Seed biologists beware: end-of-trial estimation of initial viability may be error-prone due to unexpected seed death during the experiment

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Abstract: Seed viability is routinely measured on seeds that fail to germinate at the end of an experiment. Together with the number of germinants, this is used to estimate viability of the seeds at start of the experiment (i.e., initial viability) and provides the comparative basis on which germination success is determined. We used this standard procedure on 40 *Leucadendron* species subjected to oscillating temperatures, heat and/or smoke pre-treatments to examine the extent to which they raised germination levels above that of the untreated controls. 16 species showed significantly different levels of estimated initial seed viability between treatments when they should have been unaffected. Loss of viability during the trial was an order of magnitude greater than annual loss during cold storage, which was usually negligible. Lowest levels of estimated initial viability occurred among the poorly germinating controls and confirmed that the heat and smoke treatments had little effect on viability. Species with soil-stored seeds were more vulnerable to this artefact than those with plant-stored seeds. We caution against the routine use of end-of-trial germination and viability of ungerminated seeds as an estimate of initial viability in determining germination success of various treatments. The preference is for estimates of initial viability to be undertaken on a separate sample of seeds in association with the trial.

Keywords: germination; *Leucadendron*; seed storage; seed viability estimation; viability loss

1. Introduction

Determining initial viability is a vital part of testing the germination potential and requirements of seeds, since germination success can only be compared against seeds that were capable of germinating in the first place. Researchers usually gauge seed viability at the start of a trial/experiment (initial viability) on the condition of seeds remaining at the end of the trial plus those that had germinated: a representative sample of 13 of such studies are described in Table 1. Thus, estimated initial viability [1](Gosling (2003) is given by: (viable seeds present at end of trial + germinants)/(total seeds in trial). The results are then corrected for viability: (seeds germinated)/(total seeds in trial × initial viability). This economizes on the number of seeds needed for the trial as it is not necessary to 'waste' seeds by testing for viability on separate samples before the trial begins. This can be important when seeds are scarce if the species is rare, or seeds are difficult to collect or expensive to purchase. It also removes any time-lapse loss of viability between estimating viability before the trial begins and undertaking the trial itself. Further, it expedites the testing task as only ungerminated seeds need be examined for their viability.

It also avoids the need to use a mean value obtained pre-trial to apply to all treatments that ignores sample effects on the viability of seeds used as 'actual' initial viability of the seeds used in each replicate can be estimated.

We used this standard procedure in a study of the fire-related germination requirements of 40 representative species in *Leucadendron*, an abundant shrub genus of Proteaceae in the fire-prone Cape of South Africa [2](Williams 1972, ([3]Tonnabel et al. 2018). On collating the data we noted what appeared to be differences in the calculated levels of initial viability between treatments for some species, especially among the untreated controls, when they should have been the same. This indicated that there might be an unanticipated treatment effect on the viability of seeds remaining at the end of the trial, biasing their use for estimating initial viability. We checked the literature and found that Hay and Probert [4](2013) had already warned that seeds kept for prolonged periods under (suboptimal) experimental conditions can die though they did not present supporting data. Inspection of 13 representative papers that used the end-of-trial approach in Table 1 revealed several with variable post-trial estimates of initial viability that were not commented on by the authors [5](Clarke et al. 2000, [6]Hall et al. 2017, [7]Gómez-González et al. 2017). That is, estimates of initial viability varied between treatments and, unexpectedly, were especially low among the untreated controls. As an example of the potential problem, our collation of data from Gómez-González et al. [7] highlights a case where estimates of viability for three species were anomalously low among the controls compared with the treatments (Figure 1).

Here, we compare the calculated initial seed viability of the 40 *Leucadendron* species under 4–5 treatments, and select out some to show how this varied with the levels of germination, seed storage type and duration of the trial, and compare the results against loss of viability during cold storage in the laboratory. We show that loss of viability may occur during the trial, especially among the control treatments with little germination, that will lead to erroneous estimates of germination success if this artefact is not recognized. We therefore warn against routine use of post-trial estimates of initial viability, especially if the trial is long-running.

Table 1. Examples of studies that used assessment of seed viability and germination at the end of the experiment to estimate initial viability.

Species tested	Method of determining initial viability from end-of-experiment data	Experimental conditions	Differing initial viability between treatments	Reference
21 woody native species of Chilean matorral	Viability calculated as: those germinated during the monitoring period plus non-germinated seeds identified as viable by tetrazolium test	Moist absorbent paper in petri dishes at 20/10°C with 12/12 h light/dark for 36 d	Yes (3 species), data not adjusted for viability	Gómez-González et al. [7]
13 native and one introduced species (*Acacia saligna*) of South African fynbos	Viability determined as the sum of germinated seeds and seeds appearing fresh on dissection of ungerminated seeds	1% water agar at 10/20°C with a 12 h light and dark cycle for 91 d	Yes (at least 2 species)	Hall et al. [6]
65 species commonly occurring on New England tableland (NSW Australia)	Seeds that did not germinate, but looked viable, were analysed using tetrazolium test. Viability based on treatment with highest germination plus any seeds that remained dormant but viable	Moist pad in dish at 25/15°C with a 12/12 h light/dark for 28 or 56 d	Probably (as used highest viability levels between treatments)	Clarke et al. [5]
Asterolasia buxifolia, riparian habitat of SE Australia	Embryo dissected from 20 seeds that did not germinate and viability confirmed if embryo and endosperm intact	Moist filter paper in petri dishes at 11/3°C with 12/12 h in light/dark for 77 d	Data not adjusted for viability*	Collette and Ooi [8]
10 native species of Mediterranean Basin maquis	Embryo dissected from 20 seeds that did not germinate and viability confirmed if embryo intact	Soil in trays in glass house for 84 d over winter-spring, Perth, Australia	Data not adjusted for viability	Crosti et al. [9]
46 legumes species of tropical savanna, Brazil	Initial viability equals the sum of germinated and dormant seeds in control	Moist filter paper in petri dishes at 27°C with 12/12 h light/dark for 28 d	No data at treatment level	Daibes et al. [10]
13 species of West African savanna woodland	Cut test – condition of embryo, conducted on ungerminated seeds post-trial	Moist filter paper in bell jars at 25°C light for 30 d	No data at treatment level	Dayamba et al. [11]
9 species in Brazilian Cerrado	Cut test for post-treatment seed viability	0.9% water agar in petri dishes at 25°C with a 12/12 h light/dark for 30 d	No	Fernandes et al. [12]
7 native perennial forb species of grasslands and woodlands of SE Australia	Cut test - condition of embryo on ungerminated seeds post-trial	1% water agar at 25/15°C with a 12/12 h light/dark for 56 d	No data at treatment level	Hodges et al. [13]
2 alien and 2 indigenous legume species in S African fynbos	Germination level of scarified seeds conducted at same time as other treatments	0.02% benomyl solution in petri dishes at 20°C with 12/12 h light/dark for 30 or 60 d	No data at treatment level	Jeffery et al. [14]
3 species of *Acronychia* in E Australian rainforest	Ungerminated seeds checked for firmness by pressing seed with forceps. Then firm seeds checked for viability via cut test	0.8% water agar in petri dishes at 25/10°C with a 12/12 h light/dark for 28 d	No data at treatment level	Liyanage et al. [15]
9 herbaceous species in Brazilian grassland	Tetrazolium test on ungerminated seeds	Moist filter paper in petri dishes at 20°C or 25°C at 16/8 h in light/dark for 21 d	No data at treatment level	Overbeck et al. [16]
Brassica napus	Cotyledon condition of ungerminated seeds post-trial. Necrotic cotyledons = nonviable, yellow-milky cotyledons = viable	Moist filter paper in petri dishes at 20°C in dark for 35 d	No	Shayanfar et al. [17]

*Viability 80% at 100°C but only 65% at the lower temperature of 80°C appears anomalous and might indicate an anomalous treatment effect on viability (they should have been the same) but no statistical analyses were undertaken
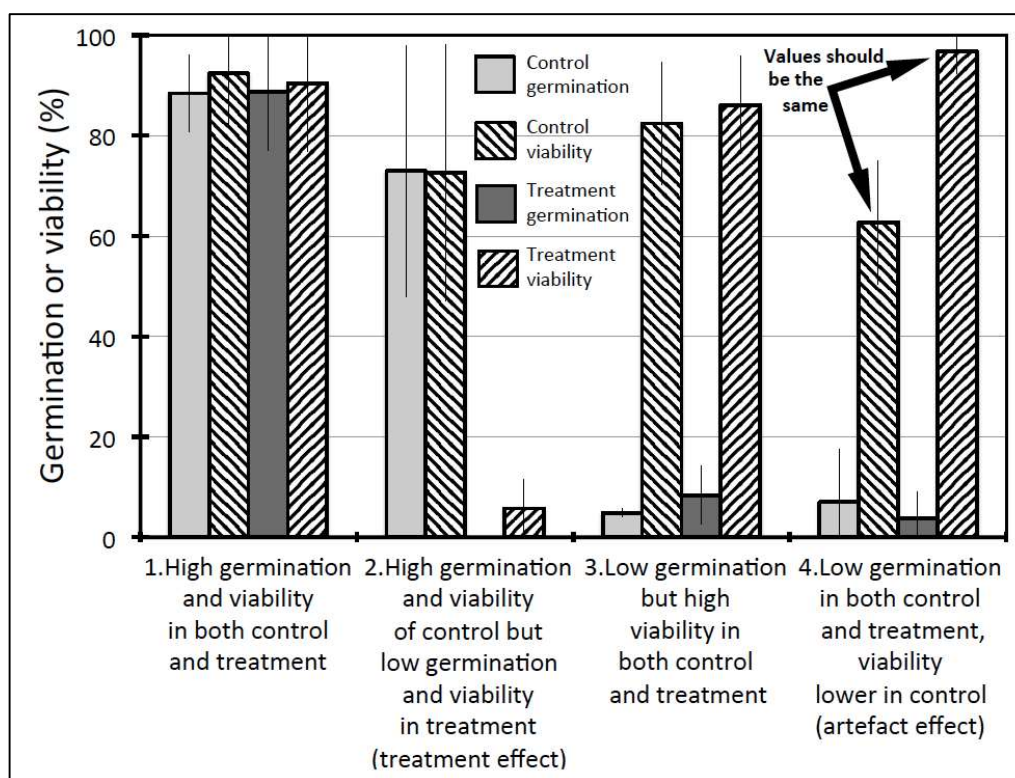
Figure 1. Example of anomalous reduction in viability of some controls.

Data collated from Gómez-González et al. [7] divided into four categories varying in germination and viability determined at the end of the trial with three species in each category. Results are means ± 95% CIs. Note the last category where viability of the control is on average 36% less than the heat treatment (100°C for 3 min), that must be an artefact of the experimental method as, at best, viability of both should be the same (categories 1 and 2) and, at worst, the treated seeds would have lower viability at the end of the trial than the controls if the heat was excessive as in category 2 (not the reverse as here). Note that the unexpected control effect occurs among species with little germination of the controls but it is not unique in that respect (for example, see category 3 that also has low % germination but without reduction in viability).

2. Results

Table A1 summarizes seed viability differences among 40 *Leucadendron* species as determined by the 'cut' test. The tests of viability carried out during 0–10 years of cold storage showed that there was zero effect on viability at the various intervals for 12 of the 17 species assessed, with a mean loss of 0.43% pa (Table 2, Figure 2). There was no trend in viability loss with time and none of the five species with non-zero values was significantly > 0. There were differential effects between the 4 treatments on estimates of viability at the start of the trial for 16 of the 40 species (Tables 2,A). The maximum difference in viability estimates between any two treatments within a species ranged from a mean of 1.2% (non-significant) to 35.8%, with a mean difference of 6.4%, and 8.7% for the 17 species in common with those in cold storage assessed (Table A1, Figure 2). Thus, seeds were likely to lose 20× more viability over the 2–5 months of the trial than during 12 months in cold storage. This can be compared with the 1.5% maximum difference in filled seeds between any two treatments that was a random effect for all but two species for which the difference was significant. Thus, the difference in viability for 14 (16 − 2) species between treatments that developed during the trial is a non-random effect.

Table 2. Loss of seed viability among 17 *Leucadendron* species air-dried and stored at −20°C for one to 120 months, and maximum differences in estimated initial viability (on embryo-filled seed basis) and embryo-filled (on total seed basis) between 4–5 treatments designed to identify conditions that break dormancy among 40 *Leucadendron* species distinguished according to whether they store seeds on the plant or in the soil. Mann-Whitney tests of A to D to put each species into its appropriate category (Table A1), followed by contingency table analyses between categories.

Where stored	On plant		In soil		Significance
Viability changes/differences	Yes	No	Yes	No	*P*
A. Loss of viability during cold storage (% pa)	0	9 (7 at 0)	0	8 (5 at 0)	>> 0.05
B. Significant maximum differences in seed viability between 4 treatments	10	21	6	3	< 0.05
C. Significant maximum differences in filled seeds between 4 treatments	1	30	1	8	> 0.05
Comparison	B		C		
Significant differences	Yes	No	Yes	No	
D. Significant maximum differences	16	24	2	38 (12 at 0)	< 0.05
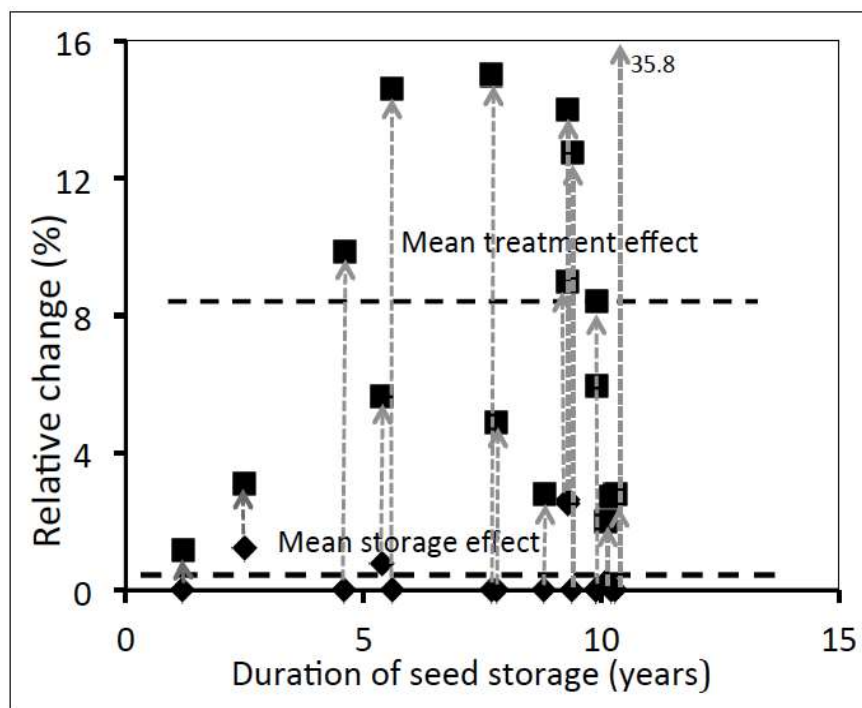
Figure 2. Comparative loss of viability during cold storage compared with during the experiment.

Loss of binomial mean seed viability (relative change) among 17 *Leucadendron* species (data incomplete for the remaining 23 species, Table A1, so omitted) during cold storage varying in duration as shown (♦, loss rate per year of storage, %) (viability remained at 100% for 11 species) compared with the same source of seeds subjected to 4–5 treatments (Table A1) over 2–5 months (■), with the greatest difference in binomial means between treatments on viability plotted. The loss rate for stored seeds was assessed on 2–3 viability tests for the period indicated.

The ten species selected showed large differences in the relationship between % germination and % initial viability (Figure 3). The logarithmic slope varied from 0.0125 (negligible relationship between germination and viability) to 0.1612 (rapid decline in viability as germination declined). A linear slope was applied to *L. foedum* as all seeds that did not germinate were nonviable ($R^2 = 1.000$). From these curves, the viability data were extrapolated back to the diagonal in Figure 3, i.e., where viability and germination levels

coincide and represent the maximum (true?) initial viability of that batch of seeds, ranging from 100% (~ six species) to 66% (*L. corymbosum*) (Table 3). At a nominated germination level of 20%, estimated initial viability fell from a minimum of 95.7% (negligible effect of the treatment on viability, despite the low germination) to a maximum of 20.0% (viability is a function of germination level on grounds that seeds which did not germinate lost viability). The net loss in viability from treatments associated with 60% to 20% germination ranged from 2.0% (negligible effect) to 40% (total dependence) (Table 3).
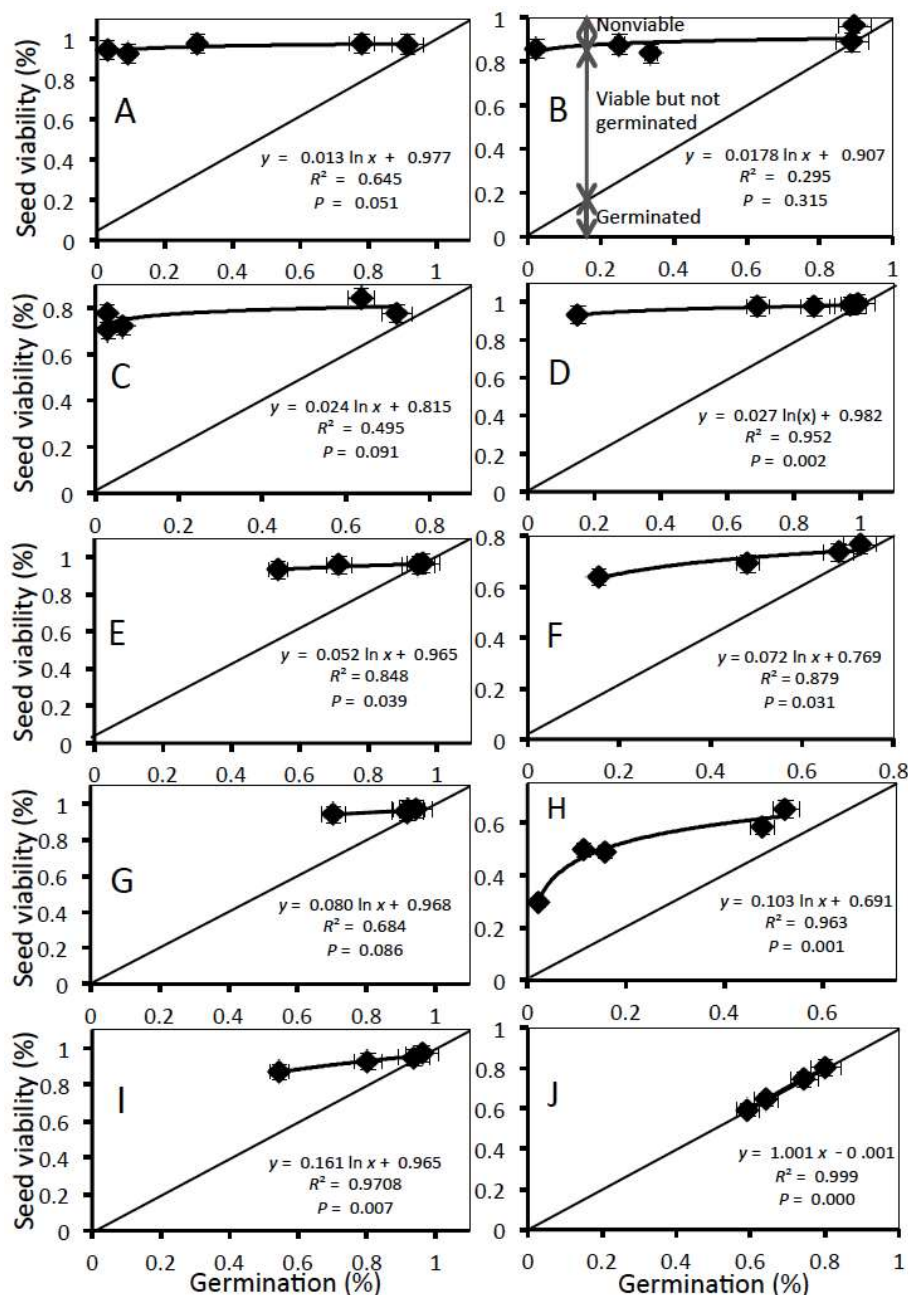
Figure 3. Level of germination (binomial mean ± binomial SE) for 10 *Leucadendron* species in 4–5 treatments as it correlates with initial seed viability (binomial mean ± binomial SE) estimated at the end of the trial. Species listed from those whose viability was least to most affected by the treatments according to the fitted slopes. The trend lines are the logarithmic relationships between the data, with the associated equation, R^2 and P values given. The diagonal is that position where all viable seeds germinate. The closer estimated viability is to the diagonal the greater it is a function of its germinability. Note that the lowest viability value for a given species is invariably the control (lowest germination). A = *L. elimense*, B = *L. laxum*, C = *L. brunioides*, D = *L. chamalaea*, E = *L. galpinii*, F = *L. thymifolium*, G = *L. dregei*, H = *L. corymbosum*, I = *L. modestum*, J = *L. foedum*.

Table 3. Relationship between estimated binomial mean initial seed viability (%) and germination (%) for 10 *Leucadendron* species from logarithmic curve fits for 4–5 treatments (except linear fit for *L. foedum*).

Leucadendron species	'True' initial viability by extrapolation to diagonal (Figure 3)	Estimated initial viability at 20% germination	Loss of viability as germination falls from 60% to 20%
elimense	98	95.7	2.0
laxum	92	87.8	2.9
brunioides	83	77.6	3.9
chamalaea	100	93.9	4.3
galpinii	98	88.1	8.3
thymifolium	76	65.3	11.6
dregei	99	83.9	12.8
corymbosum	66	52.6	13.4
modestum	99	70.6	25.9
foedum	100	20.0	40.0

As *L. corymbosum* showed the greatest difference in estimated initial viability between treatments (35.8%) (Table A1, Figure 3), it was selected for in-depth comparison of four treatments each with five replicates for any relationship between germination failure and nonviability (Figure 4). This showed that, as germination levels decreased, seed viability decreased at a similar rate, with 5–6% remaining alive but not germinating. If viability was unaffected by treatment effects on the level of germination then there would be a horizontal line running at a point equal to the 'actual' level of nonviability right of the diagonal line (nonviable seeds cannot germinate).

Duration of the trial for each replicate was compared between the treatments using the highest and lowest levels of estimated initial viability for each species. This period varied from 57 to 175 days with a mean of 131 days and the values were identical between the pairs of values for each species. Thus, there was no within-species effect on viability associated with duration of the trial. For four species with marked treatment effects on estimated viability, Figure 5 shows a linear relationship between duration of seeds in the treatment with increased loss of viability. Because of the lack of data for the 80–100-day period, the results are bimodal and do not clearly fit any unimodal relationship. Nevertheless, a linear trend can be detected, although the relationship only accounted for 12% of variation in the data. On average, 23% of seeds were nonviable if left in the treatment for 60 days and 39% were nonviable if left for 140 days, so that duration of the treatment without germination had a small but significant effect on seed viability. This means species that were slower to germinate were more likely to lose viability than those that were faster to germinate (under the treatment for a shorter time).
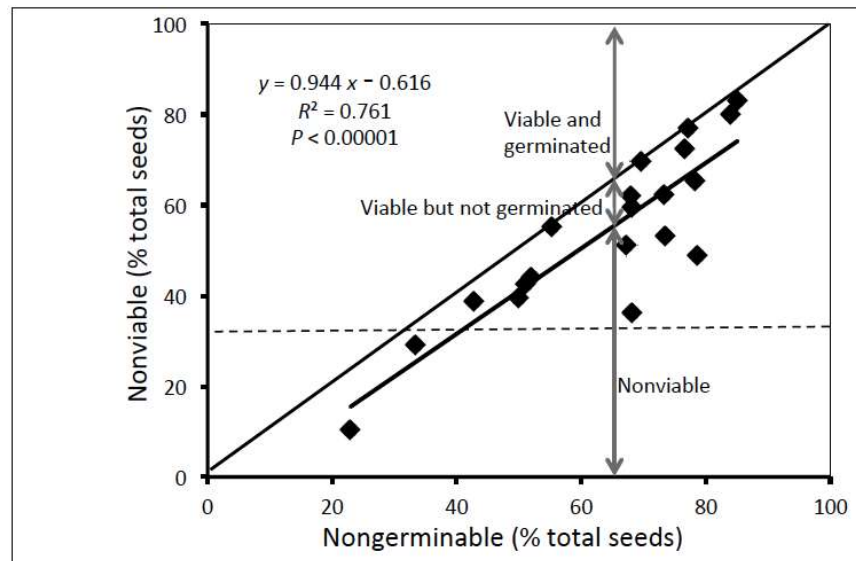
Figure 4. Estimated initial viability vs germinability of seeds of *L. corymbosum.*

Estimated initial viability vs germinability of seeds of *L. corymbosum* over four treatments each with five replicates, all plotted. If initial viability (V) was 66% (Table 2), then nonviability (NV = 100 − V, when G = 0) should follow the broken line at 34% as shown, independent of the level of subsequent germination (G). The diagonal represents the situation where all seeds that do not germinate are not viable (100 − G = 100 − V). The fitted slope represents that fraction of nongerminable seeds that was nonviable as a treatment and/or replicate effect. This shows that, as germination decreased (left to right), estimated initial viability decreased (bottom to top) is parallel to the diagonal [when it should have remained constant at 66%, as those seeds that do not germinate should re-main viable: NV (constant) = 100 − G↓ − V↑], with a constant 5–6% remaining alive but not germinating.
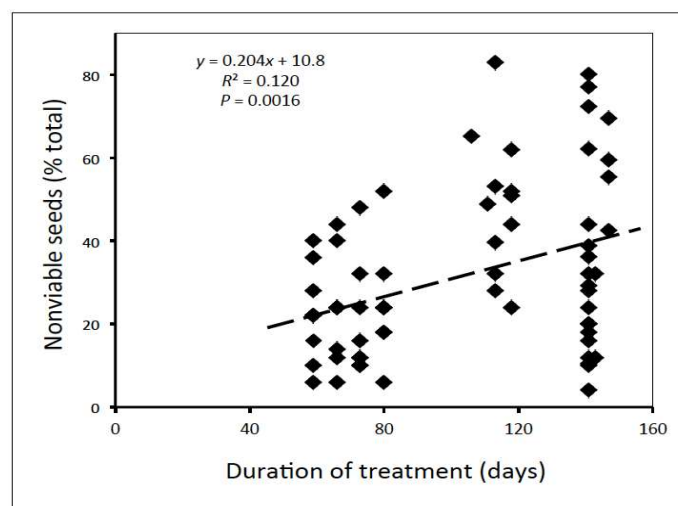
Figure 5. Nonviable seeds present at the end of the trial compared with duration of the trial.

Nonviable seeds present at the end of the trial compared with duration of the tri-al.for all replicates and treatments for four species that showed marked treatment effects on initial viability estimates (Table A1) and covered a wide range of treatment durations: *L. corymbosum*, *L. foedum*, *L. loranthifolium* and *L. laureolum*. The fitted line is broken to indicate that the data do not describe a normal curve and do not fit the assumptions of

the model so it can only be used as guide to the actual relationship. The calculated relationship is weak, accounting for only 12% of the data assuming that they were normally distributed.

3. Discussion

It is standard practice for convenience and efficient use of scarce seed resources in germination studies to estimate viability of the seeds used in a trial (i.e., initial viability) on germinants and viable seeds remaining at the end of the trail. It is assumed that the viability of seeds distributed across treatments at the start is the same, i.e., any sample effects associated with allocating individual seeds to treatments are both random and negligible (this assumption certainly applied to seeds that possessed embryos among the 40 *Leucadendron* species reported here, Table A1). However, we detected up to a 36% difference in estimates of mean initial viability between treatments for individual species determined at the end of the trial. We also noted similar marked differences in apparent initial viability between treatments in other studies (Hall et al. [6], Gomez-Gonzalez et al. [7], Figure 1). For one species (*L. foedum*), even though there was a 22% difference in the treatment effects on germination, all ungerminated seeds in each treatment at the end of the trial had died on the basis of the cut test. In all cases, this disparity must have arisen during the period that the seeds remained in the different treatments. This means end-of-trial assessment of initial viability can lead to misleading estimates of germination success if it is based on (apparent) initial viability per treatment where this varies markedly between treatments.

The reason is that the treatment itself, especially the control, may unexpectedly cause some seeds to die. In our case, the unanticipated loss of viability during the experiments could not be predicted from the (small) rate of loss of viability during cold storage. On average, the deleterious effect of experimental treatments/controls on viability was an order of magnitude greater than the effect of storage over successive 12 months and had no bearing on the extent to which different treatments affected viability during the trial.

Taking the approach of using germination as the independent variable and viability as the dependent variable, it is evident that, the lower the germination rate, the more likely ungerminated seeds will lose viability during the trial. Thus, it seems that, to some extent (Figure 5), the longer seeds sit in the medium ungerminated, the more likely they will lose viability. That is, the control treatments, where there is little germination response, are most likely to lead to viability loss (Figure 2). Since this relationship only accounted for 12% of variation in our data for four species, other factors, especially species identity (some species are more vulnerable to viability loss than others), must also contribute to the pattern. At present, we are unable to predict which species are most vulnerable to treatment effects on viability loss, other than to note that soil-stored seeds seem to be particularly susceptible (all species used by Hall et al. [6] and Gomez-Gonzalez et al. [7] were soil-stored), possibly because they may take longer to germinate, and, to a minor extent, species whose maximum viability is not high (here, *L. corymbosum*, *L. thymifolium*).

A procedural problem is indicated when the controls show lower estimated initial viability than some treatments (e.g., smoke treatment among some soil-stored species in Hall et al. [6], heat treatment of legume seeds in Gomez-Gonzalez et al. [7] – see Figure 1). As a result of these findings, we caution against the routine use of end-of-trial assessment of initial viability in determining germination success of various treatments. Values may prove to have been affected by the experimental design. This could be especially serious where a wide range of treatments may have vastly different effects on estimated viability as we show here for certain species. The problem may be especially difficult to detect when in fact some treatments, e.g., application of fire-type heat, can be expected to cause loss of viability.

Since seed viability loss has a time-dependent component (Ellis and Roberts [18]), if pretrial assessment of initial viability is not practicable, then there is a case for avoiding

prolonged duration of ungerminated seeds in the treatments. But the answer is not to terminate the trial early, as species vary greatly in their rates of germination; use some other rule, such as terminate if no further germination at twice the interval since the last germination was recorded. Note that terminating the trial at different times between treatments is not a problem as the objective is for all treatments to reach the same stage (start of asymptote) rather than choosing an arbitrary common time when this stage may not have been reached among some slow-germinating treatments. Even so, there was no clear temporal trend among studies in the brief literature survey that we undertook (Table 1). In addition, the best-fit line can be extrapolated back to the diagonal (X = Y, Figure 3) and this value (estimated Y) used for initial viability in calculating germination success. If the figure is substantially < 100%, whether there is a negligible effect of treatment on the level of estimated viability or not, then either a) this represents low viability at the start, or b) the general experimental design has led to a loss of viability. Where a wide range of related species is used and most values approach 100%, as in our case, the latter possibility seems unlikely. As a final but less satisfactory compromise, the treatment that gave the highest viability estimate is used and applied it to all treatments and control (Clarke et al. [5]) that is often near the extrapolated viability value anyway (Figure 3).

The preference is for viability estimates to be undertaken on a separate sample of seeds just before the experimental trial begins, or concurrently with it. This is especially important where pretreatments, e.g, high temperatures, are expected to kill some seeds (Liyanage and Ooi [19]). This means that, to minimize sample effects on pretrial estimates of initial viability, the number of seeds tested need be at least equivalent to the number used in the various treatments. Of course, identifying empty (no embryo), damaged or infested seeds at all stages of the trial is required (Leonard, West and Ojeda [20]), as a separate task from pretrial determination of initial viability of intact seeds. Note that there may be merit in determining the most suitable method for estimating viability before the experiment begins. Thus, Lamont and van Leeuwen [21] showed that there was no difference in estimates of viability of *Banksia tricuspis* using the cut and tetrazolium tests and thus opted for routine testing with the simpler cut test. It has not been our purpose here to compare different methods of determining seed viability (of which there are many) but we note that sometimes results are based on germinable (imbibed) seeds rather than checking directly for viability (Herranz, Ferrandis and Martínez-Sánchez [22]). However, even imbibed seeds may be nonviable (dead tissues can imbibe), a problem that is exacerbated when there are treatment effects on viability as described here.

It is an interesting final point to consider whether end-of-trial viability should also be estimated to determine if there is an experimental design effect (Liyanage and Ooi [19]. This is important as the ability of the treatment to break dormancy is usually gauged via the level of germination. If final viability is low and treatment independent, it raises the issue whether the general germination conditions imposed are unsuitable for that species, and that other approaches should be considered. Possibilities include using different germination media, such as sand; different incubation temperature regimes; different light and dark exposures, and methods that avoid anoxia due to waterlogging. The method of determining viability may also underestimate levels, e.g., meristematic tissues need to be active for responses to the tetrazolium test (Gosling [1]).

4. Materials and Methods

Seeds of 40 *Leucadendron* species (Table A1) were collected from wild plants in the Western and Eastern Cape Provinces of South Africa and sent to the Millennium Seed Bank at Wakehurst Place in West Sussex, England, and dried to equilibrium at 15% RH and 18°C. Nomenclature follows Rebelo (23). Details on the duration of storage are given in Newton et al. (24), doi.org/10.1007/s00442-021-04947-2, Supplementary file1 (DOCX 144 KB). Note that results in that paper were based on filled seeds, not viability/germination at the end of the trial as an estimate of initial viability, as discussed here. Dried seeds were stored at international gene bank standards of −20°C (FAO [25]). Viability of the pre-trial seeds was assessed by the 'cut' test after incubating seeds on 1% water-agar at

20/10°C for 2 weeks: embryos that were white, fully formed and filled the seed cavity were treated as viable and those that were discoloured, shrunken or incompletely formed were treated as nonviable. Binomial mean viabilities at various times during cold storage were calculated.

A fully orthogonal design was used to test the individual and combined effects of direct fire cues (heat and smoke) on postfire germination of 37 species. For three other species, only the combined effect of heat and smoke was examined due to limited seed availability. An after-ripening treatment to simulate exposure to summer temperatures after fire [8 weeks of dry storage at alternating temperatures of 40/20°C (12 h light / 12 h dark)] was applied to all seeds. Seeds in the control treatment received the postfire after-ripening treatment but no heat or smoke. Seeds in the heat treatment and the combined heat plus smoke treatment were heated prior to the postfire after-ripening treatment. Seeds in the smoke only treatment and the combined heat plus smoke treatment received a smoke treatment after the postfire after-ripening treatment.

4.1. Treatments

Seeds to receive the heat treatment were divided into five replicate batches of either 50 or 25 seeds per batch for each species and treatment. They were placed in open metal dishes as a monolayer in a fan-assisted, LTE Scientific Incubator with digital temperature control for 20 minutes at 80°C (VWR International, Lutterworth, UK). This treatment was chosen to avoid temperatures that might be lethal (Moreira et al. [26]) but simulate the heat from a heathland fire passing over seeds located at a depth of 1 cm. For the smoke treatments, Regen2000® Smokemaster liquid smoke solution (Batch number 11468, Grayson, Australia) was filtered through a sterile 0.2 μm Nalgene cellulose acetate ×50 filter syringe (Fisher Scientific, Loughborough, UK) to exclude microbes and large particulates prior to dilution with distilled water (1:10). At the end of the postfire after-ripening temperature period, all seeds were placed in individual Coulter® Counter containers (Scientific Laboratory Supplies, Nottingham, UK). For each species, 10–20 mL of dilute smoke solution (or distilled water) were added to containers. The containers were sealed with polyethylene lids and put into an incubator at 20°C. After 24 h, seeds were removed from the distilled water or smoke solution and blotted dry. An extra treatment was applied to seven species that were maintained at 25°C (12 h light / 12 h dark) instead of 40/20°C that assumed the seeds were not subjected to postfire summer heat before the wet season began.

4.2. Germination trial

Following the above pre-treatments, seeds were sown on 1% water-agar in 90-mm Petri dishes. These were then placed in temperature-controlled incubators set at 20/10°C (12 h light 12 h dark). Seeds were scored as germinated and removed once the radicle was longer than 2 mm. Seeds visibly contaminated with fungal mycelium were assumed to be non-viable initially and were removed (negligible in fact), while the remaining seeds were placed on fresh water-agar to prevent fungal spread. Germination tests were terminated if there was no germination in any replicate for at least four weeks. This ensured that the germination level of slow germinating species was not artificially low by terminating the trial before their asymptotes had been reached. Any seeds that had not germinated on completion of the trial were examined for evidence of viability in the same way as when tested during storage. Viability at the onset of the trial (i.e., initial viability) was determined as 100(germinated seeds + viable seeds remaining at the end of the trial)/(total seeds − empty seeds = filled seeds). Binomial means for initial viability were calculated.

The rate of viability loss (% per year) during cold storage was determined as (Viability time 2 − Viability time 1)/(time period 1 to 2 in years). Possible treatment effects on initial viability estimates were calculated as (Treatment with highest initial viability − Treatment with lowest initial viability) and were compared against treatment effect on filled seeds (Treatment with highest % filled − Treatment with lowest % filled). If treat-

ment effects were random, as they would be for filled seeds at the start of the experiment, then there would be no significant difference in the range between filled and initially viable seeds. If the post-trial estimates of initial viability were significantly different between two treatments, but not between filled seeds, this means that the treatment itself had an effect on viability.

Significant differences in estimated initial viability between treatments, or within filled seeds and initial viability of treatment pairs, were determined by the non-parametric, paired Mann-Whitney test or contingency table analysis, including Fisher's exact test where required (Vassarstats.net, ©Richard Lowry 1998–2021), that do not assume normally distributed data. % germination (G) was plotted against % initial viability (V) for ten species that showed various levels of treatment effects on estimated initial viability. Independent of treatment, we expect: $G + \Delta V = V$, where ΔV is the proportion of V (as a %) that did not germinate and V is a constant that should not be affected by the treatment and is usually close to 100%. Although these two variables are not independent (G is a component of V), we plotted them to a) show visually variations between species in the severity of treatment effects on estimated initial viability, b) determine the rate at which V can decline with a reduction in G due to treatment effects via trend lines, and c) as a device for enabling fitted curves to be extrapolated back to the diagonal where V = G provides an estimate of 'actual' initial viability. The logarithmic curve fitted most data sets so was used throughout to enable comparisons between species. Since only 4–5 data points, representing binomial means of the different treatments, were available, we were not so interested in the statistical significance of the results as to use them to show the anomalous downward trends in estimated initial viability at lower germination levels among some species when they should have been the same.

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Appendix

Table A1. Location of seeds stored by 40 *Leucadendron* species, with serial numbers, used in this study plus maximum differences in filled seeds (filled/total) among four treatments, and effect of cold storage and four experimental treatments on their estimated initial viability (viable/filled seeds) estimated with 'cut' test at end of trial. *difference significant at < 5% with Mann-Whitney test. Max = maximum. Further details in Newman et al. [24], doi.org/10.1007/s00442-021-04947-2, Supplementary file1 (DOCX 144 KB)

Leucadendron species	Millenium Seed Bank Serial No.	Stored on plant (P) or in soil (S)	Viability loss during cold storage (% pa)	Max viability difference between 4–5 treatments estimated at end of trial (%)	Max difference in seed fill between 4–5 treatments (%)
album	190013	P	2.5	9.0*	2.4
argenteum	244145	P	–	6.6*	3.2
brunioides	189761	S	2.6	14.0*	2.8
chamalaea	457752	S	0.8	5.7*	2.8
comosum	792439	P	–	6.9*	0.8
coniferum	189185	P	–	6.4*	0.0
corymbosum	214588	S	0.0	35.8*	1.6
discolor	214810	P	–	2.8	1.2
dregei	792440	P	–	2.6	3.2
elimense	215068	S	0.0	4.9	1.3
eucalyptifolium	189679	P	0.0	2.8	0.0
flexuosum	484101	P	–	1.2	2.7
foedum	486057	P	–	20.4*	0.0
galpinii	214278	P	–	3.7	4.9
gandogeri	189521	P	0.0	2.8	0.0
lanigerum	214991	P	–	3.6	1.2
laureolum	189152	P	–	10.0*	0.0
laxum	215116	S	0.0	12.7*	1.2
linifolium	189196	P	0.0	11.6*	2.0
loranthifolium	302508	S	0.0	14.6*	1.2
meridianum	327170	P	–	5.3	0.4
microcephalum	213950	P	–	4.1	0.8
modestum	389697	P	0.0	9.9*	5.7*
muirii	188672	P	–	2.4	0.8
nervosum	792462	P	–	3.2	1.3
nobilis	214234	P	–	2.8	0.0
procerum	189255	P	0.0	8.4*	0.0
rourkei	279394	P	0.0	2.7	0.0
rubrum	189750	P	0.2	2.0	1.2
salicifolium	189819	P	–	2.0	0.0
salignum	190035	P	–	4.0	1.8
sericeum	512228	S	1.2	3.1	0.4
spissifolium	189233	P	–	7.6*	1.5
stelligerum	215091	P	–	4.6	0.4
strobilinum	484042	P	–	3.2	0.0
teretifolim	214050	P	–	2.8	2.0
thymifolium	792484	S	–	12.8*	10.2*
tinctum	160283	S	0.0	1.2	0.0
uliginosum	189222	P	0.0	5.9*	1.6

xanthoconus	189141	P	–	3.4	0.0
	Overall mean for all species		0.4	6.7	1.4
	Mean for 17 species in common		0.4	8.7	1.5

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