–49% RH (Table 3). Early changes in viability were reported previously (Redden and Partington, 2019) and new data are presented here for assays conducted in 2016, 2019 and 2023 (Table 3). Average germination percentage for the accessions tested in 2023 was 89, 54 and 36% for chickpea, pea and lentil, respectively. However, there was large variation in germination among cultivars, with final germination ranging from >90 to <10% in all species, despite similar provenance, initial quality, storage time and storage environment. High germination (>90% in 2023) was noted in seven out of ten chickpea lines, one pea ('Parafield') and one lentil line ('Cumra'). Hard-seededness may play a role in the slower aging of pea cv. Parafield seeds, which may have been stored in a lower moisture environment (Redden and Partington, 2019).

To calculate a seed longevity parameter, data for germination % *versus* time at 20°C storage were fit to the Avrami equation for each AGG cultivar (Fig. 1) and time to 50% germination (P50) was determined. P50 ranged considerably within species: 11–68 years for chickpea, 13–41 years for pea and 15–20 years for lentil (Table 3). Average P50 for the species was 36, 18 and 16 years for chickpea, pea and lentil, respectively, suggesting that chickpea seeds were longer lived than pea or lentil. Regression analyses between final germination and P50 calculated using the Avrami model were somewhat weak when all species were combined: $R^2 = 0.34$, $P < 0.0001$, $P50 = 11.6 + 0.17 \times$ FinalGerm; for pea: $R^2 = 0.47$, $P = 0.016$, $P50 = 11.4 + 0.16 \times$ FinalGerm; for lentil: $R^2 = 0.49$, $P = 0.014$, $P50 = 14.4 + 0.10 \times$ FinalGerm, the relationship is not statistically significant for chickpea. Relationships between final germination and $P50^{-1}$ were stronger due to lower uncertainty when longevity is expressed as ageing rate in samples that exhibited minor deterioration within the 20-year time frame (all species combined: $R^2 = 0.57$, $P > 0.0001$, $P50^{-1} = 0.07–0.00036 \times$ FinalGerm; for chickpea: $R^2 = 0.65$, $P = 0.002$, $P50 = 0.095–0.00058 \times$ FinalGerm; for pea:

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Table 2. Effect of moisture during storage on germination of stored seeds

Storage temperature (°C)	Species	Cultivar	Germination % soon after harvest	Moisture treatment	Germination % in 2023
2°C	Chickpea	Howzat	98	High	56
				Medium	89
				Low	89
		FLIP 94-079C	100	High	100
				Medium	100
				Low	95
	Pea	Snowpeak	99	High	96
				Medium	100
				Low	93
		Kaspa	91	High	100
				Medium	100
				Low	83
	Lentil	Cumra	100	High	96
				Medium	97
				Low	91
		Cobber	100	High	100
				Medium	100
				Low	98
20–22°C	Pea	Alaska	97	52% RH	35
				43% RH	76
				33% RH	90
				13% RH	69

In the top portion of the table, seeds from two genetic lines per species were harvested in 2003 and stored at 2°C, indicating moisture levels (Table 1). The lower portion of the table shows a comparable experiment with seeds harvested in 2015 and stored at controlled RH on the lab bench (room temperature) for 7 years. Germination assays were conducted in 2023.

$R^2 = 0.62$, $P = 0.003$, P50 = 0.075–0.00037 × FinalGerm; for lentil: $R^2 = 0.47$, $P = 0.017$, P50 = 0.072–0.00044 × FinalGerm).

To test the consistency of seed longevity observed here with modelled longevity representative of the species, we calculated deterioration time courses from the viability equation (SER, 2023) using species constants (Ellis, 1988; Ellis et al., 1988; Whitehouse and Norton, 2022) and initial germination = 99%, temperature = 20°C, water content = 7.7, 9.9 and 9.6% for chickpea, pea and lentil, respectively. Viability equation models yielded P50 estimates of 29 years for chickpea and pea and 18 years for lentil, values which are relatively consistent with average P50 values calculated from Avrami curve-fitting for chickpea and lentil. No within-species variation is predicted from the viability equations given similar starting germination percentages and species constants for temperature and moisture.

RIN of stored seeds

RIN was measured in seeds stored at 2°C at different moisture levels, pea seeds stored at room temperature under precisely controlled RH, and a representative selection of cultivars stored at 20°C and 30–40% RH. RIN was assayed from seeds removed from storage in 2016 (AGG, 20°C storage only) and in 2023. RNA was extracted from individual seeds and cotyledon and embryonic axis tissues separately.

Quality of RNA extracted from seeds stored for 20 years at 2°C was excellent, with distinguishable rRNA peaks (slowly eluting region) and little evidence of short RNA fragments in fast eluting regions of the electropherogram (Supplementary Fig. S1). RIN values for the medium and low moisture treatments were not significantly different in most cases and ranged from 8.6 (pea axes) to 8.1 (chickpea axes and cotyledons) (Table 4). In contrast, RIN values for the high moisture treatment were usually lower and ranged from 7.7 (lentil cotyledon) to 7.1 (chickpea axes or cotyledons).

A trend of decreasing RIN values with increasing storage moisture was observed in pea seeds stored at room temperature in which RIN decreased from 7.6 to 5.0 in axes stored at 33–52% RH for 7 years. RIN of pea seeds harvested in 2015 and measured in 2017 was 7.8 (Fleming et al., 2019) and so comparable to those of seeds stored at 2°C (RIN = 7.8 in 2023; Table 4).

A subset of the AGG seeds stored at 20°C was removed from storage in 2016 and in 2023. Average RIN values for grains removed in 2016 were 7.7 (±0.1 SE, $n = 44$), 5.2 (±0.2 SE, $n = 81$) and 5.9 (±0.30 SE, $n = 65$) for chickpea, pea and lentil, respectively (Table 5). Average RIN decreased to 7.4 (±0.10 SE, $n = 40$), 4.8 (±0.30 SE, $n = 68$) and 5.3 (±0.40 SE, $n = 76$) for chickpea, pea and lentil, respectively, when sampled in 2023, a difference of 0.3 ($P < 0.0001$), 0.4 ($P = 0.3612$) and 0.6 ($P = 0.0299$), respectively, over 7 years (Table 5).

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Table 3. Change in the germination of 9–10 genetic lines of chickpea, pea and lentil that were stored for 20 years at 20°C and 7–10% water (Table 1, 29–47% RH)

	Variety #	Variety name	Germination in 2003	Germination in 2016	Germination in 2019	Germination in 2020	Germination in 2023	P50 (days) Avrami	P50 (days) dose.p	SE P50 dose.p
Chickpea	1	Howzat	100	92	Depleted	Depleted	Depleted	6,213	9,970	1,822
	2	FLIP 94-079C	100	33	Depleted	Depleted	Depleted	4,048	4,053	77
	3	ICCV 96836	100		93	Depleted	Depleted	9,119	7,991	780
	4	Kaniva	99	96	Depleted	Depleted	Depleted	7,374	16,086	9,776
	5	FLIP 94-090C	100	16	Depleted	Depleted	Depleted	4,863	4,322	14
	6	Tyson	100	97	88	91	96	9,446	28,956	17,271
	7	Paider-91	99	97	79	84	71	8,408	14,172	2,565
	8	WACPE 2075	100	97	95	97	90	9,426	10,816	1,254
	9	S 95342	100	90	Depleted	Depleted	Depleted	7,327	8,361	1,415
	10	FLIP 94-508C	100		89	81	100	24,745	15,190	3,209
		Average	**100**	**77**	**89**	**88**	**89**	**9,097**	**11,992**	**3,818**
Pea	1	Snowpeak	100	66	Depleted	Depleted	Depleted	4,960	4,777	58
	2	Kaspa	100	78	95	89	Depleted	7,509	8,701	664
	3	Excell	100	73	5	14	0	4,849	4,745	45
	4	Parafield	100	90	89	93	94	14,889	13,146	2,187
	5	Paravic	100	91	79	64	74	7,400	10,322	942
	6	90-158*8-1	100	80	58	39	24	5,721	5,878	101
	7	95-072*3	100	95	85	91	80	8,670	9,429	616
	8	90-131-*27-7	100	87	63	73	53	7,112	7,281	177
	9	89-036-*9-8	100	84	73	46	31	6,327	6,523	102
	10	90-166*30-5	100	73	13	6	Depleted	5,000	4,863	43
		Average	**100**	**82**	**62**	**57**	**51**	**7,244**	**7,567**	**493**
Lentil	1	Cumra	100		84	51		6,556	6,630	137
	2	Cobber	100		73	47		6,220	6,330	96
	3	Northfield	100	80	77	64	34	7,266	6,888	114
	4	CIPAL 102	100		71	60	28	7,388	6,728	95
	5	Matilda	100	82	54	16	8	5,688	5,661	64
	6	Digger	100	74	44	12	7	5,686	5,540	52
	7	Aldinga	100	Depleted	Depleted	Depleted	Depleted	3,991	6,470	101
	8	CIPAL 205	100	86	65	40	30	6,946	6,212	80
	9	CIPAL 206	100	90	8	47	36	6,377	5,311	49
	10	CIPAL 106	100	76	29	9	0	5,378	3,963	89
		Average	**100**	**81**	**56**	**38**	**20**	**6,150**	**5,973**	**88**

Germination was tested periodically, and some samples were depleted by 2016. Germination data (here and Redden and Partington, 2019) were fitted to the Avrami equation. In several cases, the model was extrapolated past 50% germination in order to calculate the longevity (P50, storage time to 50% germination) of each genetic line.

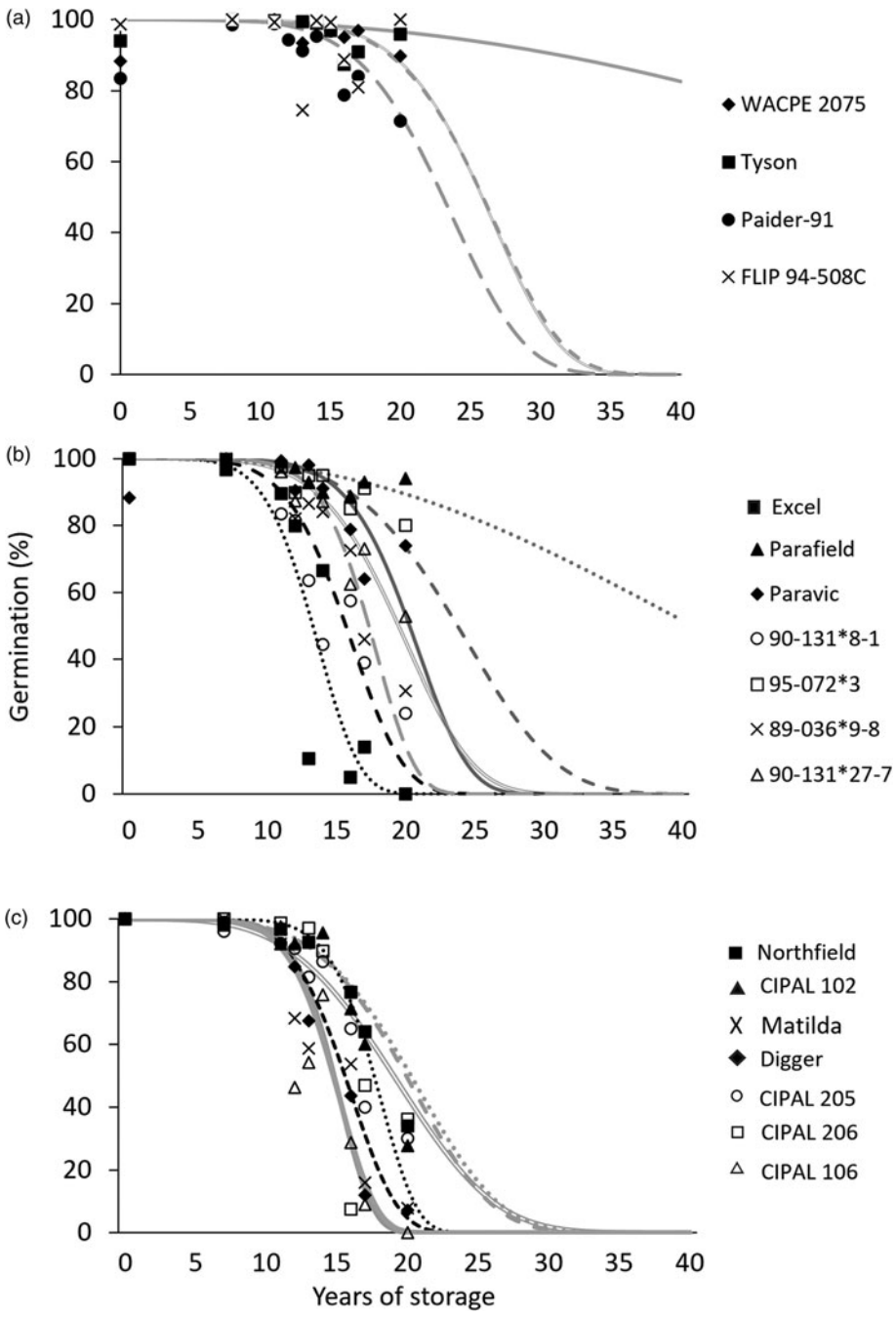

Fig. 1. Germination data and viability curves for varieties of (A) chickpea, (B) pea and (C) lentil at 20°C for 20 years with water content ranging from 0.049 to 0.11 g H_2O g^{-1} dm. Germination data for each variety measured over the 20 years of the experiment are fitted using the Avrami method.

The average RIN values from embryonic axis or cotyledon tissue from the same seed were significantly correlated for chickpea, pea and lentil ($P = 0.0017$, $P < 0.0001$ and $P < 0.0001$, respectively; Fig. 2). Although correlated, the values were not identical. Average RIN values tended to be slightly greater for embryonic axes compared with cotyledons in undegraded seeds (germination > 90%; RIN > 8.0) and then lower in embryonic axes compared with cotyledons as seeds showed symptoms of ageing (germination < 80%; RIN < 7.0, with the exception of pea cv. Parafield). This tendency is noted as points along the 1:1 line at high RIN and below the 1:1 line at low RIN (Fig. 2). This result of slightly greater RIN degradation in embryonic tissue compared with cotyledon tissue was not significantly different within species ($P = 0.6804$, $P = 0.3805$ and $P = 0.0850$, chickpea, lentil and pea, respectively).

Relationship of RIN with germination parameters

The rate of RIN decline in stored seeds is affected by the temperature and moisture of the storage environment (Tables 4 and 5). RIN decline rate also varies at the species, cultivar and tissue levels. Is there a relationship between variation in RIN decline with variation in the rate of viability loss among species and cultivars? To address this question, we expressed the rate of viability decline as final germination percentage, P50 and $P50^{-1}$, considering that all samples had similar, high, initial germination (98–100% germination), and were stored under similar conditions (20°C and 30–40% RH) for the same time (20 years). We expressed RIN decline as average RIN of either the axis or the cotyledon (presuming RIN values of freshly harvested seeds

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Table 4. Effect of moisture on quality of RNA extracted from long-term stored seeds

				Embryonic axis				Cotyledon				Average RIN (Medium and low MC)	
Storage temperature (°C)	Species	Cultivar	Moisture Treatment	RIN (average)	SE	*n*	*P*	RIN (average)	SE	*n*	*P*		
2°C	Chickpea	Howzat	High	7.2[a]	0.1	4	0.0056	7.2[a]	0.2	3	0.0002		
			Medium	8.0[b]	0.3	4		7.5[a]	0.1	4			
			Low	8.3[b]	0.1	4		8.3[b]	0.1	4			
		FLIP 94-079C	High	7.1[a]	0.1	4	0.0031	7.1[a]	0.1	3	0.0002		
			Medium	7.9[b]	0.1	4		8.1[b]	0.2	4			
			Low	8.3[b]	0.3	4		8.5[b]	0.1	4		**8.1**	**8.1**
	Pea	Snowpeak	High	6.8[a]	0.1	4	0.0004	7.3[a]	0.2	4	0.0292		
			Medium	8.7[b]	0.4	4		8.5[b]	0.1	3			
			Low	8.5[b]	0.1	4		8.4[ab]	0.1	4			
		Kaspa	High	7.8[a]	0.3	3	0.0396	7.8	0.1	2	0.5398		
			Medium	8.7[b]	0.1	4		8.2	0.1	4			
			Low	8.3[ab]	0.2	4		8.1	0.3	3		**8.6**	**8.3**
	Lentil	Cumra	High	7.5	0.1	4	0.4736	7.8[a]	0.1	4	0.0005		
			Medium	8.5	0.2	3		8.6[b]	0.1	4			
			Low	8.5	0.3	4		8.4[b]	0.1	4			
		Cobber	High	6.9[a]	0.7	3	0.0393	7.6[a]	0.1	3	0.0147		
			Medium	8.7[b]	0.1	4		8.5[b]	0.1	4			
			Low	8.2[ab]	0.2	3		8.2[ab]	0.1	3		**8.5**	**8.4**
	Pea	Alaska 2016		7.7	0.3	4		7.8	0.1	3			
20–22°C	Pea	Alaska	52% RH	5.0	0.2	7		5.5	0.2	7			
			43% RH	5.9	0.1	7		6.6	0.1	7			
			33% RH	7.6	0.2	7		7.4	0.1	7			
			13% RH	7.6	0.2	6		7.2	0.1	7			

RNA quality is expressed as the RIN. In the top portion of the table, seeds from two genetic lines per species were stored at 2°C and indicated moisture levels (Table 1) between 2003 and 2023. The lower portion of the table shows a comparable experiment with seeds stored at controlled RH on the lab bench (room temperature) for 7 years and the comparable 2016 Alaska pea seed stored at 5°C. Superscript letters indicate a significant difference among treatments for the genetic line according to the *post-hoc* Tukey's HSD test.

Table 5. The quality of RNA extracted from different cultivars of legume seeds stored at 20°C and indicated moisture (Table 1) since 2003

		Year received at NLGRP: 2016						Year received at NLGRP: 2023						
		Embryonic axis			Cotyledon			Embryonic axis			Cotyledon			
Species	Cultivar	RIN (average)	SE	*n*	RIN (average)	SE	*n*	Axis RIN	SE	*n*	RIN (average)	SE	*n*	RIN difference (RIN_{2016}-RIN_{2023}/7 years)
Chickpea	Tyson	7.5	0.1	3	7.9	0.1	12	7.5	0.1	5	7.5	0.1	5	0.2
	Paider-91	7.4	0.2	3	7.9	0.1	9	7.2	0.1	6	7.0	0.2	4	0.6
	WACPE 2075	7.7	0.1	4	7.6	0.2	4	7.8	0.1	6	7.6	0.2	5	0.0
	FLIP 94-508C	7.7	0.1	4	7.8	0.1	4	7.7	0.5	5	7.3	0.1	4	0.3
	Average	**7.6**	**0.1**	**15**	**7.8**	**0.1**	**29**	**7.5**	**0.1**	**22**	**7.3**	**0.1**	**18**	**0.3**
Pea	Kaspa	3.8	0.3	8	5.6	0.1	7							
	Excell	3.6	0.2	5	5.0	0.2	5	3.1	0.1	5	3.2	0.1	3	1.2
	Parafield	4.7	0.8	3	6.6	0.1	2	4.0	0.4	5	6.1	0.1	4	0.6
	Paravic	6.5	0.1	4	6.7	0.1	4	5.4	0.3	5	6.5	0.1	5	0.7
	90-158*8-1	4.4	0.4	4	5.9	0.1	4	4.1	0.3	6	5.0	0.2	5	0.6
	95-072*3	4.8	0.5	4	6.2	0.1	3	4.4	0.5	6	6.1	0.1	4	0.2
	90-131-*27-7	4.4	0.3	4	6.0	0.1	6	4.8	0.4	6	5.7	0.2	6	−0.1
	89-036-*9-8	3.5	0.3	5	6.0	0.1	5	3.4	0.4	3	5.3	0.4	5	0.4
	90-166*30-5	4.1	0.5	4	5.9	0.1	4							
	Average	**4.4**	**0.3**	**41**	**6.0**	**0.2**	**40**	**4.2**	**0.3**	**36**	**5.4**	**0.4**	**32**	**0.4**
Lentil	Cumra	6.0	0.1	4	6.4	0.2	4							
	Cobber	4.0	0.7	4	5.8	0.1	4							
	Northfield	7.3	0.2	4	7.0	0.1	3	6.9	0.1	6	6.8	0.1	5	0.3
	CIPAL 102	7.1	0.1	4	7.2	0.0	4	5.2	0.7	6	7.2	0.1	6	1.0
	Matilda				5.1	0.2	5	3.0	0.1	3	4.5	0.1	6	1.3
	Digger	3.5	0.3	4	6.1	0.1	4	3.2	0.2	5	4.5	0.4	5	0.9
	CIPAL 205	7.1	0.3	4	7.0	0.1	4	6.4	0.1	5	6.2	0.4	6	0.8
	CIPAL 206				6.4	0.1	5	6.5	0.1	6	6.7	0.1	6	−0.2
	CIPAL 106	3.1	0.1	4	5.4	0.1	4	2.8	0.0	5	3.1	0.2	6	1.3
	Average	**5.4**	**0.7**	**28**	**6.3**	**0.3**	**37**	**4.9**	**0.7**	**36**	**5.6**	**0.6**	**40**	**0.6**

RNA quality is expressed as the RIN. Seeds were removed from storage at 20°C in 2016 and 2023 and placed at −20°C until RNA was extracted (some in 2017, but mostly in 2023).

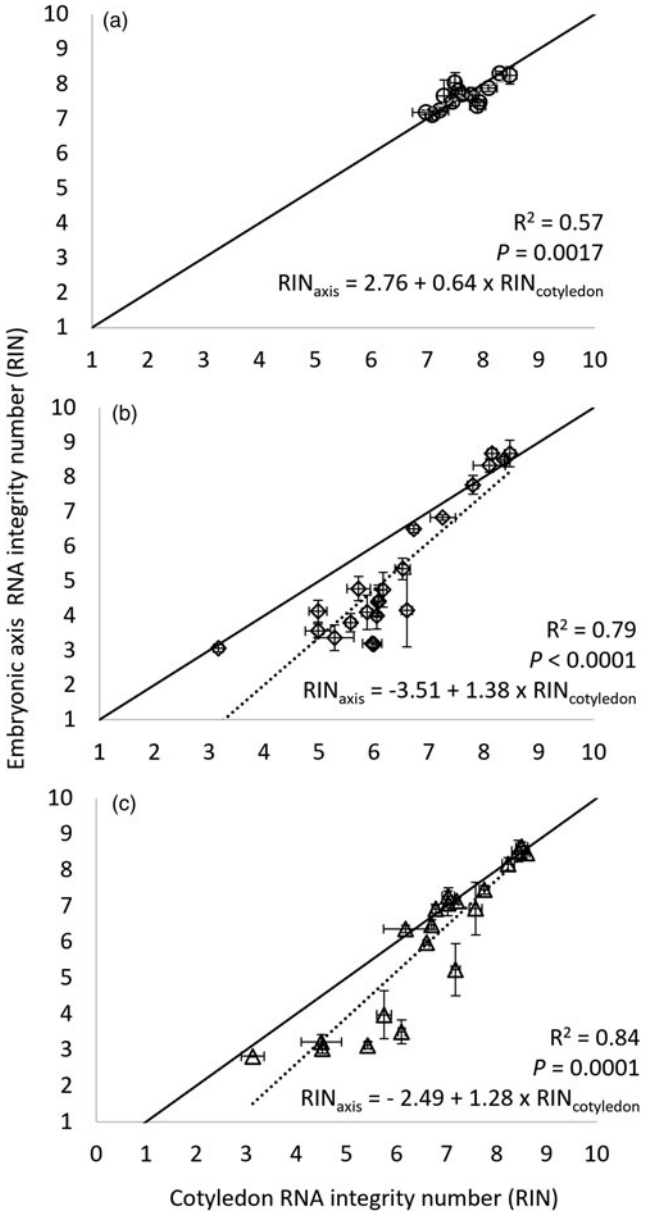

Fig. 2. RIN for total RNA extracted from both axis and cotyledon of all varieties of (A) chickpea, (B) pea and (C) lentil stored at 2°C and 20°C for 20 years. The solid line represents a 1:1 ratio if RIN from axis and cotyledon tissue from a variety are the same. The dotted line represents the linear relationship between embryonic axis and cotyledon tissue within each crop.

were similar and >8) or as the difference in RIN between 2016 and 2023 measurements when both years were tested ($RIN_{2016–2023}$). When all available samples from all species were considered in the models ($N \geq 20$), RIN decline was significantly associated ($P < 0.01$) with the three measures of viability loss in eight of the nine models (Table 6). The strongest relationships ($R^2 = 0.470$, $P < 0.0001$, $n = 22$) used RIN values from RNA extracted from cotyledons and viability decline expressed as either germination in 2023 or as $P50^{-1}$ (Fig. 3).

Regression analyses were weaker when restricted to within-species analyses. There were no significant relationships uncovered for chickpea seeds, which is to be expected because the four cultivars tested showed little sign of deterioration during 20 years of storage under the study conditions (Table 6). For pea, linear regressions between RIN from cotyledons and germination percentage in 2023 and $P50^{-1}$ were significant ($R^2 = 0.44$, $P = 0.036$, $n = 9$ and ($R^2 = 0.39$, $P = 0.055$, $n = 9$). For lentil, linear regressions between RIN from cotyledons and $P50^{-1}$ or $RIN_{2016–2023}$ and germination percentage in 2023 were significant ($R^2 > 0.67$, $P < 0.017$, $n = 9$). Including germination and RIN data from the 2°C experiment further strengthened relationships (Table 6) of pea and lentil, but the relationship between RIN and viability remained not significantly different in chickpea, presumably because most of the seeds showed little sign of deterioration.

Discussion

We seek markers of seed ageing during storage that are expressed before seeds die but portend the timing of seed death. This paper focuses on RIN as a candidate marker because RNA accumulated during seed development tends to fragment during dry storage and can be detected before seed mortality (Fleming et al., 2017, 2019; Tetreault et al., 2022). The study requires materials at various stages of degradation from known (i.e. storage environment) and unknown (i.e. initial seed quality) mechanisms. An experiment begun in 2003 provides an opportunity to explore RIN in seeds from different cultivars of chickpea, pea and lentil that were stored under controlled moisture levels at 2 and 20°C and appear to be expressing variation in longevity. We demonstrate a decline in RIN that corresponds with seed ageing.

Intuitively, we know that a seed can germinate until it cannot. The binary nature of viability creates some philosophical problems when monitoring germination potential of a population over time. Some models predict a progressive, yet oblique, decline in germination initially, based on the assumption that seed deaths are normally distributed in time, with a caution of the limitation of applying models to other seed lots with variations in storage conditions (Ellis and Roberts, 1980; Ellis, 1988). Alternatively, viability *per se* may be an inappropriate indicator of ageing if ageing results from an accumulation of minor damage that does not initially affect a seed's ability to germinate. In that scenario, measuring accumulated damage, as well as how much is required to induce a major effect, can lead to a more mechanistic understanding of the ageing process as well as better tools to measure it. In this case, models such as the Avrami equation (Avrami, 1941; Niedzielski et al., 2009) which consider cooperativity and thresholds leading up to a transition can be helpful (Fig. 1). With either scenario, a huge issue for lab testing is how a small number of seeds in a test, as well as inconsistency among tests, will limit detection of any subtle decline in germination percentage (Tetreault et al., 2022). For example, at least 68 seeds are needed for statistical confidence that a decline from 98 to 90% germination is real (Tetreault et al., 2022). Data from this study show the common observation that germination results sometimes fluctuate among tests separated by storage time, making it difficult to interpret a high and low germination result in the vicinity of the mortality threshold (Table 3).

Parameters that quantify viability and RIN decline during storage

Uncertainty about the reliability of various parameters to quantify longevity prompted us to use several options to explore significant relationships (Hay et al., 2019). The most familiar method uses germination percentage after a certain time. The time of 20 years achieved samples that varied in germination percentage. A shorter or longer storage time could have yielded all populations germinating above 90% or below 20%, respectively, which would not be

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Table 6. Correlations within and across species between viability parameters (germination percentage, P50 or $P50^{-1}$), and RIN from embryonic axis tissue, cotyledon tissue and RIN difference between 2016 and 2023 (Table 5)

Correlation test	Model variables	Slope	Intercept	R^2	F	df	n	P
Final germination and final RIN (embryonic axis)	All species	0.028	3.711	0.332	9.922	20	22	0.001
	Chickpea	0.015	6.148	0.535	2.304	2	4	0.303
	Pea	0.009	3.677	0.205	1.807	7	9	0.233
	Lentil	0.064	3.186	0.493	6.799	7	9	0.023
Final germination and final RIN (cotyledon)	All species	0.025	4.692	0.478	18.324	20	22	<0.0001
	Chickpea	0.014	6.040	0.515	2.121	2	4	0.320
	Pea	0.018	4.572	0.442	5.541	7	9	0.036
	Lentil	0.059	4.113	0.618	11.349	7	9	0.006
Final germination and RIN difference (RIN_{2016}-RIN_{2023}/7 years)	All species	−0.008	0.950	0.399	11.957	18	20	<0.0001
	Chickpea	−0.013	1.376	0.420	0.723	1	3	0.639
	Pea	−0.005	0.773	0.193	1.198	5	7	0.376
	Lentil	−0.030	1.392	0.675	10.394	5	7	0.017
P50 and final RIN (embryonic axis)	All species	0.065	3.618	0.212	5.367	20	22	0.0136
	Chickpea	0.005	7.365	0.139	0.323	2	4	0.7559
	Pea	0.014	3.828	0.028	0.199	7	9	0.8238
	Lentil	0.690	−7.183	0.689	15.484	7	9	0.0027
P50 and final RIN (cotyledon)	All species	0.049	4.825	0.211	5.354	20	22	0.0137
	Chickpea	0.000	7.327	0.000	0.000	2	4	1.0000
	Pea	0.054	4.359	0.214	1.906	7	9	0.2184
	Lentil	0.622	−5.206	0.829	33.892	7	9	0.0003
P50 and RIN difference (RIN_{2016}-RIN_{2023}/7 years)	All species	−0.011	0.808	0.099	1.975	18	20	0.1677
	Chickpea	−0.001	0.298	0.006	0.006	1	3	0.9941
	Pea	−0.006	0.649	0.020	0.104	5	9	0.9761
	Lentil	−0.112	2.749	0.218	1.396	5	9	0.3557
$P50^{-1}$ and final RIN (embryonic axis)	All species	−69.998	8.683	0.394	12.116	20	22	0.0004
	Chickpea	−9.450	7.850	0.209	0.529	2	4	0.6541
	Pea	−15.772	4.963	0.129	1.037	7	9	0.4033
	Lentil	−213.255	17.212	0.728	18.742	7	9	0.0015
$P50^{-1}$ and final RIN (cotyledon)	All species	−59.417	8.977	0.470	18.994	20	22	<0.0001
	Chickpea	−2.045	7.394	0.011	0.022	2	4	0.978
	Pea	−38.751	7.563	0.393	4.531	7	9	0.055
	Lentil	−192.556	16.808	0.878	50.472	7	9	<0.0001
$P50^{-1}$ and final RIN difference (RIN_{2016}-RIN_{2023}/7 years)	All species	14.078	−0.152	0.246	5.868	18	20	0.0109
	Chickpea	3.216	0.161	0.030	0.031	1	3	0.9704
	Pea	9.869	0.005	0.170	1.023	5	9	0.4766
	Lentil	36.857	−1.356	0.259	1.744	5	9	0.2766
2°C samples included	All species	0.042	3.350	0.503	38.443	38	40	<0.0001
Final germination and final RIN (embryonic axis)	Chickpea	0.012	6.593	0.162	1.542	8	10	0.271
	Pea	0.048	2.249	0.491	12.533	13	15	0.001
	Lentil	0.038	3.834	0.552	16.014	13	15	<0.0001
2°C samples included	All species	0.035	4.346	0.642	68.068	38	40	<0.0001
Final germination and final RIN (cotyledon)	Chickpea	0.014	6.393	0.141	1.319	8	10	0.320
	Pea	0.042	3.469	0.746	38.200	13	15	<0.0001
	Lentil	0.033	4.710	0.632	22.341	13	15	<0.0001

Shaded rows indicate a significant correlation. The top portion of the table included analyses conducted with data from seeds stored at 20°C. The lower portion of the table includes germination percentage and RIN from 2023 for seeds stored at 20 and 2°C.

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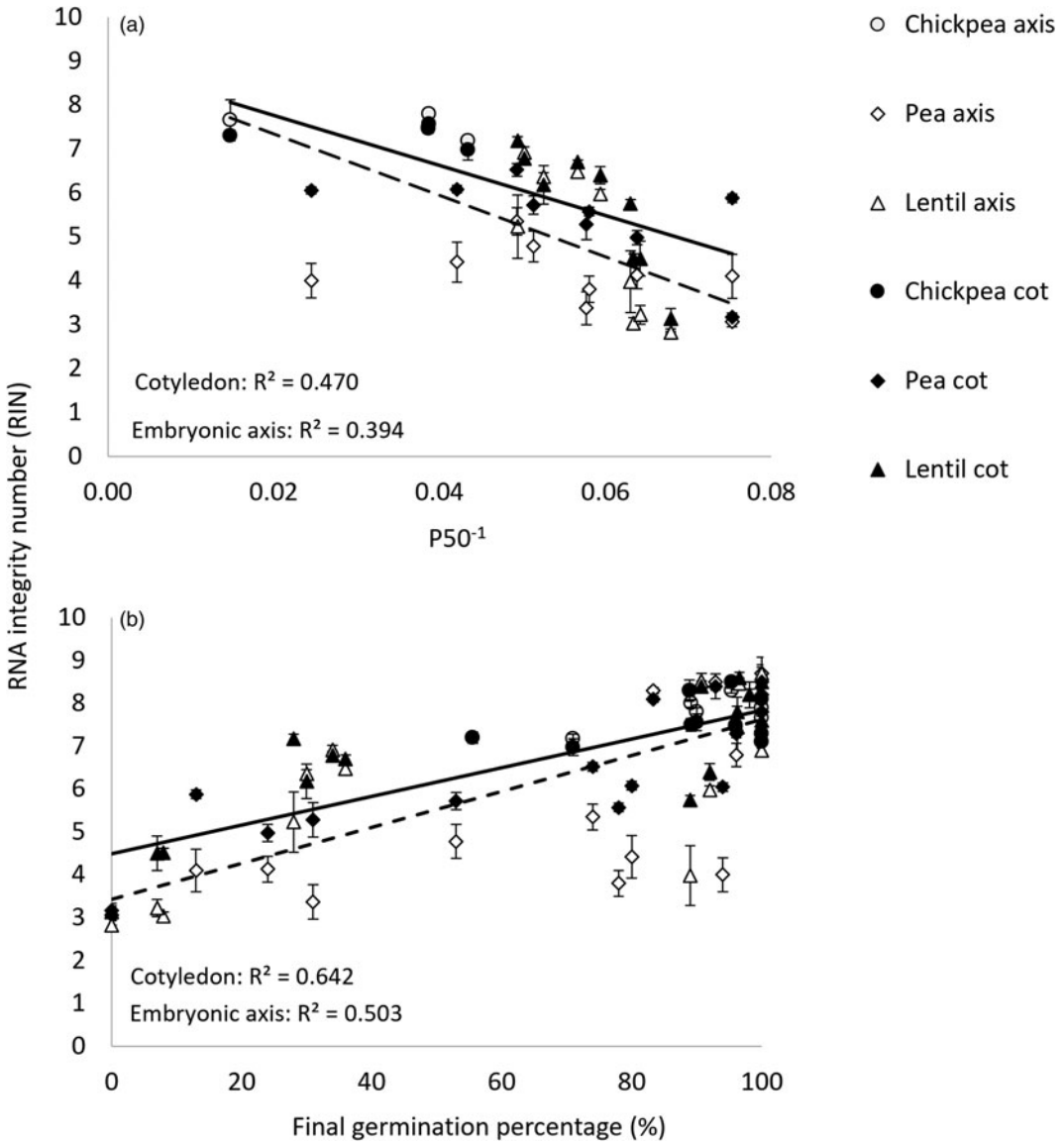

Fig. 3. Correlation of RIN with germination parameters of (A) ageing rate ($P50^{-1}$) and (B) final germination percentage for chickpea, pea and lentil in embryonic axis tissue and cotyledon tissue. (A) Data from seeds stored at 20°C and (B) data from seeds stored at both the 2 and 20°C experiments. Open symbols represent RIN values collected from embryonic axis tissue; closed symbols represent RIN values collected from cotyledons. Each data point represents an individual variety within crop included in the study (Table 1). P50 was extrapolated using the Avrami function for germination data for each variety over the 20-year experiment and $P50^{-1}$ was used to quantify the rate at which viability was lost under experimental conditions for each crop variety. Final germination is from 2023, except for samples that were depleted (Table 3), in which case 2016 final germination and RIN are depicted. Regression lines represent models including all species, solid lines represent regressions for cotyledon tissue and dashed lines represent regressions for embryonic axis tissue.

informative. However, longevity is most directly assessed as a time parameter, and this can be obtained by fitting the monitor data time series to a sigmoidal model, as we have done using the Avrami equation (Fig. 1). Our longevity calculations consider the time for germination to decrease to 50%, P50. The use of all the 20-year germination data (Redden and Partington, 2019; Table 3) evens out variation implicit in individual tests and provides a more comprehensive view than a single germination test of the storage behaviour of the sample. That said, uncertainty in P50 increases in samples that show minor to no degradation; there is a poor fit to the sigmoidal curve and P50 values tend towards infinity when germination percentage equals initial values. This problem is mitigated by expressing the reciprocal of P50 ($P50^{-1}$), which transforms a very large P50 (i.e. long-lived seed) to an ageing rate that approaches 0 (i.e. slow ageing seed). In this study of 20-year-old seeds from different cultivars of chickpea, pea and lentil, 11 of the 30 cultivars stored at 20°C exhibited P50 > 20 years, meaning that about one-third of the samples showed minor to no viability decline. Presumably, P50 at 2°C ≫ P50 at 20°C, but we were unable to estimate P50 at 2°C given just two germination tests 20 years apart that were not significantly different (Hay et al., 2019, 2022). After 20 years of storage at 20°C, germination percentages ranged from 0 to 100%; P50 ranged from 11 to 68 years; and $P50^{-1}$ ranged from 0.090 to 0.015 yr^{-1} (or −4.5 to −0.7% germ yr^{-1}).

We also provide three options for how the RIN (a quantitation of the amount of fragmentation of RNA molecules) parameter is expressed. Large-seeded legumes can be easily separated into nutritive tissues (i.e. cotyledons) and tissues that grow and

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develop into the seedling (i.e. embryonic axis). High quality RNA can be extracted from both tissue types in fresh seeds, but RNA yield per mg tissue is >10-fold higher from axes compared with cotyledons (unpublished and Fleming et al., 2017). We also had the opportunity to compare RIN values from seeds that were removed from storage 7 years apart (2016 and 2023, $RIN_{2016–2023}$) to estimate change in RIN between monitor tests. On average, RIN values were significantly lower ($P = 0.0186$) in samples removed from storage in 2023 compared with 2016 (5.5 ± 1.5 ($n = 18$) and 5.9 ± 1.2 ($n = 22$), respectively). The average rate of change of RIN was about 0.07 RIN·yr^{-1}, which leads to a predicted starting value of ≈6.9 ($6.9 = 5.5$ RIN + 0.07 RIN·yr^{-1} * 20 yr) for these samples. This prediction is lower than average RIN values measured in 2023 for seeds stored at 2°C (RIN ≈ 8), which suggests that RIN declined in 20°C-stored seeds faster than we estimated.

Correlations between viability and RIN decline during storage

We used regression analysis to test the hypothesis that RNA fragmentation occurs at comparable rates as the decline in germination percentage, P50 or $P50^{-1}$, making it a good marker of seed ageing. The hypothesis is based on the supposition that ageing is caused by incremental damage to cellular constituents that eventually lead to seed death. Moreover, the kinetics of damaging reactions are controlled by factors that affect molecular mobility in the cytoplasm (namely moisture and temperature), as well as substrate levels for reactants (e.g., reactive oxygen molecules) and protectants or 'decoys' for damaging oxidative reactions (Roberts, 1973; Ballesteros and Walters, 2019; De Vitis et al., 2020; Solberg et al., 2020). We evaluated the relationship between RIN and viability through regression analysis across all three species and within species. RIN × germination percentage data for the 2°C storage experiment was added to the correlation analysis to test the overall hypothesis of RIN decline with storage temperature and moisture data. When the correlation model combined all species and all treatments, the significance of the relationship was high ($P \leq 0.0136$, $R^2 \geq 0.21$) for all cross comparisons except $RIN_{2016–2023}$ *versus* P50 (Table 6). Relationships using P50 and $RIN_{2016–2023}$ were weakest, probably because P50 was overestimated in samples with high viability in 2023 and $RIN_{2016–2023}$ included just a subset of all the samples. When correlation models using these factors were removed, $P \leq 0.0002$ and R^2 ranged between 0.39 and 0.88 (Table 6; Fig. 3).

Relationships were expectedly weaker when individual species were considered because N was smaller and within-species variation in longevity was lower than variation across all species. In fact, a significant correlation was not gleaned for chickpea and this is likely because just two of the samples included in RIN assays showed significant indication of viability decline (i.e. cv. Paider-91 at 20°C and Howzat at 2°C, high moisture). For pea and lentil, correlations were significant when RIN from cotyledons were regressed against $P50^{-1}$; including data from the 2°C slightly aged samples strengthened the relationships ($P < 0.0001$, $R^2 > 0.63$) (Table 6; Fig. 3).

Significant relationships between viability loss and RNA fragmentation suggest these two manifestations of seed ageing interact. However, the initial slow change of viability (Fig. 1) is hard to detect, whereas the presumed linear decline in RIN (not examined here, but see Fleming et al., 2019; Tetreault et al., 2022) means that RNA fragmentation can be detected before significant mortality. For example, a monitoring interval of 10 years precedes P50 for all samples studied but in contrast would show a very detectable average RIN decline of about 0.7.

Variation in seed longevity among species, among cultivars and between tissue types

Temperature and moisture effects on seed longevity are increasingly understood, especially in the context of mobility within vitrifying cytoplasm (Walters, 1998; Walters et al., 2010; Ballesteros and Walters, 2019). This study provides a unique dataset examining an elusive factor of initial seed quality that is most likely related to the chemical composition and organization of cytoplasm in dried cells. Seeds acquire longevity during the embryogenic programme (Hay et al., 1997; Righetti et al., 2015; Whitehouse et al., 2015; Pereira Lima et al., 2017; Zinsmeister et al., 2020) and this capacity is often expressed as a species characteristic (Priestley et al., 1985; Roos and Davidson, 1992; Walters et al., 2005; Ellis and Hong, 2006; Probert et al., 2009; Merritt et al., 2014; Solberg et al., 2020). In the few studies that compare seed longevity among species, chickpea, pea and lentil are all considered to produce long-lived seeds (Priestley et al., 1985; Walters et al., 2005; Solberg et al., 2020). Chickpea seeds stored at low water content (~5%) in hermetically closed packets stored at −18°C showed relatively stable storage after 20–24years with a less than 5% viability decline compared with initial viability (Desheva, 2016). Here, the average P50 of chickpea seeds stored at 20°C was 24.9 years and it was predicted to be 28 years from the viability equations using comparable environmental conditions (SER, 2023). Pea seeds are also identified as long-lived with a P50 ~ 100 years under dry room conditions or genebanks (Roos and Davidson, 1992; Solberg et al., 2020). Here, the average P50 of pea seeds stored at 20°C was 19.5 years and the viability equations predicted a similar longevity as chickpea at 28 years (SER, 2023). Lentil seeds stored at NLGRP had an average P50 of 365 years (Walters et al., 2005); however, here (and in Priestley et al., 1985), they were less stable than the other two species with an average P50 of 17.1 years and a predicted P50 of 19 years based on the viability equations and new species coefficients (Whitehouse and Norton, 2022).

Differences in average longevity among these species are not surprising. As we have described, prediction of longevity of long-lived material requires extrapolation and associated uncertainty. Variation among labs is also likely due to the selection of cultivars used in the study. There has been increasing awareness of within-species variation in seed longevity that is due to genetic background and growth conditions (Clerkx et al., 2004; Nagel et al., 2011, 2015; Lee et al., 2019), rather than minor differences in germination capacity as modelled in the viability equations. In this study, we observed considerable variation in longevity among legume grain cultivars that were grown, harvested and stored similarly. While the underlying basis for differing longevities of seeds within a species cannot be deciphered from this study, the correlation between RIN and eventual viability loss provides a promising approach to phenotyping the seed longevity trait without having to wait for seeds to die (Hay et al., 2019).

Another novel aspect of this study is the report that the decrease in RIN appears more severe in embryonic axis tissue compared with cotyledons (Fig. 2). It was also surprising that the relationship of RIN with viability parameters (Table 6) was stronger for RNA extracted from cotyledons compared with the embryonic axis. This finding is consistent with a previous report for pea (Fleming et al., 2019), but differs from observations made

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using soybean seeds that showed no major differences between RIN measurements of RNA extracted from either cotyledons or embryonic axes (Fleming et al., 2017; Tetreault et al., 2022). We speculate that this combination of observations, as well as the extremely high yield of RNA from axes, may suggest that embryonic axes accumulate a superfluous supply of RNA and that there may be some variation in how RNA is protected within cells of the embryonic axes. These speculations can lead to inferences about RNA stability in cotyledons *versus* embryonic axes, the mechanism(s) of protection and the role of extraneous but more unprotected RNA molecules in fresh, mature seeds.

Consumption of seed during monitor testing

Hundreds of seeds were needed to conclusively show a decrease in germination with storage time (ISTA, 2003; Redden and Partington, 2019; Tetreault et al., 2022) which was not really clear until seed germination was <[85–80%] in years [12–16] depending on species. In contrast, just 4–12 seeds provided an informative estimate of RIN and deterioration of seed health.

Conclusion

The viability of lentil, chickpea and pea seeds stored at 20°C began to decline after 16–20 years. RNA integrity also declined during this time, and RIN assessments reliably distinguished grain legumes that died sooner *versus* later. Separation was largely based on species, but also significantly correlated with aging rates of seeds from different genetic lines. Assaying RIN earlier in the experiment would likely have revealed variations of ageing rates among species and lines and might help to predict which seed lots would succumb first. RIN appears to be a promising marker for seed ageing that can aid genebanks in managing the difficult task of predicting and detecting imminent seed deaths. We conclude that RNA integrity (RIN) is a feasible approach to assay the progress of seed ageing. RIN assays can be interspersed with germination assays to aid interpretation of germination assays and adjust monitoring or regeneration schedules to ensure genebanks maintain high quality germplasm. Future work correlating the rate of RIN decline with deterioration time courses may lead to more reliable prediction of the onset of rapid mortality of stored seeds.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0960258524000072.

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Conflicts of interest. The authors declare none.

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