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A power analysis for detecting aging of dry-stored soybean seeds: Germination versus RNA integrity assessments

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Abstract

Deterioration of seed during dry storage is a major problem for genebanks and seed companies. Germination tests are the gold standard to monitor seed viability; however, these prove to be insensitive during the early stage of storage when viability changes are subtle. Recent findings demonstrate that decline in RNA integrity may be an early indicator of seed longevity during dry storage. The goal of this study was to determine the sensitivity of RNA integrity, measured as RNA integrity number (RIN), regarding how soon changes can be detected and how many seeds are required. We compared the statistical power of germination and RIN assays using a well-characterized collection of ‘Williams 82’ soybean seeds, with cohorts harvested between 1989 and 2019 and stored at 5 °C. Germination was monitored in 1- to 3-yr intervals since 1989, and RIN was monitored in 1- to 2-yr intervals since 2016 providing an extensive dataset to conduct statistical power analyses. Decline in RIN can be detected in soybean seeds within 10 yr with a RNA monitor test that consumes approximately 30 seeds. In contrast, a germination test detects deterioration in 16 yr using approximately 50 seeds, and by this time, the seed lot is near the limit of longevity and has entered the phase of rapid mortality. Work from this study indicates that early detection of aging using RIN decline can be used to predict the longevity threshold to optimize viability monitoring and regeneration times, preventing loss of valuable samples by overtesting or missing the longevity threshold.

1 | INTRODUCTION

There are about 1,750 seed genebanks globally that serve agriculture, conservation, and studies of biological diversity (Hay, 2021). Genebanks must ensure stored seed lots retain at least 85% viability, according to standards aimed at maintaining the genetic identity and regeneration potential of the

sample (FAO, 2014; Reed, Bradford, et al., 2022). Moreover, standards recommend that accessions are periodically tested to affirm high viability and to, possibly, detect the onset of decline (FAO, 2014). Therefore, seed viability testing is a necessary, recurrent and costly activity that consumes precious samples that were put away and meant for safe keeping (Fu, Ahmed, et al., 2015; Hay, 2021).

Genebanks and seed industries would greatly benefit from assays that indicate the progression of aging and reliably

Abbreviation: RIN, RNA integrity number.

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predict imminent viability decline (Fu, Ahmed, et al., 2015). One common approach to allow predictions of longevity for various species is the reduction of longevity using poor storage conditions, with an assumption that longevity under good conditions can be extrapolated (Ellis & Roberts, 1980; Reed, Bradford, et al., 2022; Walters, Fleming, et al., 2020). Alternatively, accumulation of biochemical changes occurring in dry-stored seeds can be tracked and related to longevity rather than viability (because germinability changes only slightly at first) (Colville & Pritchard, 2019; Fleming, Hill, et al., 2019; Mira, Hill, et al., 2016). A major challenge for both approaches is the time frame needed to detect a decline of germinability under dry, cool conditions to allow correlations. Focus on nonlethal damage during storage provides additional benefits such as clues about the nature of reactions that occur in dry biological systems and, potentially, the mechanisms by which seeds succumb with time.

Seed viability is commonly tested using a germination assay that counts the appearance of an emergent radicle (Bewley & Black, 2013; Reed, Bradford, et al., 2022), normal development (AOSA, 2017), or vital staining in the case of dormant seeds (ISTA, 2021) relative to the total number of seeds sowed. This discrete (i.e., alive or dead) response variable does not detect subtle, nonlethal degradation, which occurs during the early stages of storage. As storage time progresses, mortality rates abruptly increase until most seeds die, yielding a sigmoidal shape for aging time courses (Ellis, 1988; Walters, 1998; Walters, Ballesteros, et al., 2010), and is best handled with logistic regression (Crawley, 2012). Genebank standards recommend testing freezer-stored samples at intervals of 10 to 20 years, and more frequently for samples stored at warmer temperatures (FAO, 2014). Rather than this generic ‘rule,’ monitoring frequencies should be optimized based on the longevity of the sample, which corresponds to the threshold separating oblique and steep rates of mortality. Too frequent or infrequent monitoring will result in significant loss of valuable seeds, either by consuming them in tests that affirm compliance to the 85% viability standard, or by inadvertently overshooting the longevity threshold, the region in which viability change is significant, and entering the aging phase when seeds die rapidly. Ideally, early seed testing might reveal proximity to the longevity threshold. However, the statistical power of viability tests, based on the binomial distribution (ISTA, 2021), limits detection of subtle changes to proportion data.

Unlike the sigmoidal decline of seed viability proportion data, RNA integrity functions as a continuous response variable that decreases linearly with storage time (Fleming, Hill, et al., 2019; Fleming, Richards, et al., 2017; Walters, Fleming, et al., 2020). This presents RNA integrity as a potential marker for biological age of seeds, but not necessarily a measure of seed viability, which is a discrete response variable (Fleming, Hill, et al., 2019; Puchta, Boczkowska, et al., 2020;

Core Ideas

- We calculate the statistical power of RNA integrity assays (RIN) for monitoring seed aging during storage.
- RIN detects changes in stored seeds earlier and with fewer seeds compared with traditional germination assays.
- Monitoring RIN presents a method to predict seed longevity with increased efficiency in seed storage operations.

Saighani, Kondo, et al., 2021; Sano, Rajjou, et al., 2020; Zhao, Wang, et al., 2020). Current research to use RNA integrity as a marker of seed aging seeks to correlate decline rates of RNA integrity with longevity across multiple species or genetic lines within a species (Saighani, Kondo, et al., 2021). To implement this potential assay as a standard in genebank operations requires it to have greater sensitivity than viability tests. In other words, the separation (i.e., minimum detectable difference) of RNA integrity measurements and sample size (i.e., number of seeds) needed to distinguish the measurements must be smaller than what is currently available with viability assays, the current gold standard.

This paper focuses on the sensitivity of RNA integrity assays in comparison to viability assessments, with the goal of predicting when stored seed samples are on the verge of rapid mortality. We used cohorts of ‘Williams 82’ soybean seeds that were harvested between 1989 and 2019, stored at 5 °C and monitored for viability in 1- to 3-yr intervals, to model the average longevity of this cultivar under these storage conditions (Fleming, Richards, et al., 2017). ‘Williams 82’ soybean seeds are used as a model to demonstrate the practical use of RIN and how it can be applied to other species as an assay for seed health. Characterization of RNA integrity, using the RNA integrity number (RIN) (Schroeder et al., 2006), began in 2016 and was repeated in about 1- to 2-yr intervals. We assessed the sensitivity of RIN measurements by calculating the experimental error, which was then used to define the optimal sample size (i.e., number of biological replicates = number of seeds) and the minimum detectable difference needed in RIN to avoid a Type I or Type II error (Zar, 1999).

2 | MATERIALS AND METHODS

2.1 | Plant material

Soybean seeds (*Glycine max* cultivar ‘Williams 82’) were obtained 4 to 5 mo after harvest from U.S. Midwest suppliers

between 1989 and 2019 and stored at 5 °C and ~35% relative humidity (Fleming, Richards, et al., 2017). The cohort is identified by an 'H' following the harvest year; for example, 1999H represents the soybean cohort harvested in 1999. Seeds are stored in bulk, and the entire sample was subjected to brief warming cycles to room temperature when removed from the refrigerator for monitoring tests; this could impact aging rate. RNA tests began in 2016 and RNA was extracted within a few months of germination assays. There were little to no detected changes in germination between monitoring intervals, therefore the impact of a few months difference between RIN and germination measurements will be negligible.

2.2 | Germination assays

Initial viability assessments for 'Williams 82' soybean seeds were conducted upon arrival, germination was subsequently monitored in 1- to 3-yr intervals. Tests used different numbers of seeds, depending on goals for detecting inviable seeds among healthy samples and viable seeds among severely aged samples. For this study, the data from monitoring assays in 2013, 2017, and 2021 were used to represent viability based 4 yr apart. Assays conducted in 2017 (1992H, 1993H, 1994H, 1996H, 1999H, and 2015H) and 2021 (all cohorts) used between 125–150 seeds. Germination tests for other cohorts at different monitoring times used 30 to 50 seeds.

Dry seeds were removed from refrigerated storage and pre-hydrated for 10–16 h at room temperature in a sealed plastic box above wet paper towels. Seeds were rolled in moist germination paper (Anchor) (25 to 35 seeds roll⁻¹) and incubated at 25 °C (Percival Scientific). After 4 d, seeds with well-developed roots were counted and after 7 d the remaining seeds were scored as "alive" if radicles extended more than 2 mm.

2.3 | RNA extraction and integrity measurements

RNA integrity was quantified using RIN for a subset of cohorts at any sampling time. Some data collected in 2016 and in 2018–2019 were previously published (Fleming et al., 2017; Walters et al., 2020), and are represented here to show time-dependent changes. This paper presents original data from 2017 RIN measurements of a subset of cohorts (1992H, 1993H, 1994H, 1996H, 1999H, and 2015H) and 2021 assessments of the entire collection as well as several retests of RNA extracted in 2016 and stored at –80 °C, a procedure used to estimate variation resulting from staffing and equipment. All RIN data collected on 'Williams 82' soybean seeds since

2016 are included in this study. In summary, RIN measurements from RNA extracted between 2016 and 2021 provide a 5-yr span over which to evaluate declining RNA integrity in soybean seeds stored at 5 °C.

In most cases, RNA was extracted from 4 to 20 seeds per harvest year (*n* values provided in Table 1). Average RIN values and standard deviation were calculated for each cohort at each monitoring time.

A whole seed, a cotyledon segment, or an embryonic axis was used for each RNA extraction: tissue from multiple seeds were never pooled. In soybean, embryonic axes and cotyledons have similar RIN values and kinetics of RNA degradation (Fleming, Richards, et al., 2017). Between 8 and 15 mg of tissue was ground in microcentrifuge tubes with a nickel/lead steel shot bead (Ballistic Products), in the presence of liquid nitrogen and 1–2 mg polyvinylpyrrolidone-40 (PVP-40; Fisher Scientific), using the TissueLyser II (Qiagen). After grinding, RNA was extracted from each sample, using the Qiagen Plant RNeasy kit (Qiagen), following the manufacturer instructions with the modification of a repeated final wash with 500 µL of RPE buffer to minimize guanidine hydrochloride carry-over. The RNA yield was quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The RNA extractions conducted in 2016 used extraction methods described previously (Fleming et al., 2017) or described above, and there was no statistical difference in RIN between the two methods for the same cohorts (data not shown). Samples were diluted to 2 ng µL⁻¹ in nuclease-free water.

The RIN of diluted RNA samples was quantified on an Agilent Bioanalyzer, using Agilent RNA 6000 Pico chips and the Plant RNA Pico assay (Agilent 2100 Expert software version B.0208.SI648 R3), following the manufacturer's protocols. Briefly, fragment sizes of the 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.

2.4 | Statistical analysis

Germination data from monitoring assays of soybean cohorts conducted in 2013, 2017, and 2021 were fit to Avrami time-courses to model the rate of viability loss (Walters, Wheeler, et al., 2005). The Avrami function (Avrami, 1941) is a classic model of cooperative kinetics providing the familiar sigmoidal form typically observed for seed deterioration time courses. We regressed $\ln(\text{time } t)$ with $\ln[\ln(\text{max germ } \alpha \text{ germination at time } t)]$, constraining maximum and minimum germination between 0.995 and 0.01, using Excel (Microsoft) 'linest' functions, which calculate the statistics for a line by using the least squares method. Storage durations required

TABLE 1 Average RNA integrity numbers (RIN) measured for cohorts of ‘Williams 82’ soybean seeds stored at 5 °C and were sampled progressively in 2016, 2017, 2018–2019, and 2021

Harvest year	Extraction year											
	2016			2017			2018–2019			2021		
	RIN	SD	<i>n</i>	RIN	SD	<i>n</i>	RIN	SD	<i>n</i>	RIN	SD	<i>n</i>
1989	5.34	1.32	17	5.14	0.18	5	4.32	0.69	8	3.64	1.49	5
1991										2.36	0.71	5
1992	7.47	0.06	3	6.30	0.46	60	5.80	0.05	2	5.79	0.27	8
1993	7.00	0.10	5	7.09	0.26	17				6.74	0.57	5
1994	6.35	0.21	2	6.17	0.28	18				5.75	0.70	5
1995	6.82	0.48	13	6.41	0.29	10	5.51	0.39	10	5.98	0.47	13
1996	5.58	0.53	4	5.78	0.58	16	5.19	0.34	3	5.03	0.30	9
1999	7.49	1.14	21	6.98	0.22	43				6.83	0.23	5
2008	6.87	1.40	10							5.93	0.39	11
2009										6.48	0.35	8
2010										6.14	0.34	4
2011	7.29	0.95	9							6.79	0.32	5
2014	7.80	0.91	69	7.25	0.37	4	5.85	0.28	10	6.32	0.21	5
2015	7.38	0.42	4	7.43	0.27	22	5.77	0.41	4	7.03	0.40	5
2016				6.50	0.04	3	6.16	0.10	5	6.52	0.21	5
2017							5.93	0.39	11	7.58	0.56	5
2018							6.47	0.35	8	7.29	0.35	10
2019										6.97	0.58	10

Note. RIN values represent least square means (lsmean), SD, and *n* (number of seeds used). Averages within each sampling time are used to calculate regression coefficients to estimate rate of RIN decline among all cohorts (Figure 2).

to reach different germination proportions (e.g., 0.85 [P85], 0.70 [P70], and 0.55 [P55]) were calculated separately for the 3 monitor years and then averaged to develop a relationship between viability (i.e., decline in germination) and storage time across a 25-to-30-yr period.

To calculate the rate of RIN decline, storage time was considered in two ways: (a) as the difference between harvest and monitoring years among all cohorts (e.g., 22 years for the 1999H cohort sampled in 2021), or (b) as the difference between the first and last monitoring dates for each cohort for which two sampling dates occurred (e.g., ~ 5 yr for the 2015H cohort sampled in 2016 and again in 2021). Linear regressions of storage time and RIN values were calculated using Excel ‘linest’ functions. We have previously shown the effect of storage time on RIN best fit a linear model (Fleming, Hill, et al., 2019; Fleming, Richards, et al., 2017) and thus have used a linear model to represent RIN versus storage time. Significance of regression lines and comparisons of slopes were tested using the ANOVA: single factor analysis in Excel at the 0.05 level.

Statistical power is the probability that the test correctly rejects the null hypothesis, that is, the probability of a true positive result. We chose 95% power (0.95) in the power analysis to estimate the necessary sample size to avoid a Type I or Type II error. Statistical power for germination proportion

data was calculated using R package ‘pwr’ using the pwr.p.test for binomial distribution data. Power curves to detect reductions in RIN were generated with G*Power (Faul, Erdfelder, et al., 2007) using the following parameters—test family: *t* tests; means: difference between two independent means (two groups); type of power analysis: a priori, compute required sample size—given α (0.05), power (0.95), and effect size (determined with sample means provided). In power analyses, viability values for freshly harvested samples were approximated as 1.0 and 7.0 for germination proportion and RIN, respectively. The term *detectable difference* describes deviation of mean values from initial values, and we modeled statistical power for deviations of 0.10, 0.15, and 0.30 for viability (i.e., germination proportions of 0.90, 0.85, and 0.70, respectively) and 0.20 for RIN (i.e., RIN changes to 6.8 to 6.0 in 0.2-unit intervals).

3 | RESULTS

3.1 | Characterizing aging rate using decline of germination or RIN

Cohorts of ‘Williams 82’ soybean seeds, harvested between 1989 and 2019, had high initial quality, with nearly all

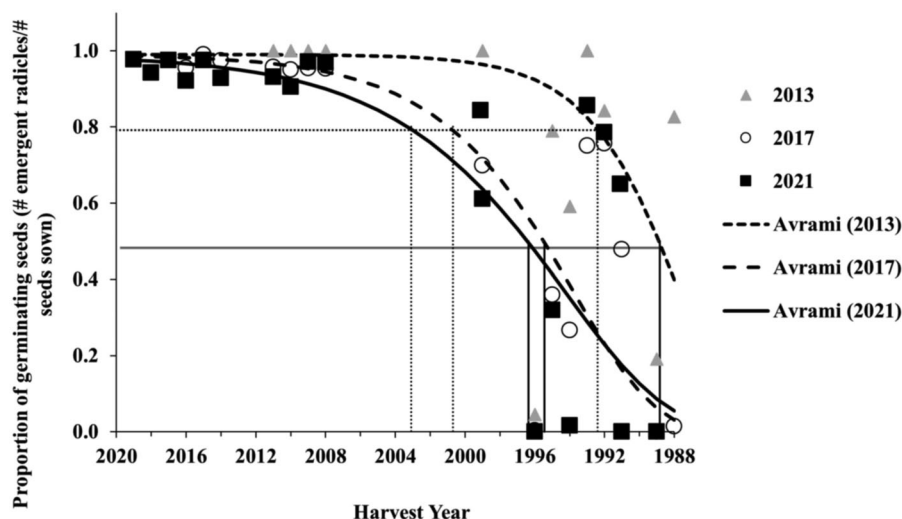


FIGURE 1 Changes in the germination of soybean seeds ('Williams 82') over time. Seeds were harvested between 1989 and 2019, stored at 5 °C and ~35% relative humidity and monitored regularly. Data for germination tests conducted in 2013 (gray, triangles), 2017 (open, circles) and 2021 (black, squares) are presented and are representative of data collected in other years which were published previously (Fleming et al., 2017; Walters et al., 2020). Two points are given for 2021 germination data from cohorts harvested in 1999 and 1991 because seeds were sampled from two separate containers; the higher value was from the container that had not been opened previously and so did not experience temperature cycling from frequent monitor-testing. Sigmoidal curves represent Avrami models fitted to data from each testing year. Each curve for the different monitoring years extend to 2020, to demonstrate the deterioration time course over a 32-yr period calculated using data from the three monitor tests. From the Avrami models the time that viability decreases to 0.8 (P80) and 0.5 (P50) were calculated. Lines from axes to curves represent P80 (dashed) and P50 (solid) and the expected harvest year corresponding to each estimation

seeds germinating within 4 d (data not shown). Germination declined with storage time and when cohorts were tested contemporaneously in 2013, 2017, and 2021, and the germination potential was usually lower in older cohorts (Figure 1). Germination proportion remained ≥ 0.90 for seed lots harvested after 2008 (i.e., cohorts less than 13 yr old in 2021), while it ranged from 0 to 0.90 for seed lots harvested before 2008 (i.e., cohorts >13 yr old in 2021). In the older seed lots, germination proportion usually declined between the repeated 4-yr monitoring intervals (2021 vs. 2013, Figure 1). Exceptions were the 1989 and 1996 seed lots, which were already dead, and the 1993 seed lot, which gave inconsistent results. In contrast, effects of storage time on the younger seed lots were barely detectable except for the 2010 cohort declining within 8 yr (Figure 1).

Coefficients derived from the Avrami function fitted to germination data (curves in Figure 1) were used to estimate when germination potential could be expected to decline. Germination potential decline is presumed to be when germination proportion declines to 0.85 (i.e., P85). The harvest year close to P85 was 1993, 2002, and 2005 for the 2013, 2017, and 2021 monitoring assays, respectively (Figure 1) (i.e., P85 $\approx$ 19.6, 14.8, and 16.1 yr, depending on the year of assay, which averages to 16.9 yr). Given this information, a prediction of decline in germination proportion of 0.15 would occur within 16.9 yr for soybean under dry storage.

RNA integrity of total extracted RNA was assessed repeatedly for some or all cohorts between 2016 and 2021, and averages ranged from 7.8 ± 0.91 (SD, $n = 69$) for the 2014 cohort measured in 2016 to 2.4 ± 0.71 (SD, $n = 5$) for the 1991 cohort measured in 2021 (Figure 2; Table 1). The slopes of the regressions between average RIN values and harvest year were $0.048 \text{ RIN yr}^{-1}$ (2016 assays, $n = 11$, $r^2 = 0.34$, $p = .041$), $0.042 \text{ RIN yr}^{-1}$ (2017 assays, $n = 10$, $r^2 = 0.68$, $p = .037$), $0.039 \text{ RIN yr}^{-1}$ (2018–2019 assays, $n = 9$, $r^2 = 0.68$, $p = .008$), and $0.082 \text{ RIN yr}^{-1}$ (2021 assays, $n = 18$, $r^2 = 0.84$, $p = 0.0002$). Slopes for different assay times were not significantly different at the $p = .05$ level (Figure 2). An average slope of $0.053 \text{ RIN yr}^{-1}$ approximated the rate of RIN decline in 'Williams 82' as a function of storage time at 5 °C.

An alternative accounting of storage time quantified RIN decline for cohorts with RIN assays sampled more than two years apart by regressing RIN values with the sampling year (Table 2) (14 of 18 cohorts). Aging rates (regression slopes) ranged from 0.46 RIN yr^{-1} (2014 cohort sampled over a 5.6-yr period [93 seeds]) to $-0.64 \text{ RIN yr}^{-1}$ (2017 cohort sampled over a 2.8-yr period [16 seeds]). Regressions were not significant when <19 seeds were used; aging rates were negative (i.e., an apparent increase in RIN with time) when the time span was < 5 yr (Table 2). The average and median aging rates among cohorts were 0.07 and 0.13 RIN yr^{-1} ,

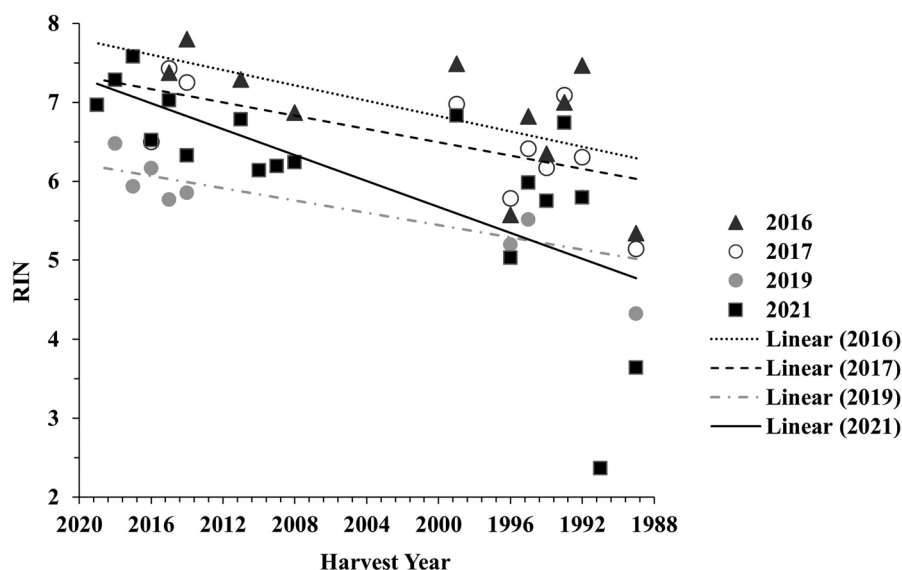


FIGURE 2 Changes in RNA integrity number (RIN) for soybean seed ('Williams 82') over time. Cohorts of 'Williams 82' soybean seeds harvested between 1989 and 2019 and stored at 5 °C and ~35% relative humidity. Sampling occurred in 2016 (black, triangles), 2017 (open, circles), 2018–2019 (grey, circles), and 2021 (black, squares). RIN values represent least square means. Lines represent linear models fit to the data within sampling year

TABLE 2 Regression analysis of RNA integrity numbers (RIN) and extraction date for 'Williams 82' soybean seeds stored at 5 °C and sampled between January 2016 and August 2021. The number of sampling times is indicated in Table 1

Harvest year	Time between first and last RNA extraction yr	Aging rate (slope of regression) RIN yr ⁻¹	r^2	n	p	95% CI	
1989	5.5	0.33	0.243	35	0.000	-0.542	-0.125
1992	5.4	0.17	0.172	73	0.000	-0.254	-0.080
1993	4.7	0.07	0.142	27	0.028	-0.147	0.001
1994	5.4	0.10	0.190	25	0.012	-0.188	-0.011
1995	5.5	0.17	0.288	46	0.000	-0.251	-0.089
1996	5.4	0.15	0.299	32	0.000	-0.235	-0.064
1999	5.5	0.15	0.085	69	0.003	-0.271	-0.030
2008	5.5	0.12	0.087	18	0.249	-0.321	0.085
2011	5.5	0.09	0.095	14	0.318	-0.275	0.088
2014	5.6	0.46	0.388	93	0.000	-0.575	-0.336
2015	5.4	0.14	0.130	35	0.013	-0.264	-0.011
2016	4.2	-0.04	0.096	13	0.345	-0.042	0.122
2017	2.8	-0.64	0.730	16	0.000	0.419	0.868
2018	3.5	-0.23	0.403	13	0.009	0.044	0.408
Average slope	0.074						
SD slope	0.258						
Median slope	0.128						
Count	14						

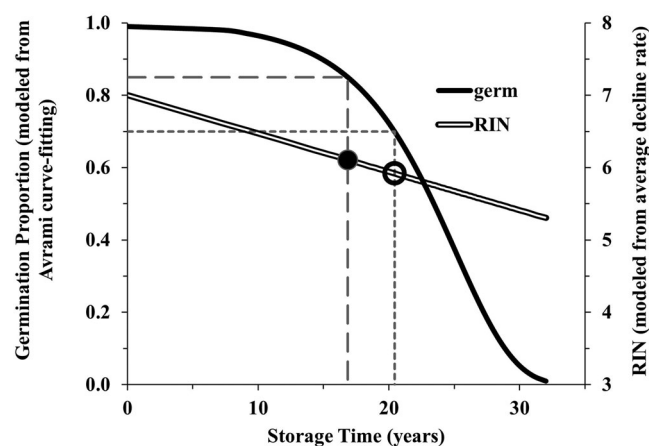


FIGURE 3 Models for viability (solid, black, sigmoidal curve) and RNA integrity number (RIN; double, black, linear line) for ‘Williams 82’ soybean seeds with storage time at 5 °C. The viability model combines all germination data (Figure 1) using storage time, which is calculated by subtracting harvest year from sampling year and fitting them to the Avrami Equation. The RIN model uses the average slope of $-0.053 \text{ RIN yr}^{-1}$ (Figure 2) and intercept of 7. Dashed lines from axes to model curves represent P85 and P70 and the years estimated to observe the decrease in viability

respectively (Table 2), which is consistent with the average slope from linear regressions of $0.053 \text{ RIN yr}^{-1}$ (Figure 2).

A simulated aging time course for ‘Williams 82’ seeds stored at 5 °C describes the general decline in germination proportion from 0.99 to 0 over a 32-year period (Figure 3). The expected decline of RIN over the same period was modeled using a starting RIN of 7 and slope of $-0.053 \text{ RIN yr}^{-1}$ (Figure 3). The overlaid models of aging, measured as either lost viability or declining RIN, allow cross-comparisons of expected change with storage time. For example, a germination decline from 0.99 to 0.85 took 16.9 yr (long dashed lines in Figure 3), and we would expect average RIN values to decrease from 7 to 6.1 in that same time (closed circle in Figure 3). Similarly, a germination decline from 0.99 to 0.70 took 20.4 yr (short dashed lines in Figure 3), during which average RIN declined to 5.9 (open circle in Figure 3). In contrast, a reduction of RIN from 7 to 6.4 took 11 yr, and germination declined from 0.99 to 0.96 in that span of time (Figure 3). The next step in understanding the differences in sensitivity of these two methods of measuring seed aging is to determine the number of seeds required to confidently detect the described changes.

3.2 | Standard error of RIN measurements among replicates within a treatment

Power analyses are used to determine the number of seeds needed to detect a change. These rely on the experimental

error of the response variable, which is known for germination data (a binomial error distribution) and is calculated in this study for RIN data. The numerous RIN measurements (Table 1), made annually or biennially since 2016, allowed us to evaluate the numerous parameters affecting RIN. Treatments included harvest year (up to 18 cohorts) and year of RNA extraction (four sampling times) in which a total of 157 (2016), 198 (2017), 61 (2018–2019), and 116 (2021) RIN measurements were used to calculate standard error (Table 3). The root-mean-square deviation (error variance) ranged from 0.98 (2016) to 0.37 (2017), and an average root-mean-square deviation of 0.56 was used in RIN power analyses for the error variance (Table 3).

3.3 | Relationship between detectable change in viability or RIN and required sample sizes

3.3.1 | Viability proportion

Power analyses for viability proportion data use the binomial error distribution and describe the relationship between total sample size (i.e., the number of seeds in a monitoring assay) needed to detect reduced germination proportions. Both the initial viability and the reduction in viability affect sample sizes needed to detect change, and so data are presented for hypothetical initial viability proportions of 0.99 (Figure 4a), 0.90 (Figure 4b), and 0.80 (Figure 4c). These power analyses demonstrate that detecting small changes in germination proportion require many seeds (AOSA, 2017; ISTA, 2021). For example, detecting a 0.10 decline in germination proportion would require a monitoring assay that used 67, 162, and 242 seeds for accessions having initial germination proportions of 0.99, 0.90, and 0.80, respectively (Figure 4a–c).

Detecting larger differences between initial and monitored germination proportions require fewer seeds. For example, only 37 seeds are needed in the monitor test to detect a 0.15 decline in germination (to < 0.85) in an initially high-quality seed lot in which 99% of the seeds germinated (Figure 4a). Detecting the same reduction in germination (by 0.15) would require 80 (Figure 4b) and 114 seeds (Figure 4c), respectively, in lower quality seed lots that exhibited initial germination proportions of 0.90 and 0.80. An allowable viability reduction by 0.30 would further reduce the number of seeds required in a germination assay to 15, 25, and 32 for seed lots with starting germination proportions of 0.99, 0.90, and 0.80, respectively (Figure 4a–c). Recall from the viability deterioration time course (Figure 3) that viability reductions of 0.1, 0.15, and 0.3 will take 14.9, 16.9, and 20.4 yr, and corresponding RIN values are expected to be about 6.2, 6.1, and 5.9 (Table 4; block labeled “viability proportion”).

TABLE 3 Analysis of variance for RNA integrity number (RIN) measurements performed in 2016, 2017, 2018–2019, and 2021 for cohorts of ‘Williams 82’ soybean seeds harvested between 1989 and 2019

RNA extraction year	RMSE	Source	df	Sum of squares	Mean square	F ratio	Prob > F
2016	0.98	Harvest year	10	100.66	10.07	10.58	<0.0001
		Error	146	138.97	0.95		
		Total	156	239.63			
2017	0.37	Harvest year	9	57.52	6.39	47.91	<0.0001
		Error	188	25.08	0.13		
		Total	197	82.59			
2018–2019	0.40	Harvest year	6	22.40	3.73	23.12	<0.0001
		Error	54	8.72	0.16		
		Total	60	31.12			
2021	0.49	Harvest year	17	157.78	9.28	37.39	<0.0001
		Error	98	24.33	0.25		
		Total	115	182.10			
Average RMSE	0.56						

Note. RMSE = root mean square error.

3.3.2 | RIN analysis

Power analyses were conducted for the RIN data presented here to establish the required replication (i.e., number of soybean seeds) required to detect a change (Figure 5). Unlike germination data, error for RIN measurements is independent of initial and final values. As a hypothetical starting point, we assumed an initial RIN = 7.0, which is the average RIN of soybean seeds when tested within 2 yr of harvest (Table 1). Using the power analyses, we calculated the number of seeds needed to detect reduction of RIN values to 6.8, 6.6, 6.4, 6.2, and 6.0 as 184, 47, 22, 13, and 9 seeds, respectively (change from initial [i.e., effect size] = 0.2, 0.4, 0.6, 0.8, and 1.0) (Figure 5, Table 4; block labeled “RIN decrease”). Assuming a RIN decline rate of 0.053 RIN yr⁻¹, these changes can be observed in 3.8-yr intervals (0.2 ÷ 0.053 RIN yr⁻¹) and correspond to viability proportions of 0.99, 0.98, 0.96, 0.90, and 0.78, respectively (Figure 3). In contrast to the number of seeds needed to detect changes in RIN, the number of seeds required to detect changes in viability are ∞ (0.99), 1,866 (0.98), 595 (0.96), 66 (0.90), and -22 (0.78) (Figure 4a, Table 4; block labeled “RIN decrease”).

To summarize, a reduction of viability from 0.99 to 0.70 will take about 20 yr and lead to a predicted reduction in RIN from 7.0 to 5.9 together this would require 15 (viability assay) or <9 (RIN assay) seeds in the monitor test (Table 4; block labeled “viability proportion”). In contrast, a reduction of RIN from 7.0 to 6.4 will take about 11 yr, lead to a reduction in viability from 0.99 to 0.96, and require 595 (viability assay) or 22 (RIN assay) seeds in the monitor test (Table 4; block labeled “RIN decrease”).

3.4 | Other sensitivity metrics that distinguish viability and RIN assays

The above comparisons of storage time, aging effects measured as reduced viability or RIN and number of seeds required to detect the aging effects indicate that RIN assays detect change earlier with fewer seeds. However, as storage time progresses and seeds enter the phase of rapid mortality, the statistical power of viability and RIN assays become comparable.

Genebanks conduct monitoring at prescribed intervals using a set number of seeds in the assay. With this approach, the questions become what can be detected using different monitoring intervals and sample sizes. In our hands, ‘Williams82’ soybean seeds stored at 5 °C are anticipated to fall below the 85% viability standard after about 17 yr of storage (Figure 3). We selected monitoring intervals of 5, 8, and 10 yr to examine how monitoring could detect change before (and after) the average sample declined below the 85% viability standard. Viability is expected to be about 0.99, 0.96, 0.90, and 0.73 when monitored in four 5-yr cycles, and RIN values are expected to decline from 7.0 to near 6.7, 6.5, 6.2, and 6.0 (Table 4; block labeled “monitoring interval”). Detecting change in viability would require ∞, 595, 66, and 17 seeds at progressive cycles; monitoring change using RIN in the same 5-yr cycles would require about ~100, ~30, 13, and <9 seeds to reliably detect change. In an 8-yr monitoring plan, viability is expected to be 0.98, 0.87, and 0.45 for the first, second, and third cycles, and RIN values should be near 6.6, 6.1, and 5.7, respectively (Table 4; block labeled “monitoring interval”). Detecting the viability change in progressive 8-year

TABLE 4 Interactions between storage time, viability, and RNA integrity numbers (RIN) calculated from composite aging time courses (Figure 3)

Genebank benchmarks	Aging indicator		No. of seeds needed to detect change at $p = 95\%$		
	Viability proportion	RIN	Viability	RIN	Elapsed storage time
					yr
Viability proportion	0.85	6.1	37	~11	16.9
	0.70	5.9	15	<9	20.4
	0.55	5.8	<15	<9	22.8
RIN decrease	0.99	6.8	∞	184	5
	0.98	6.6	1,866	47	7.3
	0.96	6.4	595	22	10.5
	0.90	6.2	66	13	15.0
	0.78	6.0	~22	9	18.8
Monitoring interval	0.99	6.7	∞	~100	5
	0.96	6.5	595×2	~30	5×2^a
	0.90	6.2	66×3	13	5×3^a
	0.73	5.9	~17 $\times$ 4	<9	5×4^a
	0.98	6.6	1,866	~40	8
	0.87	6.1	~50 $\times$ 2	~10	8×2^a
	0.45	5.7	<15 $\times$ 3	<9	8×3^a
	0.96	6.5	595	~30	10
	0.73	5.9	~17 $\times$ 2	<9	10×2^a
Sample size (i.e., number of seeds)	~0.89	6.2	60	13	15.3
	~0.84	6.1	40	~10	17.2
	~0.76	6.0	20	9	19.2
	0.99	~6.7	∞	60	5
	0.98	~6.6	1,866	40	7.3
	0.93	~6.3	~120	20	13

Note. The required number of seeds to detect the modeled change at 95% probability is calculated from power analyses for viability (Figure 4a) and RIN (Figure 5) assuming a starting viability = 0.99 and RIN = 7. Calculations are given for different genebanking benchmarks relating to viability, RIN, monitoring intervals and seed numbers (values in bold).

^aRepeated monitoring in 5-, 8- and 10-yr intervals.

monitoring intervals would require 1866, ~50 and about 15 seeds; detecting change in RIN would require about 47, ~10, and ~8 seeds. Monitoring in 10-year cycles would yield similar results as the 2nd and 4th cycles of the 5-yr monitoring interval.

Genebanks might also limit the number of seeds used in a test, and so we calculated the amount of change that could be detected and when that change occurred for various sample sizes. Detection of viability reductions from 0.99 to about 0.89, 0.84, and 0.76 is possible using 60, 40, and 20 seeds, and these changes are anticipated in approximately 15, 17, and 19 years (Table 4; block labeled “sample size”). In contrast, 60, 40, and 20 seeds could detect RIN reductions from 7.0 to 6.7, 6.6 and 6.5 which are modeled to occur in about 5, 7, and 9 years.

3.5 | Differences among seed lots

The seed lots in this study did not age at the same rate. In the 2021 assay, cohorts harvested in 1992, 1993, and 1999 had exceptionally high viability, whereas cohorts harvested in 1989, 1991, 1994, and 1996 appeared to die prematurely (Figure 1). The RIN values partially follow a similar pattern in that cohorts from 1992, 1993, and 1999 harvests also had higher than expected RIN values and cohorts from 1989, 1991, and 1996 had lower than expected RIN values (Figure 2). The RIN values for the 1994 harvested cohort were relatively high and do not follow the same pattern. Going forward, the change in RIN with time (slopes in Table 2) may be a more sensitive indicator of aging rates. However, only a 0.2 to 0.3 difference in RIN is expected in the 5 yrs since utilizing

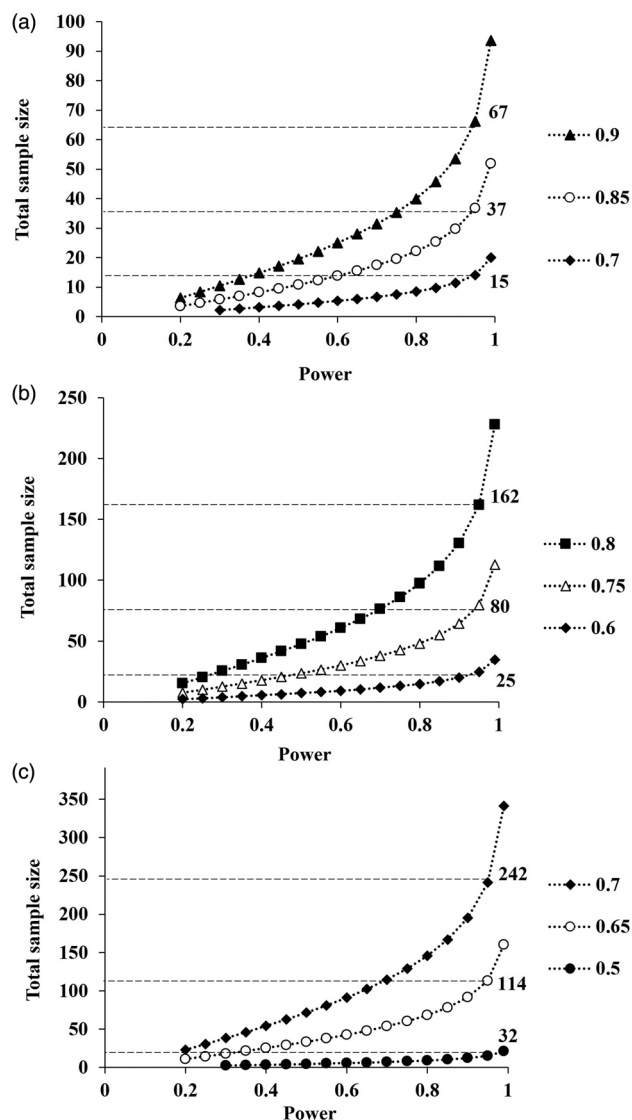


FIGURE 4 Simulated power curves for germination data showing sample size (i.e., number of seeds) needed to detect a decrease of 0.1, 0.15, and 0.3. Initial proportions are (a) 0.99, (b) 0.90, and (c) 0.80 germination proportions. Dashed lines and numbers represent the sample size needed for 95% power. Proportion power was calculated for a binomial distribution (arcsine transformation) using R package pwr using the function `pwr.p.test`

the RIN assay, which is below the limits of detection according to the power analyses, especially for the small sample sizes (usually <40 seeds) (Table 2).

4 | DISCUSSION

Germination assays are currently the “gold standard” used to detect aging in seeds during storage. In this paper, we compare the efficacy of germination and RIN assays in terms of detection time and the number of seeds required to register a decline in seed quality. We used a seed collection of ‘Williams

82’ soybean cohorts stored at 5 °C to make these comparisons because aging kinetics have been tracked for about 30 yr and seeds are large enough to provide 1:1 characterizations of seeds consumed in germination and RIN assays. We found that RIN assays detected significant decline in less time using fewer seeds compared with viability assays. For example, RIN assessments detected a decline in seed health after 12 yr in storage using approximately 22 seeds (Figures 3 and 5); while thousands of seeds would be needed to detect a decline in seed health in a similar time interval using viability assays (Figures 3 and 4a). In addition, change in RIN is detectable in 7 yr using a sample of 40 seeds, whereas the same sample size requires nearly 15–17 yr (germination declines from 0.99 to 0.87–0.84) to detect change using viability assessments. These results are independent of the model shape that RIN (linear) and germination (sigmoidal) classically produce (Figure 3).

The data in this paper suggest, but do not link, reduction of RIN with seed longevity. Power analyses show that an 8–12 yr interval is needed to confidently compare RIN measurements using approximately 10 seeds at both initial and final assays. This study uses a 5-yr time frame (2016–2021), which is just below detectable limits. That said, these experiments using dry (35% relative humidity) storage at 5 °C provide a rare insight about deterioration under conditions normally used by genebanks and seed companies; they do not rely on assumptions about how to translate experiments conducted under more humid or warmer conditions, which are more commonly conducted because detection times are considerably shorter (Hay, Valdez, et al., 2018). A major impediment to understanding seed aging under cold, dry conditions is a dearth of experimentation using these conditions, and hopefully a quicker assay will lead to more studies of reaction kinetics under dry conditions (Fu, Ahmed, et al., 2015; Hay & Whitehouse, 2017). In combination with other studies showing high correlation of RIN decline with longevity (Fleming, Hill, et al., 2019; Puchta, Boczkowska, et al., 2020; Saighani, Kondo, et al., 2021; Walters, Fleming, et al., 2020), this study suggests that, unlike viability tests, early monitoring using RIN assays may be a powerful tool to reliably predict seed longevity during dry storage.

The kinetics of reactions causing RNA to fragment, which we detected using RIN, appear to be related to the kinetics of reactions that eventually cause seed mortality. This does not necessarily imply a direct cause–effect relationship between RNA integrity and seed capacity to germinate. Like changes observed within other classes of molecules, such as volatile emission (Han, Fernandez, et al., 2021; Mira, Hill, et al., 2016) and thermal behavior of lipids (Mira, Nadarajan, et al., 2019), the kinetics of RIN decline can be viewed in the context of general molecular mobility within dry systems (Fleming, Hill, et al., 2019; Walters, Fleming, et al., 2020; Zhao, Wang, et al., 2020). The linear relationship of

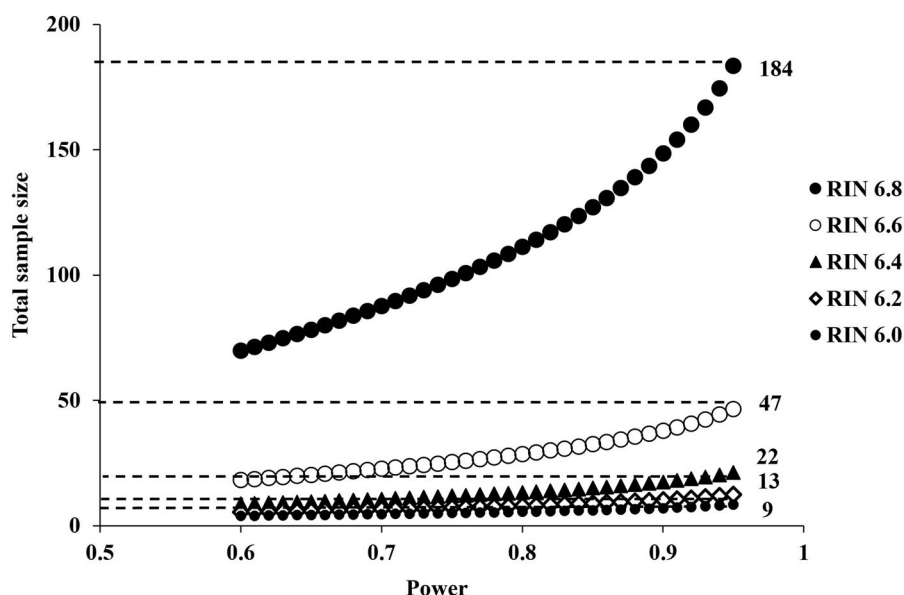


FIGURE 5 Simulated power curves for RNA integrity number (RIN) data showing sample size needed to detect a decrease of 0.2, 0.4, 0.6, 0.8 and 1.0. Dashed lines and numbers indicate the sample size needed to detect a difference with 95% power for a monitoring test. Power curves were generated using G*Power (Faul et al., 2007)

RIN with time (Figure 2) is intriguing because it suggests constant assaults on biochemical integrity during storage. Yet, seeds tolerate these changes to a point, and then die almost synchronously during the phase of rapid mortality (Figure 1). Does this suggest that seeds tolerate accumulation of small lesions to a particular threshold or that attack of a specific essential biomolecule is a one-in-a-million chance? These questions are highly related in a mathematical context in which RIN assessments can serve as a biological clock.

For genebank management purposes, the linear decline of RIN means that strategic spacing of RIN assays during initial storage can reveal seeds approaching the threshold when rapid death will occur while also not consuming large numbers of seeds (Table 4). The number of seeds consumed in RIN assays highly depends on the seed size, because sample mass directly impacts RNA yield. Viability assays alone consume many seeds and do not guarantee that a sample is flagged for regeneration before it decays past the 85% standard (FAO, 2014). We present scenarios in which the sample is viability-tested $3 \times$ (5-yr interval), $2 \times$ (8-yr interval), and $1 \times$ (10-yr interval) before the viability declines rapidly and demonstrate the facility of over-shooting the longevity threshold because of the steep slope when seeds start dying rapidly (Table 4). The slope, representing the synchrony of mortality in a seed lot, is an unexplored component of the seed aging kinetic (Walters, 1998). Variation in aging rate among seed lots is apparent, even among cohorts of the same genetic line (Figure 1) (Ellis, 2019; Hay, Davies, et al., 2022; Nagel, Vogel, et al., 2009; Walters, Fleming, et al., 2020). Seed longevity is frequently characterized at the species level (Probert, Daws, et al., 2009;

Walters, Wheeler, et al., 2005), but the unexplained variation within species is a critical feature of aging and the reason that monitor-testing is mandated at genebanks. Incorporating RIN assays for genebank management purposes has the potential to conserve valuable seed while the initial costs and technical infrastructure to implement such assays need to be considered.

The accessions stored in genebanks are highly valuable and depleting samples by viability testing or lost viability is a major problem. RNA integrity measurements detect changes in dry-stored seeds sooner than viability tests and require fewer replicates (i.e., seeds). The efficacy of RNA integrity measurements for early characterization of aging rates provide insights about regulation of reactions that occur in dry seeds that may contribute to seed deaths during storage.

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AUTHOR CONTRIBUTIONS

Hannah Tetreault: Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing. Margaret Fleming: Conceptualization; Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing. Lisa Hill:

Conceptualization; Data curation; Methodology; Writing – review & editing. Emma Dorr: Data curation; Formal analysis; Methodology; Writing – review & editing. Kathleen Yeater: Conceptualization; Formal analysis; Writing – review & editing. Christopher Richards: Conceptualization; Formal analysis; Methodology; Writing – original draft, Writing – review & editing. Christina Walters: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Writing – original draft; Writing – review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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