

Assessing the RNA integrity in dry seeds collected from diverse endangered species native to the USA

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In Review

Scope Statement

This study tests the feasibility of using RNA integrity to detect aging of seeds from wild species collected across different habitats in the United States. The RNA integrity assay is a promising tool to detect aging of seeds from cultivated species and can be used as an extra metric in genebanks during viability monitoring. Unlike seeds from native species, seeds from cultivated species tend to be large, uniform, filled and have water-permeable seed coverings. We compared the quality of extracted RNA from seeds of wild species and tested the effects of seed size, coverings, fill (empty or no embryo), maturity on RNA quantity and quality. RNA was compared from seeds that were recently harvested to those of the same species that have been genebanked for 16 to 41 years. RIN (RNA Integrity Number) values were usually high across all samples and variables, attesting to both the quality of newly collected material as well as its maintenance under genebanking conditions. However, stored sa

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Credit Author Statement

Christina Walters: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. **Hannah M Tetreault:** Conceptualization, Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. **Joyce Maschinski:** Conceptualization, Writing – review & editing. **Katherine D Heineman:** Conceptualization, Funding acquisition, Writing – review & editing. **Lisa Hill:** Conceptualization, Methodology, Writing – review & editing. **Shaimaa Ibrahim:** Data curation, Writing – review & editing. **Zoe Zingerman:** Conceptualization, Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing.

Keywords

RNA integrity, Seed quality, Seed aging, Native seeds, Genebank, Wild plant populations, viability

Abstract

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Dry seeds do not show obvious signs of life and so testing for viability, health and life expectancy can be challenging. Usually testing seed quality involves adding water and measuring metabolic capacity or growth potential by vital staining or germination assays. Importantly, most laboratory seed tests are intended to assay immediate viability, while most genebanks need tests that predict seed performance in the distant future. All currently available assays require considerable a priori knowledge of germination conditions and seeds large enough to dissect. Germination conditions are often unknown for seeds produced from wild species and are an important criterion for seed testing. We aimed to test the feasibility of adapting a new seed quality assay that measures RNA integrity and appears promising for cultivated species, to seeds from wild species. Most of the 100 wild species we included are rare or endangered and in need of preservation through genebanking, thus tests with long-term predictive value are needed. To determine the feasibility of measuring RNA integrity in seeds from wild populations, we compared the quality of RNA extracted from seeds that were recently harvested to those of the same species that have been genebanked for 16 to 41 years. Using standardized methods (i.e., commercially available kits), we demonstrate reliable characterization of RNA quality across a diverse group of plants, despite variation in germination requirements, seed morphology or composition. RIN (RNA Integrity Number) values were usually high across all samples and variables, attesting to both the quality of newly collected material as well as its maintenance under genebanking conditions. This study conclusively demonstrates the feasibility of reliably extracting and characterizing RNA from dry seeds collected from wild populations, regardless of a variety of seed traits and morphologies. Relationships between RNA quality and seed age and viability require further exploration.

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In review

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15 viability

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20

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25 Importantly, most laboratory seed tests are intended to assay immediate viability, while most
26 genebanks need tests that predict seed performance in the distant future. All currently available
27 assays require considerable *a priori* knowledge of germination conditions and seeds large
28 enough to dissect. Germination conditions are often unknown for seeds produced from wild
29 species and are an important criterion for seed testing. We aimed to test the feasibility of
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31 cultivated species, to seeds from wild species. Most of the 100 wild species we included are rare
32 or endangered and in need of preservation through genebanking, thus tests with long-term
33 predictive value are needed. To determine the feasibility of measuring RNA integrity in seeds
34 from wild populations, we compared the quality of RNA extracted from seeds that were recently
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37 characterization of RNA quality across a diverse group of plants, despite variation in germination
38 requirements, seed morphology or composition. RIN (RNA Integrity Number) values were
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40 material as well as its maintenance under genebanking conditions. This study conclusively
41 demonstrates the feasibility of reliably extracting and characterizing RNA from dry seeds
42 collected from wild populations, regardless of a variety of seed traits and morphologies.
43 Relationships between RNA quality and seed age and viability require further exploration.

44

45 **Introduction**

46 Germination tests are the “gold standard” to evaluate seed health. Seeds from most
47 domesticated species germinate within a few days after sowing and so laboratory germination
48 tests can quickly and reliably assess viability, normal growth and germination speed (AOSA,
49 2019). Laboratory tests are more labor intensive and less informative for seeds of many wild
50 species, which tend to germinate slowly (weeks to years) and asynchronously, have high
51 interspecific variability (Hamasha and Hensen, 2009, Zhang et al., 2020) and complex dormancy
52 status (Kildisheva et al., 2019, Pedrini and Dixon, 2020, Baskin and Baskin, 2021). Unlike crop
53 species, many wild plant species display some degree of seed dormancy. Seed dormancy is a
54 natural mechanism that regulates germination through various physical or physiological means
55 imposed by the seed coat or within the embryo (Baskin and Baskin, 2000). Treatments to
56 stimulate germination of seeds from wild species, at species and population-specific levels, are
57 needed to increase testing reliability and increased stand establishment for wild plants used as
58 genetic resources, restoration or evolutionary biology questions (Etterson et al., 2016, Coyne et
59 al., 2020). In many seed testing labs, time allowed to complete a germination assay is too short
60 for wild-collected samples and the assay is prematurely terminated by testing viability of
61 ungerminated seeds using a vital stain, such as tetrazolium that indicates metabolic or respiratory
62 capacity (Miller, 2005, França-Neto and Krzyzanowski, 2019).

63
64 Plant genebanks, commonly referred to as “seed banks,” use seed testing as a standard
65 operation to assess initial quality, develop procedures for recovery and growth, and ensure that
66 germination capacity and genetic integrity of the stored material is maintained (Hay and Probert,
67 2013, FAO, 2018, De Vitis et al., 2020). Despite cold, dry storage conditions used to prolong
68 viability of seeds maintained in genebanks indefinitely, aging is inevitable (Walters et al., 2010,
69 Hay et al., 2022). A genebank needs to know the sample’s ‘expiration date’ so that it can be
70 used or replaced before its utility is compromised. Hence, the design of laboratory tests used by
71 genebanks must accommodate comparisons across time, with sufficient statistical power to
72 detect small change that occurs over decades. Germination assays may not be efficient or
73 effective at detecting subtle changes of a stored sample. Germination assays provide categorical
74 (nominal) dichotomous data (i.e., normal/abnormal or alive/dead), requiring large sample sizes to
75 detect minor increases in mortality (Tetreault et al., 2023). High variability among germination
76 tests of wild-collected seeds confounds comparisons separated by time. Also, repeated testing
77 depletes the sample -- a critical problem when genebanking germplasm from small or remote
78 populations having low fecundity. Moreover, the nature of dichotomous designation of alive or
79 dead does not reveal nonlethal changes that precede mortality. Because they are the culmination
80 of aging, lost germination capacity or mortality are particularly insensitive symptoms of aging.
81 Assays that mark deteriorative progress toward a mortality threshold would better serve
82 genebank needs.

83
84 RNA integrity is an emerging technique that detects degradation in seeds from
85 domesticated species before large scale mortality occurs (Fleming et al., 2017, Fleming et al.,
86 2019, Walters et al., 2020, Zhao et al., 2020, Tetreault et al., 2023). The assay relies on RNA that
87 accumulates during seed maturation and slowly degrades in dry storage because RNAases appear
88 inactive in dry cytoplasm (Spanò et al., 2007). Non-enzymatic oxidation of RNA over time leads
89 to steadily increasing fragmentation that is visible during electrophoresis, especially in the 25S
90 and 18S rRNA fractions (Brocklehurst and Fraser, 1980, Kranner et al., 2011, Fleming et al.,

91 2019, Walters et al., 2020). Damage to DNA structure occurs as well; however DNA is a more
92 stable molecule than RNA and does not appear to fragment until long after death (Walters et al.,
93 2006). The extent of RNA fragmentation can be quantified by RIN (RNA Integrity Number),
94 which considers the relative size of both rRNA peaks and other features from the
95 electropherogram. RIN is now an industry standard to characterize RNA quality because it is
96 reliable, reproducible and easily standardized; the formula calculating RIN is proprietary
97 (Mueller et al., 2004, Schroeder et al., 2006). In dry seeds, RIN appears to function like a clock
98 that ticks at different rates depending on how fast seeds age. Hence, RIN declines linearly with
99 time in contrast to the time courses for viability loss which are sigmoidal (Fleming et al., 2019,
100 Walters et al., 2020, Tetreault et al., 2023). Before now, application of RIN to seed aging has
101 only been reported using domesticated seeds that were included in legacy collections with highly
102 controlled provenance.

103
104 Using RIN to detect seed aging offers advantages of a standardized procedure that detects
105 early stages of degradation that is independent of seed dormancy. Adapting RIN assessments for
106 use in genebanks will require general knowledge of the applicability of these assays to diverse
107 species. Importantly, RIN decline over time should reflect aging rate or longevity, with kinetics
108 relatable to sample expiration dates. This is an unusual application of RIN, which is normally
109 used to confirm that an extraction yields RNA of sufficient quality for sequencing; a low RIN is
110 mostly interpreted as a flawed extraction. In this proposed new application, observed differences
111 of RIN in extractions of seeds separated by storage time could either reflect artifacts created
112 during extraction or electrophoresis. Alternatively, low RIN may be the desired signal of seed
113 aging. To determine the risk of artifact, we have aimed to determine the contribution of storage
114 time to RIN assessments in seeds, relative to other factors that contribute to variation of RNA
115 quantity and quality, such as diverse physiologies or morphologies as well as RIN assay
116 uncertainty.

117
118 In this work, we compared RNA quality of paired seed cohorts of over 100 diverse wild
119 plant populations harvested from the same locales after 2021 and before 2005 to allow paired
120 comparisons of RNA extraction and characterization parameters. The seeds were from wild
121 populations, predominantly rare or endangered species, native to the US representing diverse
122 botanical families, geographic origins, seed morphologies, chemical compositions, and
123 physiologies. We evaluated whether 1) RIN was lower in the stored seeds compared to the newly
124 harvested ones; 2) low concentration or purity of extracted RNA contributed to low RIN values
125 or anomalous results; 3) particular seed traits interfered with RNA extraction or characterization;
126 and 4) anomalous results could be resolved by additional tests.

127 128 **Materials and Methods**

129 **Seed Material**

130
131 Seeds from approximately 100 native plant species of conservation interest were identified, and
132 collections were obtained from the same population at two time points, referred to as “recently
133 harvested” (sampled between 2021 and 2024) and “stored” (sampled before 2005, with mean
134 harvest year occurring in 1995 ± 6 yrs) (Table 1). An “accession” refers to a seed sample of a
135 particular species from a particular sampling year (i.e., cohort). The harvest year for a cohort is
136 denoted with an ‘H’ after the year; for example, seeds collected in 2001 will be identified as

137 2001H. Storage conditions mostly reflected international recommendations for genebanking (low
138 RH and -18°C) (FAO, 2018), though some seeds were placed under refrigerated (5°C) or
139 cryogenic (~ -180°C) conditions. Seeds were stored either at botanical gardens within the Center
140 for Plant Conservation (CPC) network or at the National Laboratory for Genetic Resources and
141 Preservation in Fort Collins, CO. Analyses used a bulk sample consisting of seeds combined
142 from several maternal lines.

143

144 **Sample Preparation**

145

146 Samples were prepared to address how variation in seed size, embryo presence (i.e., seed fill),
147 thick or water-impermeable seed coverings (i.e., hard seeds), and seed maturity, type of tissue
148 and lipid content affect RNA quality and RIN values. Between 1 and 66 mg (average of $12.3 \pm$
149 6.2 mg) of seed tissue were used per biological replicate in RNA extractions. The median
150 number of seeds used in an extraction was 13, but as many as 500 seeds were used in a single
151 extraction for the smallest seeds (e.g. *Kalmiopsis fragrans*, 0.012 mg/seed; *Leiophyllum*
152 *buxifolium*, 0.025 mg/seed). All efforts were made to use a clean seed sample that lacked plant
153 debris. The number of biological replicates per accession ranged from 1 to 16 (average of $5.6 \pm$
154 3.2). For accessions with few or tiny seeds we used fewer biological replicates. Because it is
155 possible that RNA yield in a sample could have been affected by non-seed material (e.g., chaff,
156 flower parts), we explored the quantity of RNA yield and subsequent RIN score in seed parts
157 (seed coats, embryos, cotyledons) and in samples cleaned to varying degrees.

158

159 *Seeds lacking embryos:* Seeds harvested from wild populations often have outer coverings that
160 appear normal, but there is no embryo inside. The incidence of “empty” or “unfilled” seeds in
161 this study varied among species and cohorts from 0 to 99%, with an average of 20% (Walters et
162 al., unpublished). We reasoned that seeds with and without embryos may have different RNA
163 properties and that the inability of empty seeds to germinate would confound comparisons of
164 RIN with germination tests. For this reason, empty seeds were excluded from RNA extractions
165 when an accession presented greater than 20% empty seeds and the mass per seed was greater
166 than 0.2 mg. Sorting filled from empty seeds involved the laborious step of dissecting seeds
167 under a microscope, carefully pulling out filled seed. To test if this step affected RNA yield,
168 purity or RIN, we identified a subset of 15 accessions having greater than 35% empty seeds and
169 compared RNA parameters for samples in which the empty seeds were and were not removed
170 prior to RNA extraction.

171

172 *Seeds with thick outer coverings:* Many of the seeds in this study set were covered by thick
173 layers of maternal tissues such as remnant fruit or floral parts, or had thick, probably water-
174 impermeable seed coats (e.g., seeds from Fabaceae). In some cases, seed cleaners at botanical
175 gardens removed maternal tissues from one of the cohorts but not the other. To be consistent
176 between cohort pairs, the same tissues were used in RNA extractions, which sometimes required
177 removing pods or external layers (e.g., *Amorpha herbacea* var. *crenulata* (2003H), *Hymenoxys*
178 *texana* (2005H) and *Osteomeles anthyllidifolia* (2021H and 2000H)). For very large seeds, it
179 made sense to use only the embryo which could be dissected out and used for RNA extraction
180 (e.g., *Arctostaphylos catalinae* (79 mg/seed), *Castela emoryi* (36 mg/seed), *Rhus kearneyi* spp.
181 *kearneyi* (45 mg/seed) and *Ziziphus celata* (511 mg/seed)). Apart from embryo-only samples,
182 between 3 and 85% (average of 40%) of the grain mass of seeds was removed by careful peeling,

183 cutting or scraping under a microscope in seeds that weighed more than 0.3 mg and had
184 apparently thick outer layers. To test if this affected RNA yield, purity or RIN, we identified a
185 subset of 28 accessions having less than 25% empty seeds and compared RNA parameters for
186 samples in which outer coverings were and were not removed prior to RNA extraction.

187
188 *Seed maturity:* Seeds from wild populations typically do not mature synchronously and so there
189 can be a range of maturities within an accession. Most of the accessions in this study appeared to
190 contain seeds that were fully mature with appropriately dark coloring and low incidence of
191 shriveling. However, there were four accessions that presented within-sample variation in
192 coloring reminiscent of slight variation in seed maturity at harvest (Supplemental Figure 1). To
193 test if this apparent difference in seed maturity affected RNA yield, purity or RIN, we compared
194 RNA parameters for replicates prepared using seeds having only dark or light coloring.

195
196 *Tissue type:* To examine if tissue types and relative contribution of tissue mass to seed size
197 impacted RNA yield, purity or RIN, we dissected embryonic and nutritive tissues from *Abies*
198 *fraseri* (both cohorts), *Amaranthus pumilus* (1987H) *Astragalus magdalenae* var. *peirsonii*
199 (2021H), *Lupinus westianus* var. *aridorum* (2021H), and *Rhus kearneyi* ssp. *kearneyi* (both
200 cohorts) and compared RNA parameters among the different tissues.

201
202 *Lipid content:* The amount of lipid in seeds was inferred from the enthalpy of lipid melting
203 transitions, which was measured in a separate study using differential scanning calorimetry
204 (DSC) (Walters et al., unpublished). Briefly, samples were cooled and warmed to and from -
205 150°C at 10°C/min and enthalpy was calculated by the size of melting transition during heating.
206 A melting enthalpy of 60 J/g oil was used to translate melting enthalpy based on sample mass to
207 g lipid per g of seed. To relate RNA yield, purity or RIN to the lipid content of seeds within the
208 dataset we measured melting enthalpy from known seed mass.

209
210

211 **RNA extraction and integrity assays**

212
213 RNA was extracted from seed samples prepared as described above using approximately 10 mg
214 of dry tissue per sample. The sample was prepared by combining the dry tissue and ~1mg of
215 polyvinylpyrrolidone-40 (PVP; Fisher Scientific, Fair Lawn, NJ) in a 2 ml test tube with a
216 nickel/lead steel shot bead (Ballistic, Inc., Hamel, MN), then flash frozen in liquid nitrogen
217 before using the TissueLyser II (Qiagen, Hilden, Germany) to grind tissue to a fine powder. For
218 RNA isolation, the Qiagen Plant RNeasy kit (Qiagen, Hilden, Germany) and Takara Nucleospin
219 RNA kit with Fruit-mate (Takara, Düren, Germany) were used per the manufacturer's
220 instructions. The DeNovix DS-11 FX+ Spectrophotometer (DeNovix, Wilmington, DE) was
221 used to determine RNA yield and purity. A subsample was used immediately for electrophoresis
222 and the remaining sample was archived at -80°C.

223
224 Subsamples used for electrophoresis were diluted to 2 ng μL^{-1} in nuclease-free water and
225 electrophoresed using the Agilent Bioanalyzer (Agilent, Waldbronn, Germany) with Agilent
226 RNA 6000 Pico chips and the Plant RNA Pico assay (Agilent 2100 Expert software version
227 B.0208.SI648 R3) per the manufacturer's protocol. RNA concentration, rRNA ratio and RIN
228 were quantified using Agilent RNA 6000 Pico chips and the Agilent 2100 Expert software which

229 analyzes peak and fragment sizes and calculates RIN using a proprietary formula (Fleming et al.
230 2017; Tetreault et al. 2022).

231
232 The ratios of absorbance at 260 and 280 nm and at 260 and 230 nm were used to assess the
233 purity of extracted RNA. Ratios near 2.0 reflect a successful extraction with relatively pure
234 RNA. A 260/280 ratio near 1.7 indicates higher than desired levels of DNA and absorbance
235 ratios less than 1.7 usually indicate contamination by proteins, polysaccharides or reagents used
236 during extraction.

237
238 **Statistical analysis**
239 Analysis of variance tests were done within species to determine significant difference in RIN
240 when special treatments were applied to each species. Analysis of variance tests were calculated
241 using JMP 12.2.0 (SAS Institute Inc., 2015).

242
243

244 **Results**

245 246 **Factors associated with reliable electrophoresis**

247
248 RNA was extracted from over 200 accessions of wild seeds from diverse species that were harvested
249 recently (103 species) or stored for at least 15 years under genebanking conditions (105 species). More
250 than 1200 extractions were performed and characterized with over 1350 electrophoresis runs (Table 1).
251 On average, 4.8 (\pm 2.0) and 4.9 (\pm 2.7) RNA extractions were performed per species for recently
252 harvested and stored treatments, respectively. A RIN result was deemed reliable if its value was within 2
253 units of the average of 3-4 replicate runs for the accession. Overall, 96% of the RNA extractions
254 provided reliable RIN values on the first or second electrophoresis run (Fig 1A).

255
256 Most (93%) of all RNA extractions provided a reliable RIN value on the first electrophoresis run using
257 the bioanalyzer (Fig 1A). Experimental error was initially apparent in about 7% of the electrophoresis
258 runs or 79 of the 1210 RNA extractions (Fig 1B). Electrophoresis “failed” in a total of 87 of 1365
259 electrophoresis runs, in that either no RIN value was calculated (37 runs) or the calculated RIN was
260 inconsistent with the average determined for the accession (50 runs). Most of the 37 runs in which the
261 Agilent software failed to calculate RIN were due to mis-calling the location of the 25S and 18S rRNA
262 peaks. Thus, about 3% of all electrophoresis runs resulted in no RIN data (Fig 1C). That did not affect
263 overall RIN averages, but did risk further depletion of the accession by requiring another replicate. We re-
264 ran electrophoresis in 26 of the 37 original extractions from samples that had been archived at -80°C and
265 found that the problem was resolved in all but 4 samples. Anomalously low (48 runs) or high (2 runs)
266 RIN values were obtained in 50 of the 87 “failed” electrophoresis runs (or 4% of all runs) and there is a
267 risk that these might be attributed to seed quality rather than an extraction or electrophoresis mishap. We
268 tested the possibility of an error during electrophoresis by re-running 36 of the 50 original extractions
269 from samples that had been archived at -80°C and found that the problem was resolved in all but 4
270 samples. In summary, of the 87 electrophoresis runs initially deemed to provide unreliable or no RIN
271 data, 54 could be resolved by re-running an archived sample, 23 were not re-run, and 8 RIN outcomes
272 reflected a problematic extraction. From this analysis of experimental error, we concluded that, in our
273 hands, about 1% of RIN determinations may falsely indicate a deteriorated sample and about 0.5% of
274 extractions may not provide a RIN measurement. Also, at least 60% of detected errors could be corrected
275 by repeating electrophoresis a second time.

276

277 We examined spectroscopic properties of RNA extractions to determine whether RNA concentration or
278 purity contributed to failed or suspect electrophoresis runs that gave either no or anomalous RIN values.
279 RNA concentration fell within the 25 to 500 ng/μl range recommended by the manufacturer for RNA
280 integrity characterization (Schroeder et al., 2006) for most of the extractions (874 out of 1210), with 129
281 and 206 extractions giving RNA concentrations above or below, respectively, the manufacturer's
282 guidelines (Figure 1A and Table 1). There was about a 3% failure rate (incidence of no or anomalous
283 RIN values) in extracts at or above the recommended RNA concentration and over 60% of these could be
284 resolved by a second electrophoresis run of the same extract. In contrast, the failure rate in extracts
285 containing less than 25 ng/μl was 28% and only about 1/3 could be resolved with a second electrophoresis
286 run. In other words, roughly 3/4 of failed electrophoresis runs in this study can be attributed to RNA
287 concentrations that were too low (Figure 1C). Notably, valid RIN assessments were obtained in extracts
288 despite extremely low RNA concentrations of 0-5 ng/μl range (Fig 1A and 1B). There appeared to be no
289 difference between RNA concentration and failed electrophoresis runs in recently harvested and stored
290 seeds. There was only one extraction in which RNA was not detected by absorbance at 260nm (a stored
291 accession of *Remirea maritima* (Cyperaceae) in which all biological replicates contained less than 10
292 ng/μl RNA despite being cleaned to remove most of the empty seeds).

293
294 There was little indication that RNA purity, indicated by absorbance ratios at 260 and 280nm or 260 and
295 230 nm contributed to the reliability of RIN determinations (Figs 2 and 3). The majority of extracts (1094
296 out of 1210) had A260/280 ratios between 1.7 to 2.2 indicating relatively high purity without protein or
297 polysaccharide/phenolic contaminants (Figure 2A and 2B). About 4% of these failed to give reliable RIN
298 results (Fig 2A) and half of these also had low RNA concentrations (< 30 ng/μl) (not shown). There were
299 116 (of 1210) extractions that gave A260/280 ratios outside the recommended 1.7 to 2.2 range. Of these,
300 80% also had low RNA concentration (< 30 ng/μl) and 32% failed to give reliable RIN results (Fig 2C).
301 In other words, low RNA concentration was also a common factor in over 90% of extracts with low
302 purity based on A260/280 ratios. A secondary marker of RNA purity, the ratio of absorbance at 260 and
303 230nm (e.g., A260/230), indicated the general presence of contaminants in extracts with a relatively broad
304 distribution of samples giving A260/230 between 0 and 3 (Fig 3A and 3B). The highest proportion of
305 failed electrophoresis occurred in samples with A260/230 ratios in the 0 to 1 range (Figure 3C). Over
306 80% of these extracts also had low RNA concentrations (< 30 ng/μl) and about 60% also had A260/280
307 ratios less than 1.7 (not shown). This suggests that contamination may interfere with reliable RIN
308 determinations, but that low RNA concentration is also associated with low purity.

309
310 Peaks representing elution of ribosomal RNA (25S and 18S) dominate electropherograms when RNA is
311 intact because rRNAs are the most abundant, as well as largest, RNAs in the cytoplasm (Leaver, 2018).
312 Hence, rRNA peaks have historically been used to evaluate RNA quality (Schroeder et al., 2006). In high
313 quality RNA, the ratio of electropherogram peak areas for the 25S and 18S rRNAs should be about 2, and
314 this ratio should decline as RNA fragments. In this study set, the rRNA ratio was between 1 and 4 in 1051
315 out of 1210 RNA extractions performed. The rRNA ratio was higher and lower than this range in 41 and
316 145 extractions, respectively (Figure 4). The majority (1003 or 98%) of 1051 extracts with rRNA ratios
317 between 1 and 4 gave reliable RIN determinations (Fig 4A and 4B). Low RNA concentration (< 30 ng/μl)
318 was noted in all failed electrophoresis runs when rRNA ratio ≥ 1 (18 extracts) and more than half of the
319 failed electrophoresis runs when rRNA ratio < 1 (42 extracts). From this assessment, the occurrence of
320 extracted samples having both RNA concentration less than 30 ng/μl and rRNA ratios less than 1 is rare
321 (~5%), but the occurrence of RIN anomalies (71%) is highest in this fraction. In contrast, the fraction of
322 extracted samples having both RNA concentrations greater than 30 ng/μl and rRNA ratios greater than 1
323 is largest (73%), and the occurrence of RIN anomalies within this fraction is minor (0.5%).

324
325 Unlike the spectroscopic parameters measuring RNA concentration and purity (Figs 1-3), accession age
326 (i.e., recently harvested vs. stored) has a significant effect on measured rRNA ratios (Fig 4A; $p = 0.05$).
327 There are more accessions from the stored cohort that have rRNA ratios < 1 compared to counterparts that

328 were recently harvested ($p = 0.0014$). Importantly, the incidence of RIN anomalies occurs at similar rates
329 in the recently harvested vs stored accessions (Fig 4C). However, the nature of the detected errors appears
330 to differ (not shown): in stored samples, RIN anomalies usually present as no-RIN-computed errors,
331 which do not affect average RIN among replicates. In contrast, RIN anomalies in freshly harvested
332 samples usually present as computed, but low, RIN values that are inconsistent with other replicates and
333 lower the average RIN value when included.

334

335 Because the RIN calculation is anchored by the prominence and position of rRNA peaks, we expect a
336 strong correlation between rRNA ratio and RIN in this study set (Fig 5). Correlations for these
337 relationships for non-anomalous RIN values and rRNA ratios < 4 were significant at $P \ll 0.0001$. The
338 coefficients of the linear regressions vary for recently harvested and stored seeds, owing mostly to the
339 greater number of points with rRNA ratio < 1 from stored seeds, as noted in Fig 4. RIN calculations are
340 considered more reliable than rRNA ratios, especially in borderline cases when rRNA ratios < 1 (values
341 for RIN range from 1 to 7) or when rRNA ratio = 0 (a RIN value is sometimes calculatable) (Fig 4B).

342

343

344 **Do seed characteristics affect RNA extraction and characterization?**

345

346 Our analysis of characteristics of RNA extracts that do and do not signify successful electrophoresis
347 (previous section) revealed that low RNA concentration was an important factor. The mass of individual
348 seeds varied considerably in this study (between 0.01 and >400 mg per seed with a median seed mass of
349 0.9 mg per seed). Samples containing few, tiny seeds or focusing on specific tissues with low RNA
350 content are, therefore, at risk for providing unreliable RIN results. Moreover, variation of RNA quality
351 among seed parts may confound assessment of its stability during dry storage. To address these
352 questions, we assessed RNA yield and RIN for seed and seed parts in a cross section of species.

353

354 RNA content within seeds was approximated by dividing RNA yield by the number of seeds used in the
355 extraction. RNA content ranged from 0.1 to >900 ng RNA/seed and averaged ~ 90 ng RNA/seed. RNA
356 yield (RNA concentration (ng/ μ l)/ sample mass (mg)) was significantly higher in seed parts with growing
357 potential (i.e., the embryonic axis) than with tissues that served mostly as food reserves (i.e., nutritive
358 tissues -endosperm or megagametophyte) for half of the species tested for this treatment, *Lupinus*
359 *westianus* ($p = 0.0203$), *Abies fraseri* ($p = 0.0450$), and *Rhus kearneyi* ($p = 0.0310$) (Table 2). RIN was
360 significantly higher in nutritive tissue compared to embryonic tissue for one species, *Amaranthus pumilus*
361 ($p = 0.0245$; Table 2).

362

363 Many of the seeds in the study had thick outer-coverings, likely remnants of degraded maternal tissues.
364 The presence of the thick seed coat reduced RNA yield in 10 of the 28 accessions studied and RIN was
365 lower in 2 of the 28 accessions studied (Table 2). The two accessions that had a lower average RIN when
366 seed coverings remained on the seeds prior to extraction were *Amelanchier nantucketensis* ($p = 0.0344$;
367 70% mass removed) and *Ceanthus cyaneus* ($p = 0.0355$; 3% mass removed).

368

369 Seed filling was tested to determine if extracting RNA from a sample that was cleaned for empty seed
370 versus a sample that bulked filled and empty seed impacted the overall average RIN for a given species.
371 There were 15 species that had greater than 36% empty seed in a sample (Table 2), with an overall
372 average of 56% empty seed. Of 10 samples tested statistically, four had higher RIN in the cleaned rather
373 than the bulked sample (*Abies fraseri* 2023H ($p = 0.0011$), *Bidens torta* 2022H ($p = 0.0036$), *Oxypolis*
374 *canbyi* 2022H ($p = 0.0176$) and *Solidago plumosa* 2003H ($p = 0.0224$)). An additional five samples were
375 not tested statistically, due to low seed numbers, but suggested an impact of empty seeds on RIN. RNA
376 yield but not RIN was lower in 5 of the remaining 6 samples (Table 2). This extra step of cleaning seed
377 had mixed effects on RIN values. For example, cleaned and uncleaned samples of recently harvested
378 seeds of *Abies fraseri* (95% empty) provided average RIN values of 7.9 and 2.9, respectively. While the

379 same procedure in recently harvested seeds of *Dubautia menziesii* (89% empty) presented RIN values of
380 8.2 and 7.6 for cleaned and uncleaned samples, respectively.

381
382 Homogeneity in seed maturity is also difficult to control in seeds collected from wild populations. There
383 were four species with mixtures of brown (mature) and green (immature) seeds in a given sample
384 collection. When RNA was extracted and characterized for the two phenotypes separately, there was no
385 evidence of an effect on RNA yield, but half of these taxa had significant differences in RIN average
386 (Table 2). *Geum geniculatum* 2009H, had a slightly lower average RIN for immature seed than mature
387 seed ($p = 0.0486$) and *Physaria obcordata* 1987H had a higher RIN for immature seeds ($p = 0.0182$.)
388

389 Discussion

390 We examined a laboratory test that detects aging in seeds, particularly seeds from wild populations.
391 Traditional germination tests are widely used by genebanks and seed companies for crop seeds, for which
392 germination behavior is well characterized and amenable for testing. Numerous features of non-
393 domesticated seeds complicate seed testing. For example, germination cues are not known, seeds are
394 difficult to acquire and in low supply, test results are frequently inconsistent due to uncontrollable
395 features of the seed and asynchronous germination. In this study, we demonstrated that assaying RNA
396 integrity is a promising new test of seed quality that is quantitative, standardizable, sensitive to time-
397 dependent changes and adaptable to future automation. We evaluated RNA integrity number (RIN)
398 between pairs of seeds from over 100 wild, endangered species. Seeds from one cohort were harvested
399 recently (2022 ± 1 yr) and seeds from the older cohort were harvested at least 16 years ago (1995 ± 6
400 years) and stored in a genebank (mostly at -18°C , but a few at 5°C and a few cryogenically). We used
401 standardized techniques and commercially available kits to assess RIN from all groups. With few
402 exceptions, RIN values among species in the recently harvested cohort were consistently high, despite
403 high diversity among accessions. In contrast, there was wider variation and lower RIN values measured in
404 the stored cohorts, indicating that time-dependent fragmentation had occurred in dry seeds.
405

406 In general, there was sufficient RNA within dry seeds to analyze for RIN. An RNA concentration of 25
407 ng RNA μl^{-1} or more is recommended for reliable RNA characterization (manufacturer's protocol).
408 This was easily obtained for most seeds using a sample mass of 9 to 12 mg. Variation in RNA yield was
409 broad, due to uncontrolled factors during extraction as well as seed traits. We noted that samples smaller
410 than 9 mg yielded more than sufficient RNA in about $\frac{1}{4}$ of the species, suggesting that we, perhaps, used
411 more seeds than needed to glean reliable RIN values. We also noted that RNA yields were consistently
412 low in some seeds, for example, seeds from Ericaceae and Cyperaceae tended to have lower RNA yields
413 compared to other species; sample sizes between 15 to 20 mg per replicate may be preferable for seeds in
414 these families. For tiny seeds, (seed mass < 0.08 mg seed $^{-1}$; e.g., *Leiophyllum buxifolium* (formerly
415 *Kalmia buxifolia*) and *Vaccinium crassifolium* ssp. *sempervirens* (Ericaceae) or *Cyanea angustifolia* and
416 *Clermontia kakeana* (Campanulaceae)), total mass of the accession was small, and we needed to either
417 reduce the standard 9-12 mg sample size for each replicate or reduce the number of replications, or both.
418 RNA extractions from seeds within Campanulaceae appeared to yield reliable RIN values despite small
419 sample size and low replication; however, data produced for seeds within Ericaceae tended to be
420 problematic (discussed below). More than 100 seeds were needed per replicate for some species
421 producing tiny seeds. The number of seeds used for a RIN determination declines as seed mass increases,
422 and seed consumption for RIN and standard germination tests are both about 50 seeds when seed mass is
423 about 0.8 mg seed $^{-1}$. Genebanks are deeply concerned about depleting accessions by testing needed to
424 meet curatorial standards (FAO, 2013).
425

426 RNA quality was usually high in recently harvested seeds from the 103 wild species we studied. Average
427 RIN values ranged between 7 and 9 for 88 species (Table 1), which is slightly higher than RIN reported
428 for recently harvested commercial seed lots (Fleming et al., 2017, Fleming et al., 2019). Some of that
429 difference may be explained by seed collectors' speed and care when processing seeds from endangered

430 species. Average RIN was less than 5 for 4 species from the recently harvested cohort (*Packera*
431 *franciscana*, *Polemonium occidentale* ssp. *lacustre*, *Remirea maritima*, *Remya kauaiensis*), all of which
432 had relatively high proportions of unfilled seeds (> 50% empty) as well as relatively small seeds (< 0.4
433 mg seed⁻¹) that precluded dissection – an extra step we took for larger seeds to enrich the filled-seed
434 fraction. High incidence of empty seeds (e.g., *Dicerandra immaculata* (80% empty); *Lycium sandwicense*
435 (30% empty) and/or low yielding RNA (Cyperaceae) explain some of the moderate RIN values (between
436 5 and 7) in the remaining species of the recently harvested cohort.

437 RIN values that are lower (or higher) than expected present questions of the reliability of the assay. The
438 more common application of RIN assessments – to alert when an extraction yields RNA too degraded to
439 sequence (Mueller et al., 2004, Schroeder et al., 2006)– suggests that experimental errors during
440 extraction and electrophoresis might bias interpretation of the extent of RNA fragmentation *in planta*. We
441 found that simply repeating the electrophoresis run using archived extracts resolved most problems with
442 missing data or ambiguities, while not consuming more seeds. Our initial error rate of about 7% (of 1365
443 runs) was reduced to about 3% by a second electrophoresis of the extract that yielded suspect data
444 initially. The incidence of RIN errors was significantly higher in samples containing low RNA
445 concentration (< 30 ng µl⁻¹). RIN errors were also higher in samples with low rRNA ratio (<1). Extracts
446 that had both low RNA concentration and low rRNA ratio accounted for 71% of RIN errors.
447 Experimental errors occur at the same frequency in both recently harvested and stored accessions, and so
448 we do not anticipate these to be influential in assessing the effects of storage. Overall, flawed extractions
449 affecting average RIN occurred in about 0.5% of the RIN measurements presented in the study.

450 Aside from low RNA concentration, we found few other factors that consistently affected the reliability of
451 RIN measurements. Standardized procedures, with few adjustments for varying seed traits, led to few
452 anomalies or ambiguities in the data and attest to broad applicability of using RIN across diverse seeds
453 that vary by phylogeny, morphology, composition and germination behavior. In contrast, germination
454 assays require conditions tailored for individual species (Hay and Probert, 2013); often the optimal
455 conditions are unknown for seeds of wild species, leading to ambiguous calls on seed quality. Collectors
456 do their best to optimize harvest times; even still, some samples may include seeds at various levels of
457 maturity (Hay and Probert, 1995). This study demonstrated that tissue type or prevalence, including bulky
458 or water-impermeable seed coverings and embryonic axis vs nutritive tissues may affect RNA
459 concentration in some species but not RIN outcomes (Table 2). This conclusion is consistent with
460 previous reports using legume crop seeds, (Fleming et al., 2017, Fleming et al., 2019, Walters et al.,
461 2020) and provides reassurances that seed dissections are rarely needed.

462
463 The general insensitivity of RIN to the presence of bulky, low RNA-containing tissues might make it an
464 unreliable test for seed fill. In accessions with high prevalence of empty seeds, we found that additional
465 steps to remove seeds lacking nutritive tissue or embryo tended to increase RNA yield. As described
466 earlier, low RIN was observed in some recently harvested accessions with low seed fill and seeds too
467 small for dissection (e.g., *Packera franciscana*, *Polemonium occidentale* ssp. *lacustre*, *Remirea maritima*,
468 *Remya kauaiensis*); however, the opposite was also encountered (e.g., *Castilleja kaibabensis*,
469 *Chenopodium oahuense*, and *Chrysopsis floridana*). The inconsistent effect of unfilled seeds on RIN may
470 be related to the presence/absence of residual seed tissues with degraded RNA in under-developed
471 embryos. It will be difficult to conduct paired comparisons of RIN among populations or cohorts without
472 accounting for how seed fill affects RIN.

473
474 Of all the variables tested to determine potential influence on RIN, the difference among cohorts was the
475 most clear and consistent ($p = 0.0037$). Difference of average RIN between recently harvested and stored
476 cohorts was greater than 1 in 42 of the 101 species, and less than -1 for just 5 species, of which 4 had
477 confounding problems of seed fill (e.g., *Packera franciscana*, *Polemonium occidentale* ssp. *lacustre*,
478 *Remirea maritima*, *Remya kauaiensis*). In a previous paper, we showed that a RIN difference of about 0.5

479 is needed to establish significance with 6 replicates (Tetreault et al., 2023). Actual significance of RIN
480 differences between cohorts will be presented in a subsequent paper that also compares germination
481 results (Walters et. al. *in progress*). The comparisons attempt to detect aging in stored seeds and this
482 requires the assumption that RIN values for the recently harvested cohorts are representative of initial
483 RIN values for the stored cohorts (RIN technology was not available in the 1990s when most of the stored
484 cohorts were harvested). That assumption appears safe considering the great efforts to collect from the
485 same populations and process the seeds in the same way, as well as the high quality of seeds obtained
486 (with a few notable exceptions) and the consistency of RIN in the recently harvested seeds.

487
488 This work provides important confirmatory evidence that RNA quality declines with storage time and
489 shows the applicability of the assay for a large and diverse set of seeds from wild populations native to
490 the US. Demonstrating that RNA degradation is a marker for seed aging is challenging. Aging is currently
491 measured in seeds as increasing mortality after an initial period of no viability change. In other words,
492 aging in seeds is defined by lethal effects, not by the accumulation of nonlethal damage. RIN is likely to
493 reflect non-lethal damage, and because it declines linearly with time (Fleming et al., 2017, Fleming et al.,
494 2019, Tetreault et al., 2023) it can be detected before viability changes are noted. Thus, RIN cannot be
495 used as a viability assessment per se and in fact, we expect a poor correlation of RIN and viability.
496 However, detecting degradation before mortality can be powerful if the rate of degradation (i.e., the slope
497 of a linear time course) correlates with the *duration* that the seed stays viable (i.e., longevity). Hence,
498 developing this work further requires continued monitoring of seed quality using both germination and
499 RIN assays.

500 501 *Summary*

502 Genebanking seeds collected from wild species is an important conservation strategy that maintains
503 viable, genetically representative germplasm for restoration or research. A key challenge is maintaining
504 viability over decades, especially when there are no guidelines for survival duration. To address this
505 major unknown, most genebanks monitor viability periodically using germination tests (Hay and Probert,
506 2013, Van Treuren et al., 2013, Solberg et al., 2020). Germination tests of wild seeds can be especially
507 time-consuming and yield ambiguous results if germination protocols are not available. Moreover,
508 germination tests provide a snapshot of the seed quality and do not provide insights for an expiration (or
509 best used by) date. Thus, genebanks are consigned to test at frequencies that may be too broad and miss
510 detection of a viability threshold, or too narrow and unnecessarily deplete a valuable seed sample.
511 While most of the work to-date exploring RIN as a marker for seed aging uses commercial seed lots, here
512 we show that RIN assessments may be uniquely amenable for quality assessments of seeds from wild
513 populations. First and foremost, RIN may provide information that can be translated to seed longevity,
514 which will help optimize quality monitoring frequencies as well as to estimate when seed accessions
515 should be used or regenerated. A complete RIN assessment can be completed within a week, while
516 germination assays for wild seeds can take 6 months or more (when a protocol is available). RIN
517 procedures are highly standardized (using purchased kits), despite wide variation among species, and
518 results are highly reliable, especially electrophoresis can be repeated on samples if data appear
519 anomalous. Germination tests provide the “gold standard” assessment on how to grow out valuable
520 germplasm, and seedlings resulting from a germination test can be re-purposed if the need is dire. The
521 value of quality assessments in genebanked samples is generally recognized, but the cost of consuming
522 valuable seeds by testing is lamented. Plant fecundity and seed size are important factors in optimizing
523 curation strategies to ensure genebanking goals are met.

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532

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615 Figure 1. Number of RNA extractions in each class of RNA concentrations ($\text{ng } \mu\text{l}^{-1}$) that (A) were
616 considered successful and included as RIN data or (B) had a suspect RIN value in the first
617 electrophoresis run however provided a reliable RIN value after electrophoresis was repeated, and (C)
618 the number of samples that were considered to give an unreliable RIN value after reanalysis from a
619 failed first run.

620 Figure 2. Number of RNA extractions in each class of 260/280 ratio that (A) were considered
621 successful and included as RIN data or (B) had a suspect RIN value in the first electrophoresis run
622 then provided a reliable RIN value after electrophoresis was repeated, and (C) the number of samples
623 that were considered to give an unreliable RIN value after reanalysis from a failed first run. A reliable
624 RIN value has a $260/280 = 2.0$.

625 Figure 3. Number of RNA extractions in each class of 260/230 ratio that (A) were considered
626 successful and included as RIN data or (B) had a suspect RIN value in the first electrophoresis run
627 then provided a reliable RIN value after electrophoresis was repeated, and (C) the number of samples
628 that were considered to give an unreliable RIN value after reanalysis from a failed first run.

629 Figure 4. Number of extractions in each class of rRNA ratio that (A) were considered successful and
630 included as RIN data, (B) had a suspect RIN value in the first electrophoresis run then provided a
631 reliable RIN value after electrophoresis was repeated, and (C) the number of samples that were
632 considered to give an unreliable RIN value after reanalysis from a failed first run. A reliable rRNA
633 ratio is between 1 and 4.

634 Figure 5. The relationship between RNA integrity number (RIN) and rRNA ratio in RNA samples
635 from (A) recently harvested (closed circles) and (B) stored seed extracts (open circles). $P < 0.0001$ for
636 both recently harvest and stored.

637 Supplemental Figure 1. Immature and mature seed for (A) *Astragalus tyghensis* and (B) *Geum*
638 *geniculatum*.

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655 Table 1. The number of species, harvest year and number of replications/species included in the large
 656 study. Summary of Nanophore diagnostics gathered from the spectrophotometer on RNA purity and
 657 on fragmentation of RNA from the Agilent bioanalyzer.

	average \pm std dev or count	
factor	Recently harvested	Stored
# species	103	105
harvest year	2022 \pm 1	1995 \pm 6
# replications/species	4.8 \pm 2.0	4.9 \pm 2.7
Nanopore diagnostics		
[RNA] (ng/ μ l)	202 \pm 185	194 \pm 187
260/280	2.01 \pm 0.16	1.91 \pm 1.44
260/230	1.67 \pm 0.60	1.61 \pm 0.65
Agilent bioanalyzer diagnostics and data		
RNA area	388 \pm 188	382 \pm 203
[RNA] (pg/ μ l)	2252 \pm 959	2496 \pm 1862
rRNA ratio	2.07 \pm 1.09	1.82 \pm 1.53
RIN	7.7 \pm 1.3	6.8 \pm 1.7
# electrophoresis runs	685	655
# suspect	45	44

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In review

Table 2. Species used for the different treatments tested for differences in RNA quality. Percent (%) empty, indicates the amount of seed that was determined empty in a given seed lot for that harvest year.

Treatment	Species	Harvest year	% Empty	seed mass (mg)	Cleaned					Not cleaned					Difference between treatments		[RNA]/mean sample mass	RIN
					mean RNA sample mass (mg)	mean [RNA] ng/μl	[RNA]/mean sample mass	mean RIN	std dev RIN	mean RNA sample mass (mg)	mean [RNA] ng/μl	[RNA]/mean sample mass	mean RIN	std dev RIN	RIN	std dev RIN	pvalue	pvalue
Cleaned for empty seed	<i>Sidalcea nelsoniana</i>	2021	36	2.583	16.4	679.7	41.5	8.2	0.2	19.2	478.8	25.0	8.3	0.3	-0.1	-0.1	0.0319	0.6532
	<i>Rhus kearneyi ssp. kearneyi</i>	2022	52	48.994	16.2	414.7	25.7	8.3	0.1	18.0	237.0	13.2	8.3	0.3	0.0	-0.1	0.0025	0.7698
	<i>Remirea maritima</i>	2021	53	0.404	5.9	2.8	0.5	3.4	N/A	5.1	4.8	0.9	3.2	N/A	0.2	N/A	N/A	N/A
	<i>Packera franciscana or Senecio franciscanus</i>	2022	94	0.313	13.9	115.8	8.3	2.4	N/A	9.6	195.7	20.4	2.0	N/A	0.4	N/A	N/A	N/A
	<i>Oxypolis canbyi</i>	2022	42	6.321	11.1	209.4	18.9	7.8	0.3	15.0	138.9	9.3	6.7	0.7	1.1	-0.4	0.1263	0.0176
	<i>Linum carteri var. carteri</i>	2021	69	0.351	8.1	39.5	4.9	8.2	N/A	6.4	41.4	6.4	2.3	N/A	5.9	N/A	N/A	N/A
	<i>Dubautia menziesii</i>	2021	87	0.806	18.6	194.5	10.5	8.2	0.9	34.5	65.2	1.9	7.6	0.1	0.6	0.9	0.1255	0.3467
	<i>Chrysopsis floridana</i>	2021	64	0.420	10.1	334.8	33.2	8.7	0.5	4.5	35.5	7.9	9.1	0.1	-0.4	0.4	0.0071	0.2411
	<i>Bidens torta</i>	2022	41	1.488	11.3	243.3	21.5	8.6	0.2	16.8	223.7	13.3	7.4	0.6	1.2	-0.4	0.1174	0.0036
	<i>Abies fraseri</i>	2023	48	7.608	9.5	145.7	15.4	7.8	1.3	23.1	16.1	0.7	2.9	0.8	5.0	0.4	0.0033	0.0011
	<i>Solidago plumosa</i>	2003	47	0.387	4.8	208.4	43.2	8.0	0.3	7.4	269.0	36.3	7.5	0.2	0.5	0.1	0.1090	0.0224
	<i>Remirea maritima</i>	2003	57	0.559	6.3	3.6	0.6	5.6	N/A	6.1	6.4	1.1	2.6	N/A	3.1	N/A	N/A	N/A
	<i>Lycium sandwicense</i>	2006	47	0.273	9.7	125.0	12.9	6.0	N/A	11.8	114.6	9.7	4.9	1.5	1.1	N/A	N/A	N/A
	<i>Echinocactus horizontalonius var. nicholii</i>	1991	52	3.368	10.6	165.6	15.7	7.8	0.5	15.7	144.6	9.2	7.7	0.4	0.1	0.1	0.5531	0.0530
	<i>Cirsium pitcheri</i>	1991	49	9.837	11.6	325.5	28.2	8.3	0.6	15.3	234.7	15.3	8.2	0.3	0.1	0.3	0.0330	0.7157
<i>Amaranthus pumilus</i>	1987	52	2.212	3.7	35.8	9.6	5.6	1.4	13.6	51.2	3.8	5.9	1.2	-0.2	0.2	0.1320	0.4911	
Seed coverings removed	<i>Tephrosia angustissima var. corallicola</i>	2022	0	8.369	10.8	620.6	57.2	7.5	0.1	14.8	205.8	13.9	7.4	0.5	0.1	-0.3	0.0001	0.7908
	<i>Ornithostaphylos oppositifolia</i>	2022	17	4.028	11.0	39.7	3.6	8.7	N/A	10.1	155.7	15.5	8.6	0.1	0.1	N/A	N/A	N/A
	<i>Lomatium bradshawii</i>	2021	0	7.099	10.4	279.8	26.9	8.2	0.8	15.2	246.3	16.2	8.8	0.1	-0.6	0.7	0.0245	0.2137
	<i>Hibiscus dasycalyx</i>	2021	22	11.026	13.8	678.6	49.0	7.1	0.4	18.4	479.7	26.0	7.0	1.3	0.1	-0.9	0.0151	0.4494
	<i>Eryngium aristulatum var. parishii</i>	2022	27	0.710	10.7	235.7	22.0	8.4	N/A	9.5	144.8	15.2	8.6	0.3	-0.2	N/A	N/A	N/A
<i>Eriogonum cusickii</i>	2022	4	1.127	13.2	48.0	3.6	8.5	0.6	15.8	231.1	14.6	8.4	0.2	0.1	0.4	0.0679	0.8336	

<i>Echinocactus</i>																		
	<i>horizontalthalonius</i> var. <i>nicholii</i>	2022	14	6.948	11.1	36.4	3.3	7.3	0.5	13.5	50.3	3.7	7.7	1.4	-0.4	-0.8	0.7265	0.5151
	<i>Dodonaea viscosa</i>	2022	3	5.151	11.1	243.7	22.0	8.2	0.2	17.1	290.6	17.0	8.2	0.1	0.0	0.1	0.2723	0.8294
	<i>Cirsium pitcheri</i>	2021	21	5.664	9.2	226.1	24.6	8.8	0.3	15.2	235.5	15.5	9.1	0.4	-0.2	-0.1	0.2704	0.4220
	<i>Cimicifuga elata</i>	2022	0	2.150	11.7	137.4	11.7	7.9	0.2	15.5	254.3	16.4	7.9	0.2	0.0	0.0	0.0345	0.9650
	<i>Ceanothus cyaneus</i>	2021	26	2.206	17.1	133.8	7.8	8.5	0.1	34.9	230.4	6.6	8.1	0.2	0.4	0.0	0.7907	0.0355
	<i>Carex comosa</i>	2022	23	0.978	10.4	1.4	0.1	7.0	1.0	13.5	1.5	0.1	6.8	N/A	0.1		N/A	N/A
	<i>Astragalus linifolius</i> or <i>Astragalus rafaensis</i>	2023	0	6.088	11.7	789.9	67.3	7.5	0.7	15.3	213.3	13.9	7.1	0.3	0.3	0.4	0.0011	0.5799
	<i>Astragalus bibullatus</i>	2021	0	5.978	9.8	558.3	57.0	6.9	0.3	16.2	62.3	3.8	7.1	0.2	-0.2	0.1	0.0006	0.3297
	<i>Argemone glauca</i>	2022	12	3.294	14.1	6.7	0.5	7.7	0.9	13.8	11.3	0.8	7.7	0.1	0.0	0.8	0.0617	0.4626
	<i>Arctostaphylos catalinae</i>	2023	12	89.592	11.9	18.2	1.5	8.2	0.8	56.6	18.8	0.3	8.0	0.9	0.2	-0.1	0.1326	0.7861
	<i>Amsonia tharpii</i>	2023	3	18.795	12.0	202.4	16.9	7.4	0.1	16.4	114.8	7.0	7.0	0.4	0.4	-0.3	0.2803	0.5555
	<i>Amelanchier nantucketensis</i>	2023	7	5.737	10.8	80.8	7.5	8.9	0.2	34.1	8.2	0.2	7.0	1.8	2.0	-1.6	0.0060	0.0344
	<i>Amaranthus pumilus</i>	2022	5	2.875	13.2	24.3	1.8	8.4	0.7	12.1	40.4	3.3	8.4	0.4	-0.1	0.3	0.0360	0.4438
	<i>Actaea arizonica</i>	2022	5	0.851	12.3	208.8	16.9	8.5	0.4	17.3	342.2	19.8	8.5	0.6	0.0	-0.2	0.3048	0.9855
	<i>Rhus kearneyi</i> ssp. <i>kearneyi</i>	1986	25	42.672	9.9	269.8	27.3	7.4	0.4	31.3	189.8	6.1	7.2	0.2	0.3	0.2	0.0110	0.8930
	<i>Penstemon clutei</i>	1991	15	0.430	19.5	18.9	1.0	2.8	N/A	17.5	31.4	1.8	2.6	0.0	0.2		N/A	N/A
	<i>Eutrema penlandii</i> or <i>Eutrema edwardsii</i>	1988	7	0.307	10.1	621.7	61.6	8.8	N/A	6.7	645.4	96.3	7.8	0.1	1.0		N/A	N/A
	<i>Eriogonum cusickii</i>	1983	15	1.024	13.0	62.3	4.8	6.3	0.3	16.2	61.5	3.8	4.2	1.1	2.0	-0.8	0.5776	0.7184
	<i>Dodonaea viscosa</i>	1990	0	5.482	10.4	208.4	20.0	6.1	0.7	15.9	319.8	20.1	5.0	2.2	1.1	-1.4	0.9843	0.3009
	<i>Astragalus magdalenae</i> var. <i>peirsonii</i>	2003	0	20.416	9.9	937.8	94.3	6.9	0.5	31.3	73.2	2.3	7.6	N/A	-0.7		N/A	N/A
	<i>Astragalus albens</i>	1995	0	2.352	10.1	495.3	49.2	8.5	0.6	13.9	283.2	20.4	7.8	0.1	0.7	0.5	0.0357	0.1514
	<i>Abronia umbellata</i> var. <i>breviflora</i>	1990	1	13.457	16.2	292.2	18.0	7.8	0.4	15.4	77.9	5.1	7.4	0.3	0.4	0.0	0.1664	0.0970
<div style="display: flex; justify-content: space-around;"> Mature Immature Difference between treatments </div>																		
Maturity	<i>Amorpha herbacea</i> var. <i>crenulata</i>	2021	0	3.712	4.7	274.7	58.9	7.0	0.0	4.2	236.8	56.8	6.7	0.3	0.3	0.3	0.7600	0.1318
	<i>Physaria orbicordata</i>	1987	0	3.933	5.5	180.3	32.9	4.3	2.4	6.8	168.1	24.8	6.8	0.0	-2.5	-2.4	0.4608	0.0182
	<i>Geum geniculatum</i>	2009	19	1.276	18.9	136.8	7.2	7.3	0.2	12.2	80.8	6.6	6.1	1.0	1.2	0.7	0.9454	0.0486
	<i>Astragalus tyghensis</i>	2000	3	4.061	10.8	434.4	40.1	7.6	0.2	9.0	409.1	45.4	7.4	0.3	0.2	0.1	0.3681	0.1866

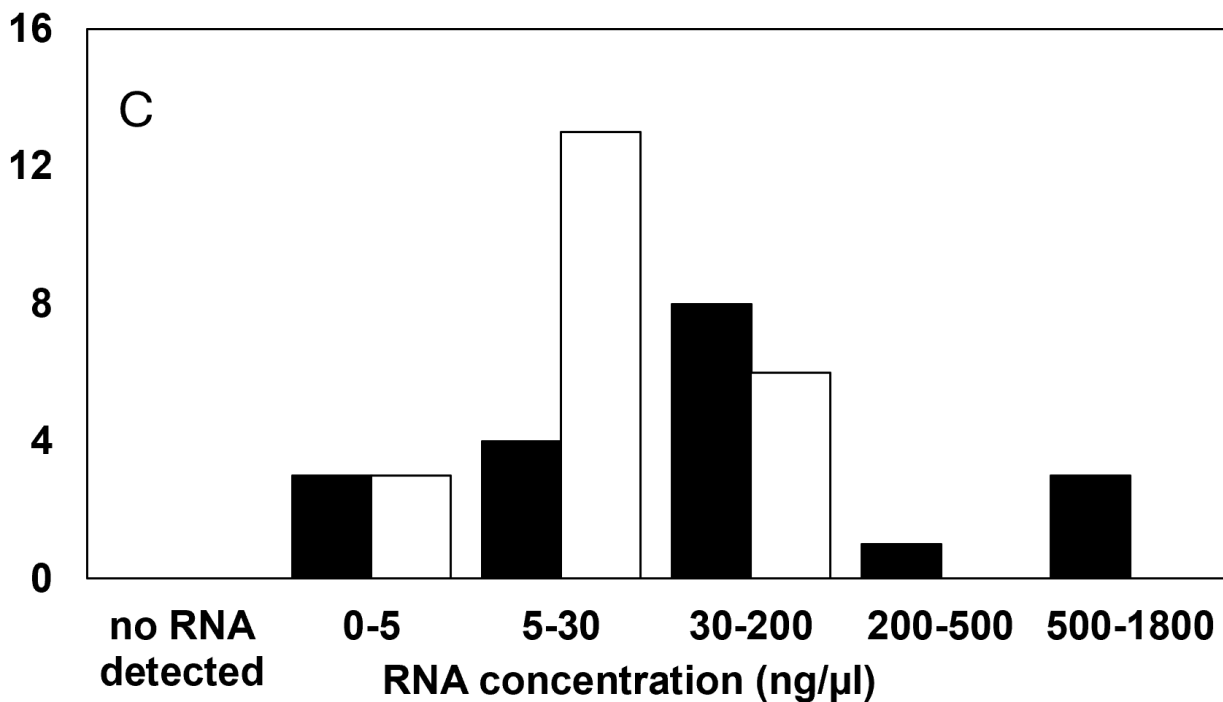
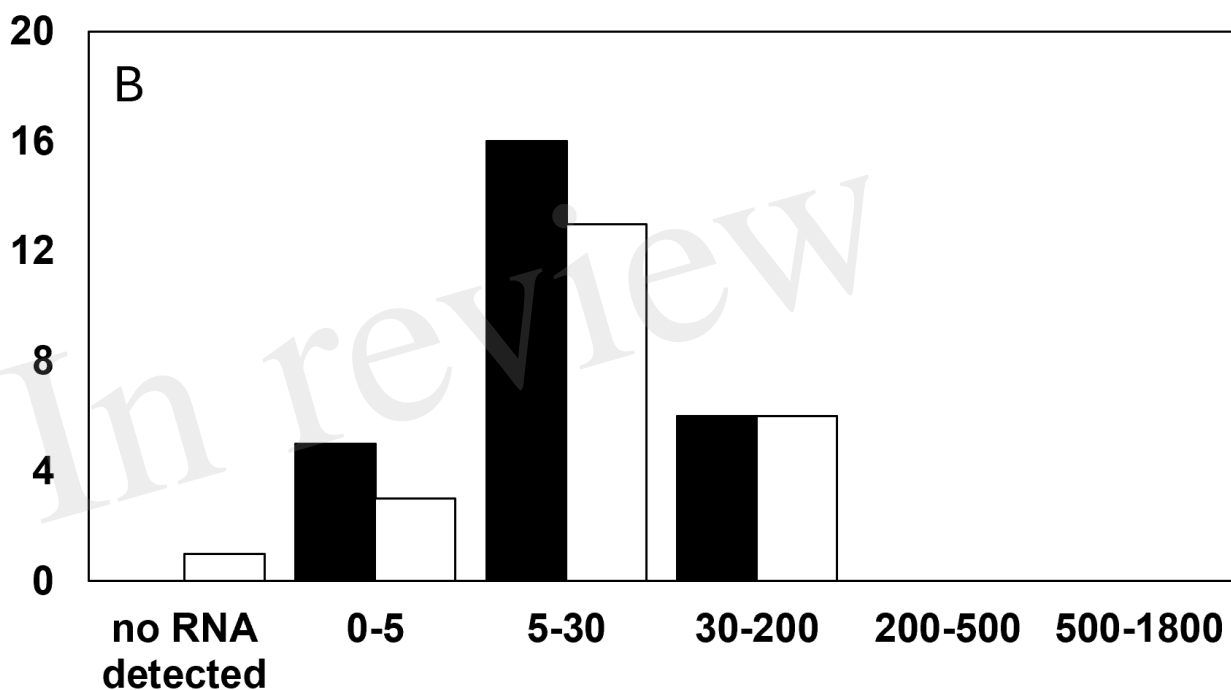
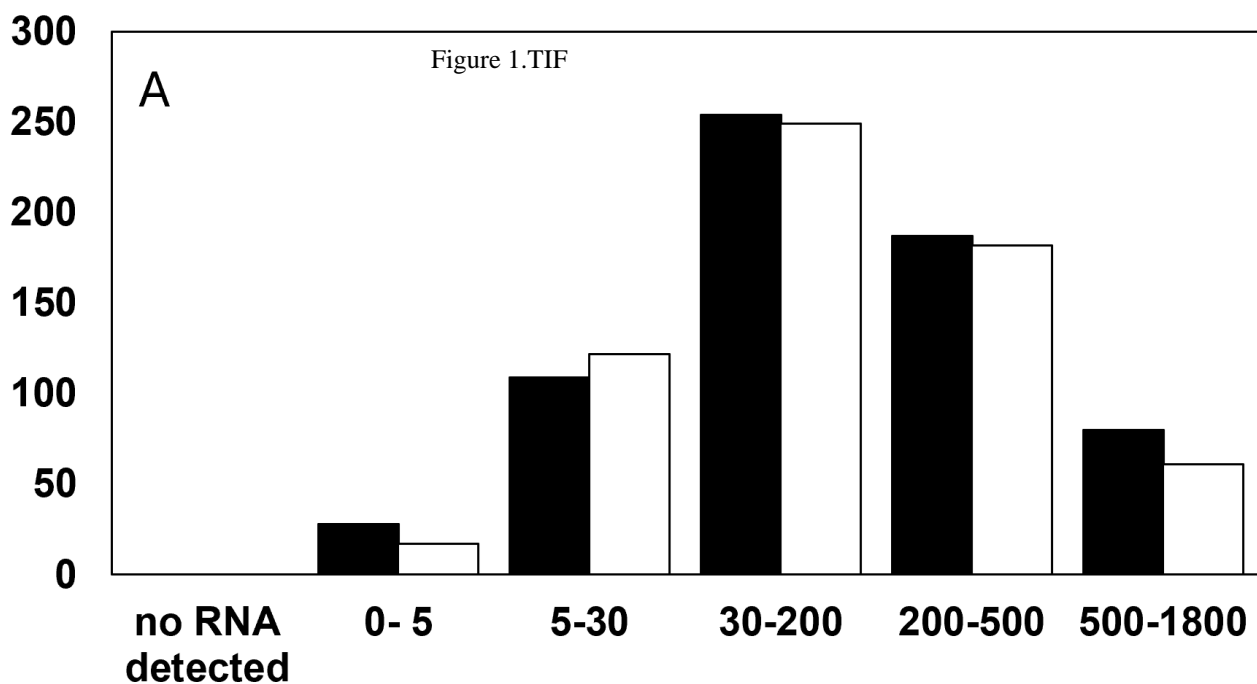
			Embryo							Nutritive Tissue					Difference between treatments			
Tissue type	<i>Rhus kearneyi</i> ssp. <i>kearneyi</i>	2022	52	48.994	7.2	173.5	24.0	8.2	0.1	20.8	403.9	19.4	8.5	0.2	-0.3	-0.1	0.1513	0.4534
	<i>Lupinus westianus</i> var. <i>aridorum</i>	2021	0	16.152	2.1	171.2	80.2	7.4	0.2	10.2	368.2	36.2	7.3	0.6	0.0	-0.4	0.0203	0.4929
	<i>Astragalus magdalenae</i> var. <i>peirsonii</i>	2021	0	22.111	3.6	288.3	80.1	8.1	0.5	17.3	927.3	53.5	7.5	0.2	0.7	0.2	0.2522	0.0723
	<i>Abies fraseri</i>	2023	48	7.608	2.1	144.5	69.6	8.0	1.3	10.2	83.6	8.2	7.2	2.5	0.9	-1.2	0.0450	0.4838
	<i>Rhus kearneyi</i> ssp. <i>kearneyi</i>	1986	25	42.672	1.5	86.5	59.0	7.7	0.4	13.5	199.0	14.7	7.7	0.3	0.0	0.0	0.0310	0.4427
	<i>Amaranthus pumilus</i>	1987	52	2.212	2.0	52.9	26.4	6.6	0.4	6.3	10.3	1.6	4.3	1.3	2.3	-0.9	0.0682	0.0245
	<i>Abies fraseri</i>	2006	95	5.879	1.4	10.0	7.1	2.5	N/A	6.1	9.9	1.6	N/A	0.5			N/A	N/A

665

In review

Figure 1.TIF

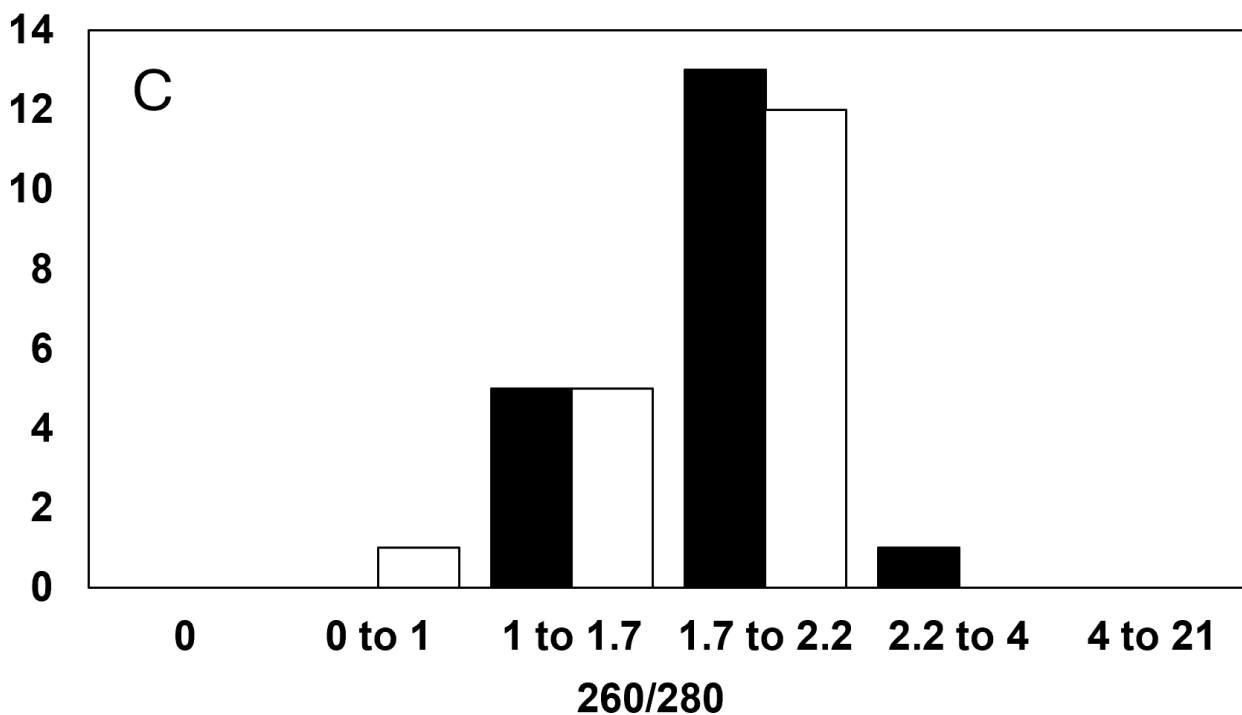
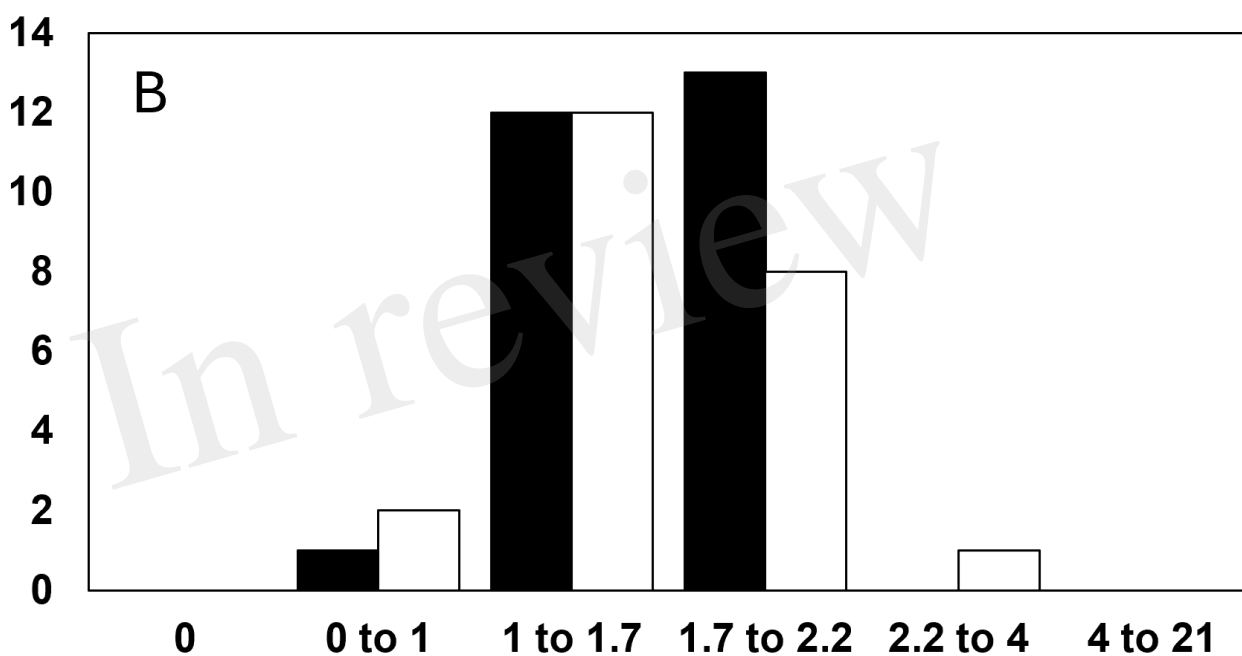
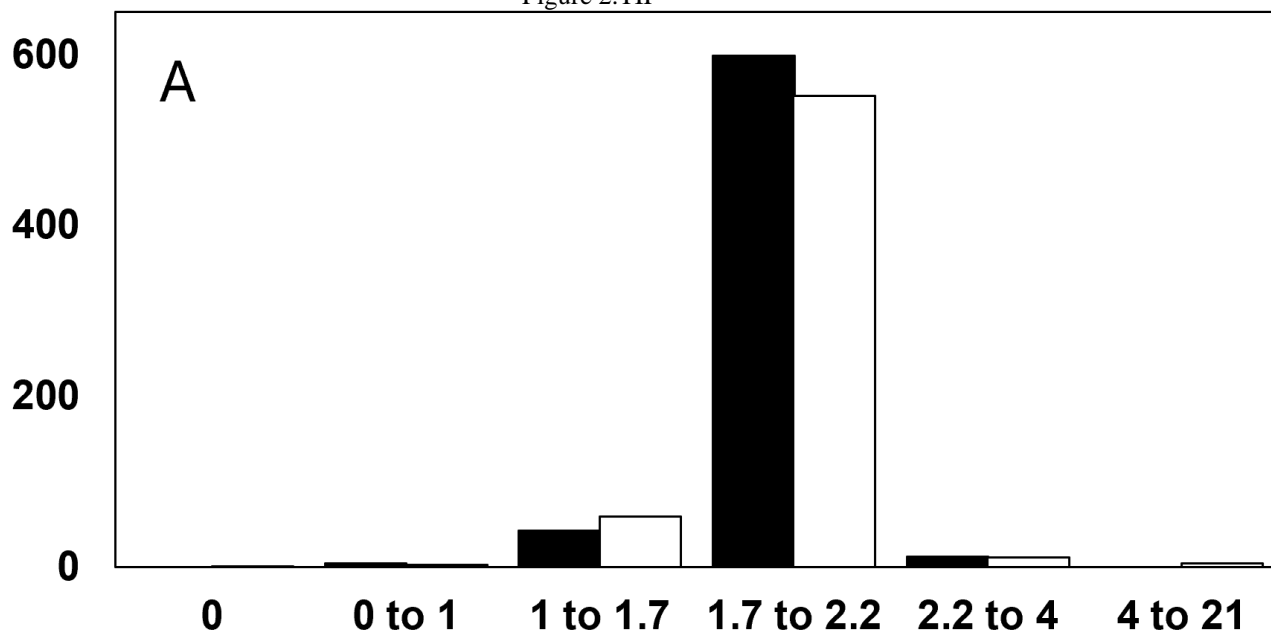
Number of RNA extractions in each class



■ fresh □ stored

Figure 2.TIF

Number of RNA extractions in each class



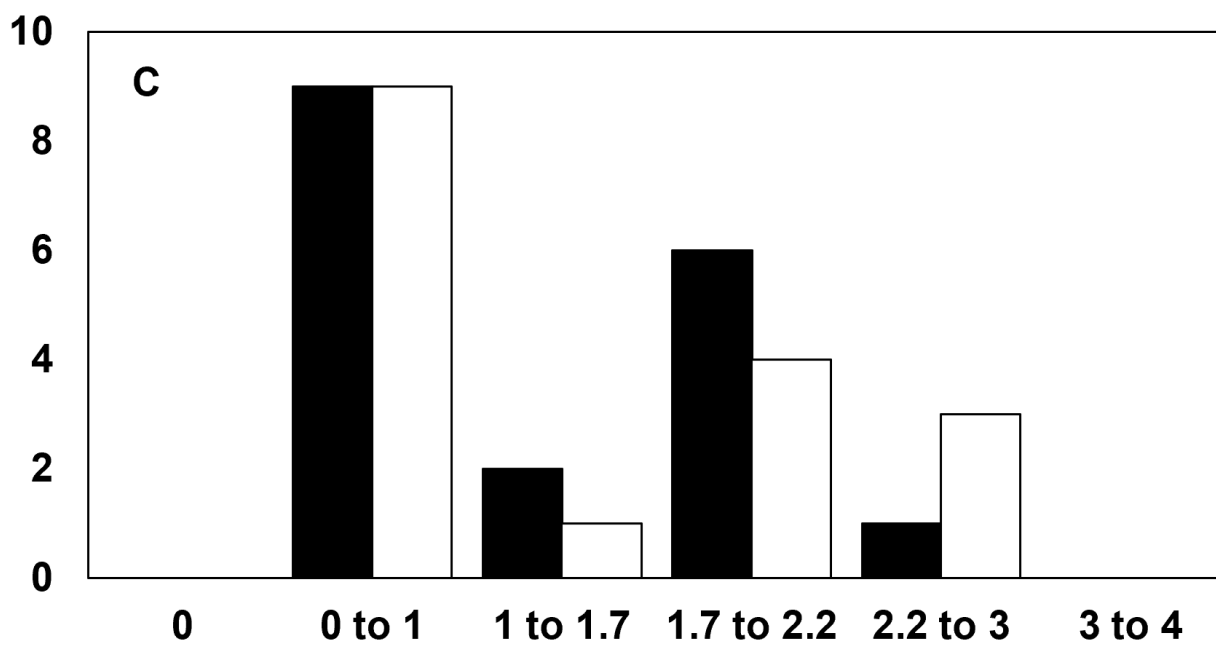
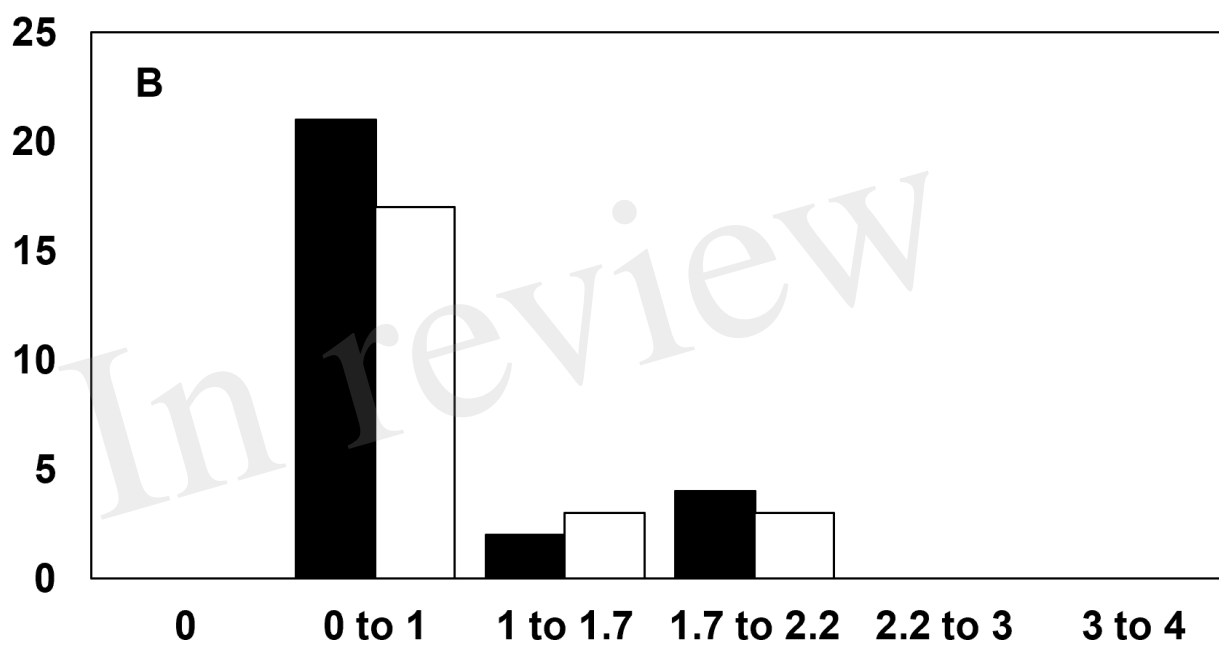
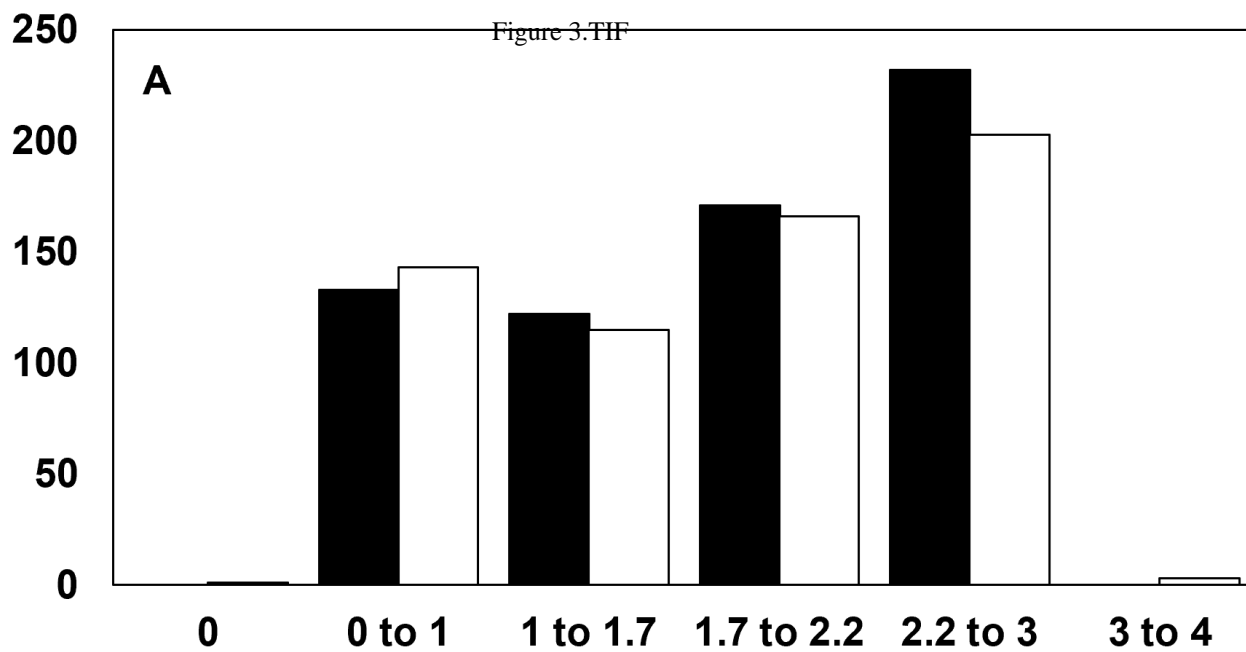
260/280

■ fresh

□ stored

Figure 3.TIF

Number of RNA extractions in each class



260/230

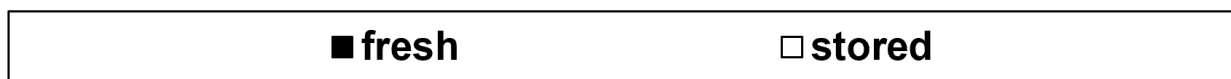


Figure 4.TIF

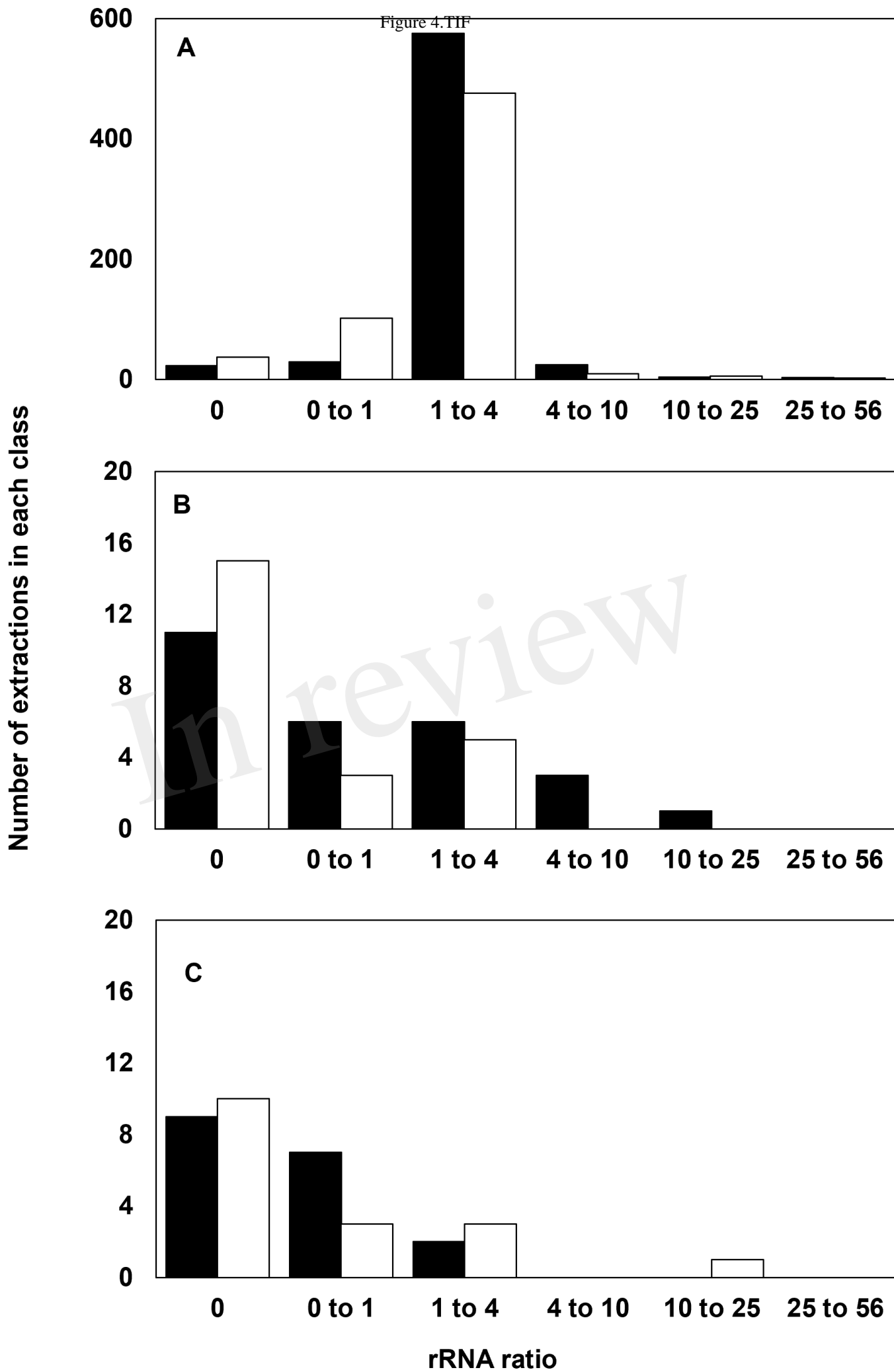
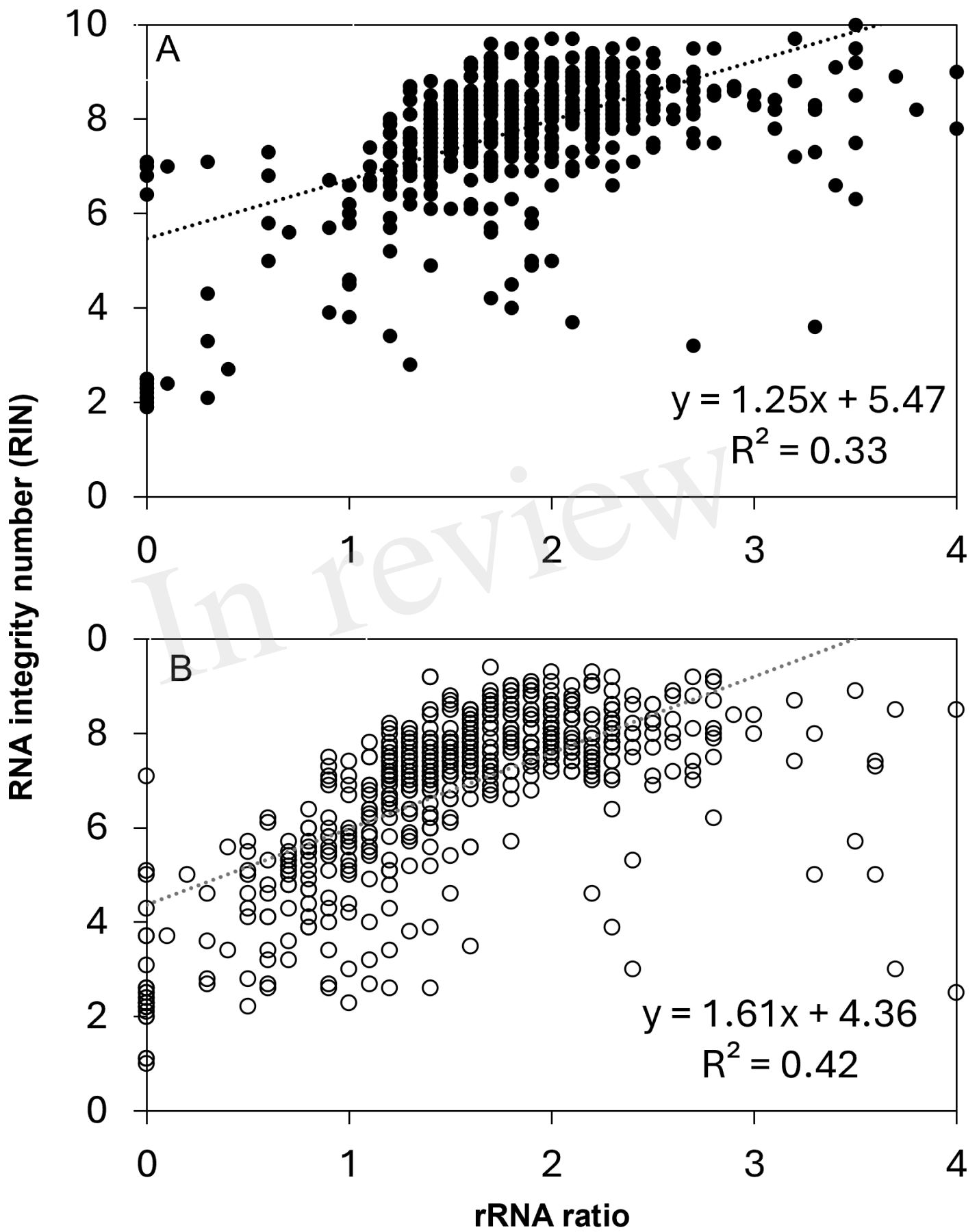


Figure 5.TIF



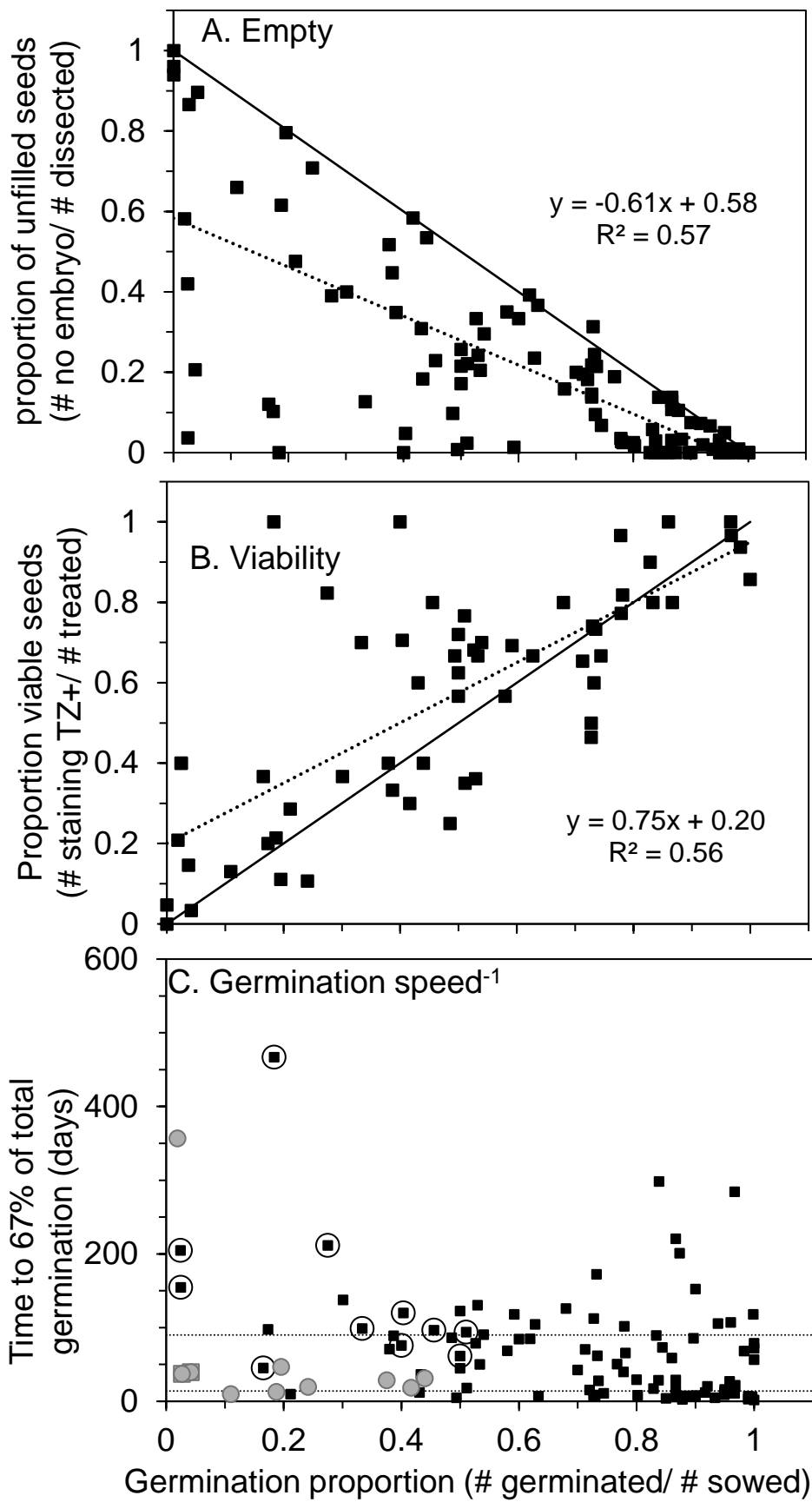


Figure 1

Figure 1. Relationships between germination proportion and other factors of seeds quality for recently harvested seeds: proportion of empty seeds (A), proportion of viable seeds based on TZ staining (B) and germination speed⁻¹ (time for $\frac{2}{3}$ of the total seeds to germinate in an assay) (C). Regression (dashed) and 1:1 (solid) lines are plotted in A and B ($r^2 = 0.57$ (n=105) and 0.56 n=59), respectively; $P \ll 0.01$). The numerous points below the 1:1 line in A (shaded portion) demonstrate additional factors besides empty seeds play a role in low germination. Points well above the 1:1 line in B suggest that, despite low germination, seeds are viable and likely dormant. The dotted horizontal lines in C demark germination times that are considered fast (within 15 days) and slow (more than 90 days). Encircled points represent seeds that are believed to retain dormancy according to TZ assay results (Fig 1B) and gray points have high proportions of empty seeds: ≥ 0.8 (circles) and $0.5 < \text{prop. empty} < 0.8$ (squares). Points that show low germination (< 0.25) and low viability, but were filled are *Polemonium occidentale* ssp. *lacustre* (10 days), which had shriveled embryos, and *Ptilimnium nodosum* (98 days).

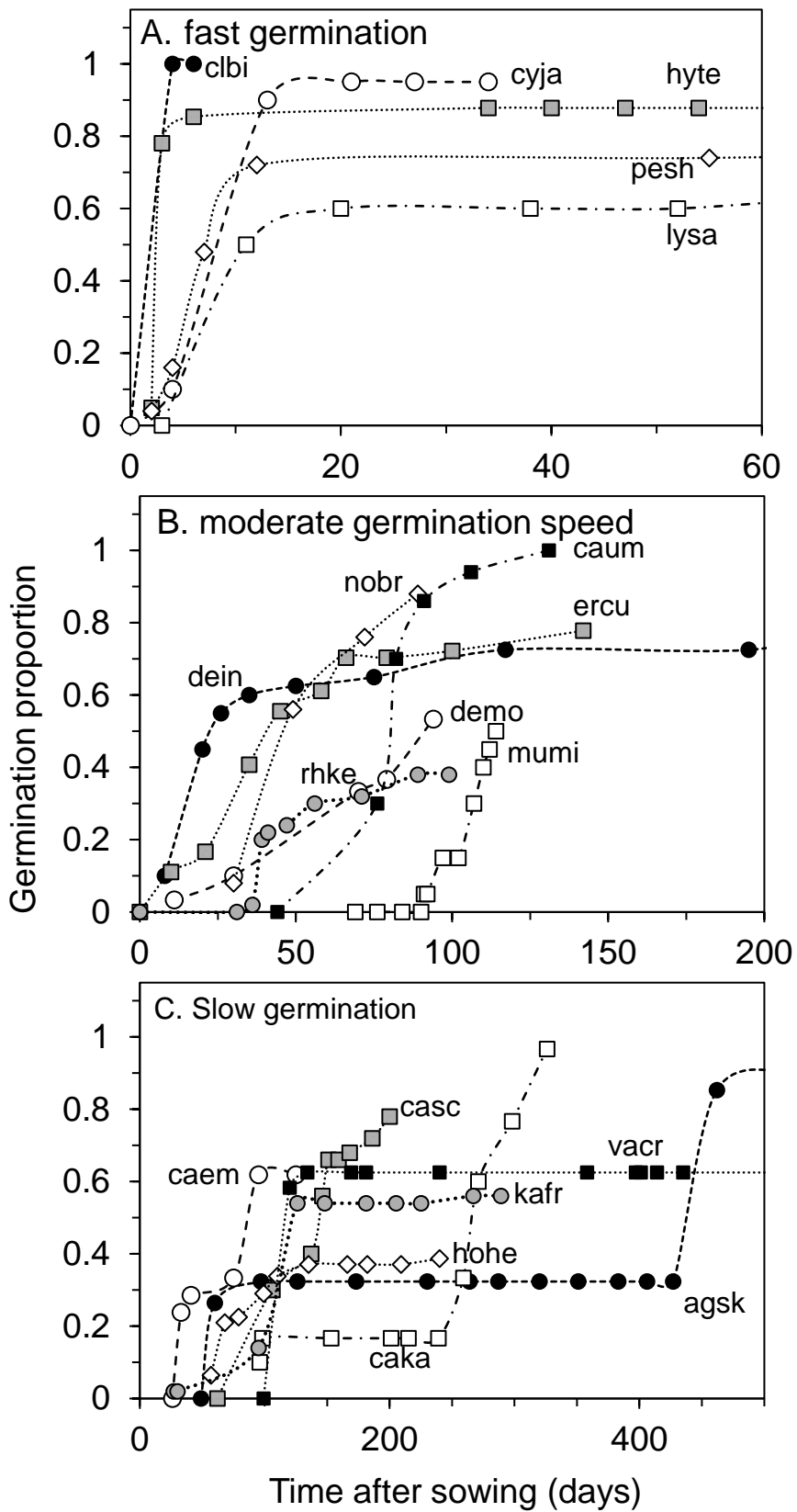


Figure 2

Figure 2. Germination time courses for recently harvested seeds that germinate quickly (A), at moderate rates (B) and slowly (C) according to the categories described in Fig 1C. Germination speed⁻¹ is calculated from the number of days for 67% total radicle emergence. Species are indicated by initials as listed in Table 2. Time courses mostly reflect germination on blotter paper at temperatures indicated in Table 4 for the indicated species with few additional treatment with the exception of rhke (B) and hohe (C) in which the upswing in germ is associated with transfer from 5C to warmth and mume (B) and vacr (C) in which the upswing is associated with transfer to warmth and/or GA3 treatment.

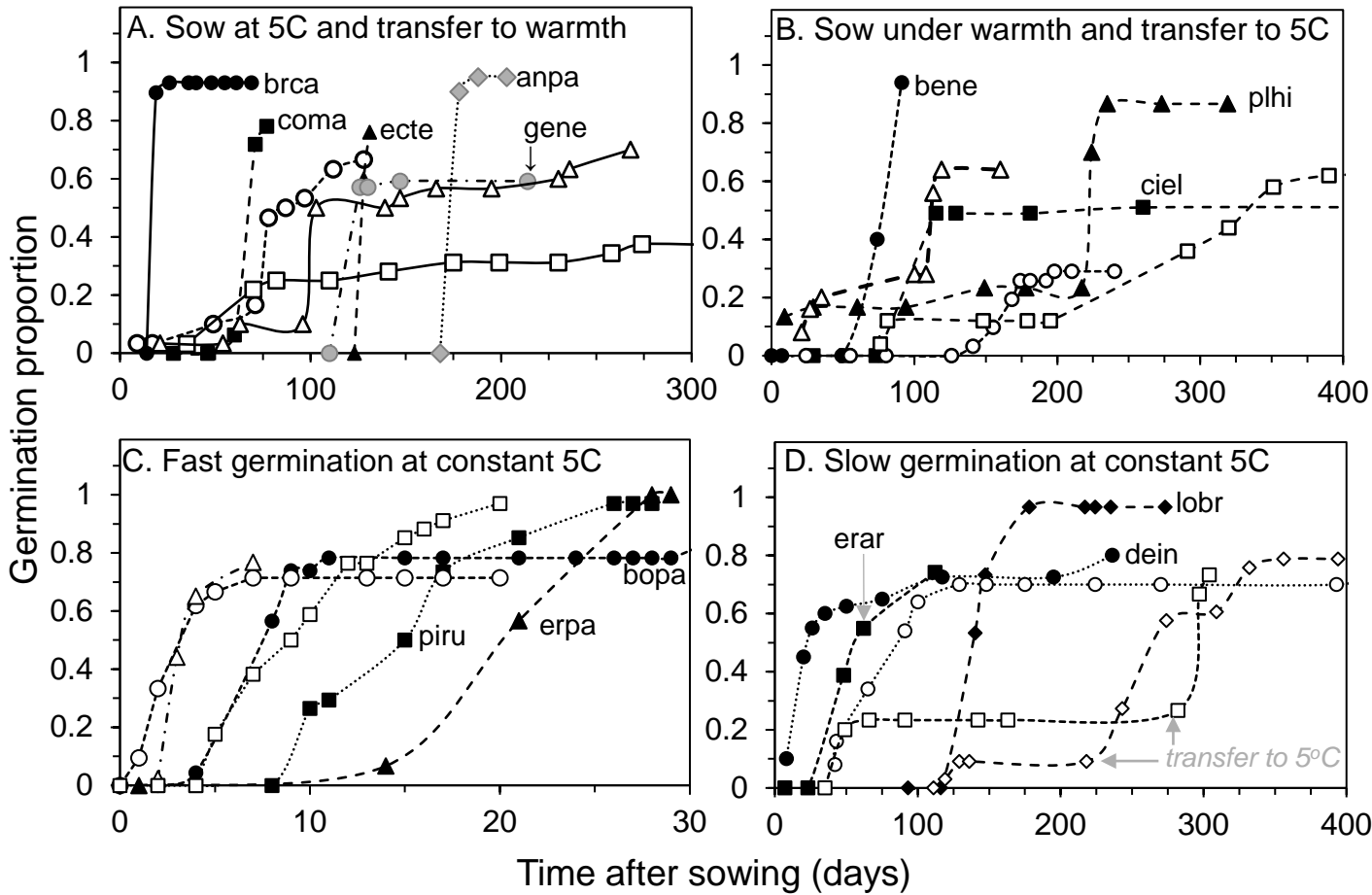


Figure 3. Germination time courses for recently harvested seeds that are stimulated to germinate during or after exposure to 5C. Seeds may be placed at 5C upon sowing and then transferred to warmth (A) or placed at warm temperatures at first and later germinate when transferred to 5C (B) (black symbols). An alternative treatment of warmth alone shows slower germination (open symbols with same shape corresponding to same species). In some cases, germination appears induced by temperature transfers, but alternative treatments were not given due to sample availability (gray symbols). Warm treatments for *ecte* (open triangles in A) also included GA3 at day 96 and peeling outer coverings at day 138. There is a spike in germination (black and gray symbols) following transfer to warmth or cold in (A) and (B), respectively. In (C), seeds germinate quickly at 5C (black symbols) but also at 20C (open symbols of the same shape represent the alternative treatment for that species) (C). In (D), seeds germinate slowly at 5C (black symbols), but transfer to warmth generally gives lower and slower germination (open symbols). Species are indicated by initials as listed in Table 2; the warm temperature treatment and time to 67% total germination for the treatment giving higher germination are listed in Table 4.

Figure 3

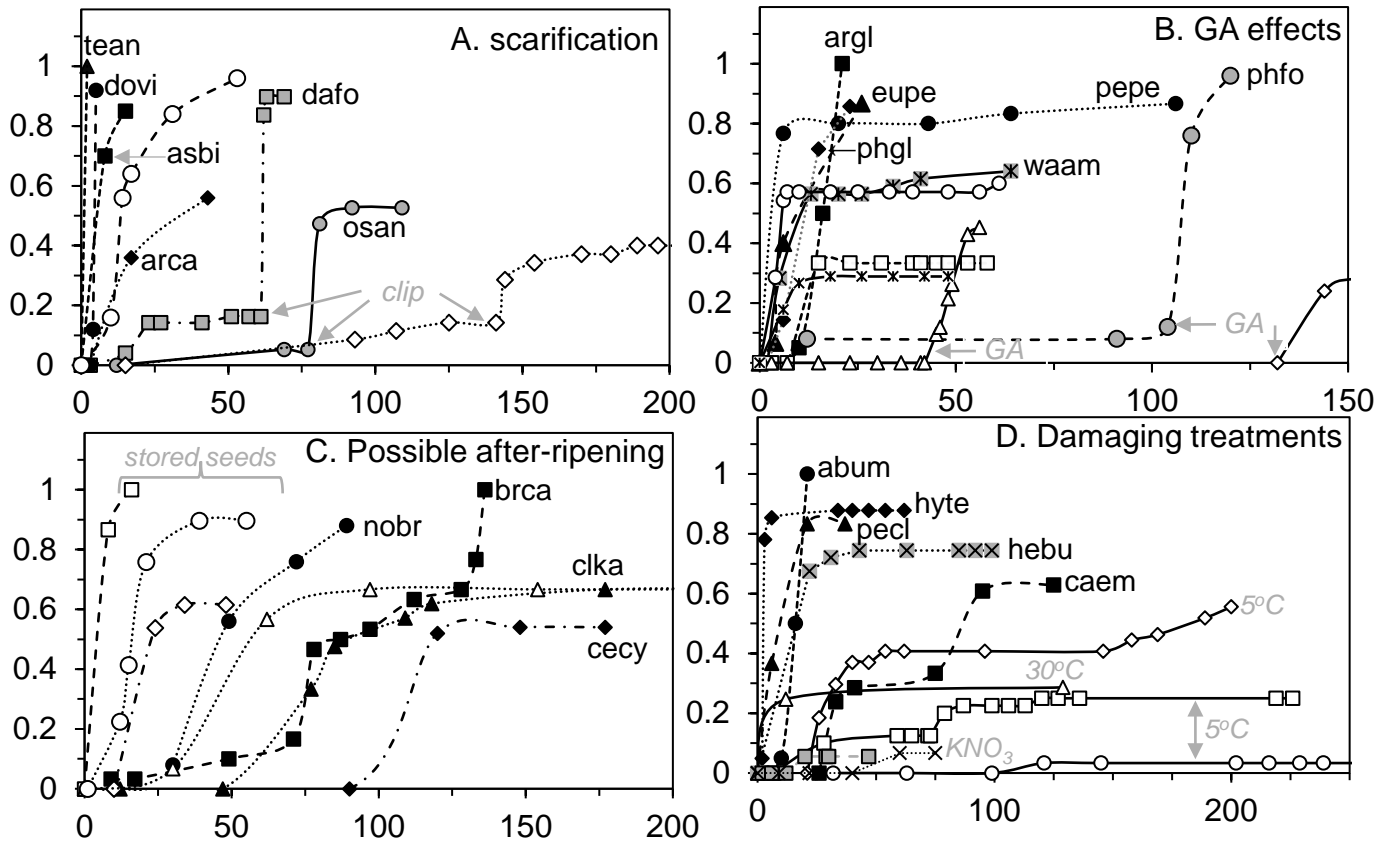


Figure 4. Time courses for germination showing stimulation or inhibition of recently harvested seeds by various treatments. Some seeds are stimulated to germinate following scarification (i.e., abrasion of seed outer layers by clipping or peeling) (A). Scarified seeds germinate quickly, whether it occurs before sowing (black symbols) or later during incubation (open and gray symbols) (black and open symbols of the same shape represent the same sample treated at different times). Some seeds are stimulated to germinate following exposure to GA₃ (B). Some seeds that are hydrated with GA₃ solutions during sowing germinate quickly (black symbols); germination is delayed or lower if GA₃ is delivered later (gray symbols; open of the same shape represent the same sample treated at different times), for example *Physaria globosa* (*phgl*, diamonds) germination proportion is 0.44 at day 240 when exposed to GA₃ on day 144. Some seeds germinate faster after storage than recently harvested counterparts which may be an after-ripening effect (C). In this case, onset of radicle emergence occurs sooner in stored seeds (open symbols) than recently harvested counterparts (black symbols). Some samples appeared damaged by treatments we believed might stimulate germination (D, open and gray symbols). Most often negative responses involved exposure to 5°C (e.g., *hyte*) or too long exposure to 5°C (*abum* or *caem*). Seeds of *Abronia umbellata* var. *breviflora* partially recover from 5°C treatment with germination proportion of 0.43 after 330 days (open circles beyond the scale of the x-axis). Other damage results from peeling away outer coverings inviting mold growth (*caem*, gray squares) or by hydrating *hebu* with dilute KNO₃ solution (D, x symbols). Seeds of *pecl* were highly sensitive to exposure to 30°C (seeds were germinated at 25°C (black) or 20/30°C (open) triangles). Species are indicated by initials as listed in Table 2. Stimulatory or inhibitory effects of these treatments are indicated “+” or “-” symbols in Table 4.

Figure 4

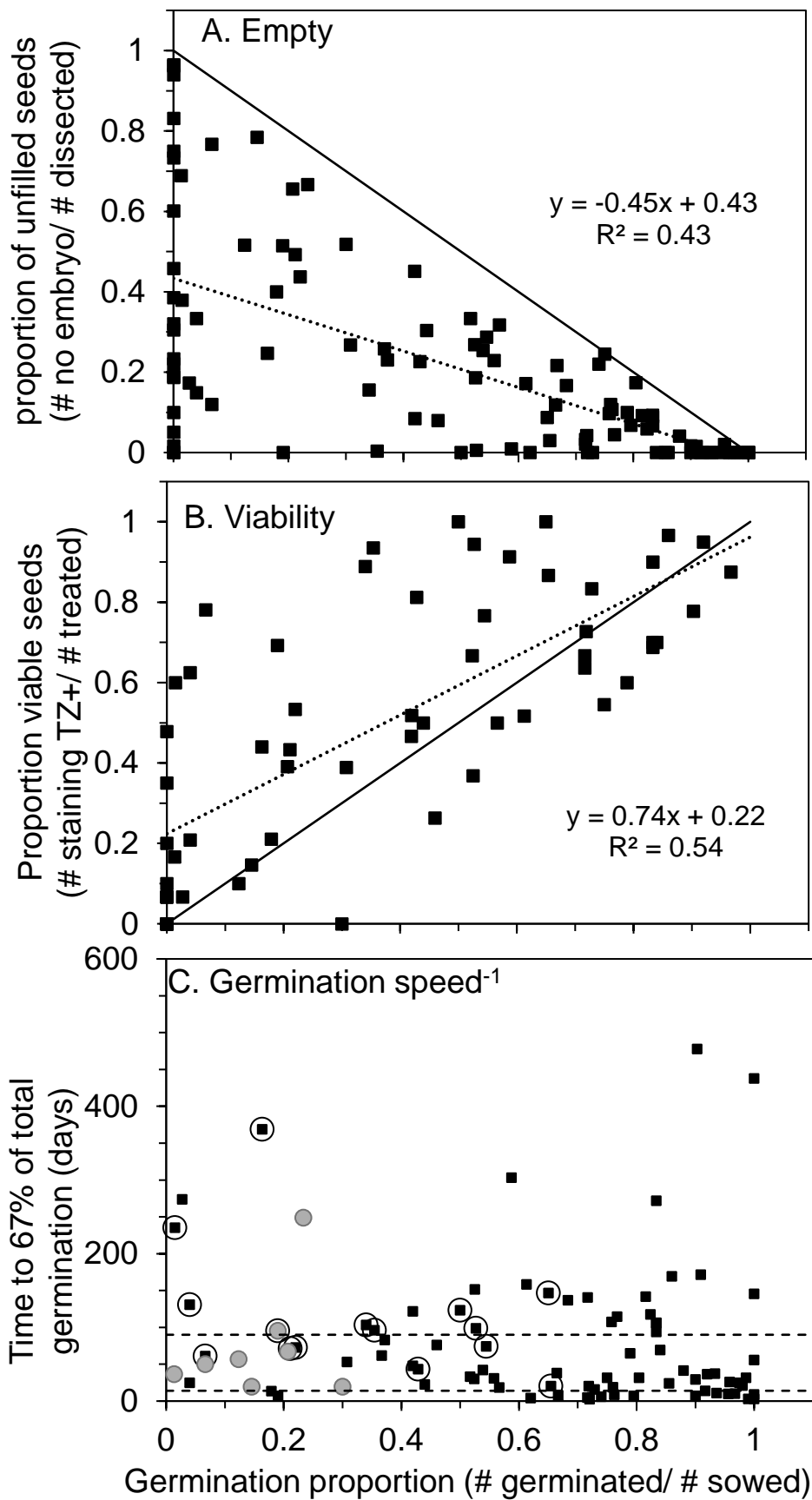


Figure 5

Figure 5. Relationships between germination proportion and other factors of seeds quality for stored seeds: proportion of empty seeds (A), proportion of viable seeds based on TZ staining (B) and germination speed⁻¹ (time for ⅓ of the total seeds to germinate in an assay) (C). Regression (dashed) and 1:1 (solid) lines are plotted in A and B ($r^2 = 0.43$ (n=108) and 0.54 n=59), respectively; $P \ll 0.01$). The numerous points falling below the 1:1 line in A demonstrate additional factors besides empty seeds play a role in low germination. Points well above the 1:1 line in B suggest that, despite low germination, seeds are viable and likely dormant. The dotted horizontal lines in C demark germination times that are considered fast (within 15 days) and slow (more than 90 days). Encircled points represent seeds that are believed to retain dormancy according to TZ assay results (Fig 5B) and gray points have high proportions of empty seeds: ≥ 0.5 (circles).

30°C