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Fiona R. Hay^{1,2*} , Rachael M. Davies³ , John B. Dickie³ ,
David J. Merritt^{4,5}  and Dustin M. Wolkis^{6,7} 

Research Opinion

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***Author for Correspondence:**

Fiona R. Hay,
E-mail: fiona.hay@agro.au.dk

¹Department of Agroecology, Aarhus University, Forsøgsvej 1, Slagelse 4200, Denmark; ²School of Agriculture, Policy and Development, University of Reading, Earley Gate, Whiteknights Road, PO Box 237, Reading RG6 6EU, UK; ³Seed and Lab-Based Collections, Royal Botanic Gardens Kew, Ardingly, Haywards Heath, Sussex RH17 6TN, UK; ⁴Department of Biodiversity, Conservation and Attractions, Kings Park Science, Biodiversity and Conservation Science, Kings Park, WA, Australia; ⁵School of Biological Sciences, The University of Western Australia, Crawley, WA, Australia; ⁶Department of Science and Conservation, National Tropical Botanical Garden, Kalāheo, HI, USA and ⁷Natural History Museum of Denmark, Faculty of Science, University of Copenhagen, Copenhagen, Denmark

Abstract

Understanding the relative longevity of different seed lots, perhaps of different species or genotypes, but also following production under different environments or using different cultivation methods, or following different post-harvest treatments, is relevant to anyone concerned with the retention of seed lot viability and vigour during storage. However, different scientists over the years have used different conditions to assess seed lot longevity, as well as different variables as the measure of ‘longevity.’ Here, we give some of the backgrounds to how two standard protocols, with an open and closed system respectively, were derived, and explain why we consider p_{50} , defined as the time during storage when seed lot viability, as measured through a germination test, has declined to 50%, is a suitable longevity trait parameter.

Introduction

‘How should we measure seed longevity across species, genotypes or seed lots?’ has been a perennial question in seed science over many years, and raised at a number of meetings of the International Society for Seed Science (ISSS). This has perhaps been driven by the recognition many decades ago, that there is wide variation in the longevity of seeds of different species, but at the same time, relatively little variation in the longevity of seeds from different seed lots within a species (Justice and Bass, 1978; Ellis and Roberts, 1980b; Priestley et al., 1985; Roberts and Ellis, 1989), and thence a desire to know the full extent to which seed longevity can vary across diverse taxa and to apply that knowledge to plant conservation and a better understanding of plant reproductive biology and evolution.

As a trait, seed longevity expresses the retention of seed lot viability and vigour during storage, key quality parameters of commercial seed lots (Leprince et al., 2017). Seed lots which are harvested with high physiological quality are likely to have both high storability and, dormancy notwithstanding, germinate well (fast, uniformly and to a high germination percentage) under both laboratory and field conditions (Powell and Matthews, 2012). The seed longevity trait is also important in the context of managing orthodox seed accessions in genebank and conservation seedbank collections, theoretically meaning that seed lots of different accessions or different groups of accessions could be tested for viability at different frequencies. This is still mainly theoretical, since, to a large extent, many genebanks still follow the standard regime of testing every 5 or 10 years (FAO, 2014), but it is certainly not beyond reach. In the context of genebank management, a better understanding of seed longevity will also contribute to better ‘stock management’: predicting when individual accessions might need regenerating or recollecting to ensure availability of seeds with viability above the threshold viability, for distribution to end-users and for the conservation of biodiversity. Measurement of seed longevity is perhaps particularly relevant when it comes to identifying desiccation-tolerant species that are short-lived under long-term conventional genebank/seedbank conditions (stored at approximately -20°C after drying to equilibrium with 10–25% relative humidity (RH) and $5\text{--}20^{\circ}\text{C}$, respectively; FAO, 2014; Royal Botanic Gardens Kew, 2015), for which alternative conservation strategies, such as cryobiotechnologies, for example, cryo-storage of seeds or vegetative tissues, may be more effective in the long-term (Davies et al., 2016; Walters and Pence, 2020; Breman et al., 2021).

Agreement on how seed longevity should be measured has, however, never been reached, and many laboratories have adopted their own ageing conditions (and terminology) to compare the longevity of different seed lots within and/or among species, leading to wide variation in the ageing temperature and moisture content/equilibrium RH of the seeds and, even, the gaseous environment used (e.g. as illustrated in Hay et al., 2019). In this opinion article, we describe what is perhaps the most widely adopted protocol that has been used to measure

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seed longevity across very diverse species, the Millennium Seed Bank (MSB) Partnership's 'Comparative longevity protocol' and suggest how it might be modified for different contexts.

The MSB comparative longevity protocol

In the 2000s, at the MSB of the Royal Botanic Gardens Kew, a 'comparative longevity' protocol was established (Newton et al., 2009, 2014; Fig. 1A), with the aim of applying it across diverse species stored in the MSB, to get an understanding of the variation in seed longevity among MSB accessions and hence prioritise management interventions. There were two parts to the discussions on this comparative longevity protocol: (1) the actual experimental protocol, that is the ageing conditions, sampling times and number of seeds to use and (2) the parameter to consider as the measure of seed longevity. In settling on the ageing environment for the tests, an open storage system was adopted: ageing seeds of different species at the same RH and temperature, so that these factors were the same across species and could easily be maintained for the duration of the storage experiments. Certainly, ageing seeds of different species at the same moisture content would not make sense, since the water activity (or equilibrium relative humidity, eRH) of the seeds would vary depending on the species' seed oil content (Cromarty et al., 1982), that is, it would not be comparing like with like. In terms of the actual RH and temperature adopted for the MSB comparative longevity protocol, first, it was important to be in the moisture range where it is expected that there is a linear relationship between seed longevity and moisture content (see Fig. 1A); 60% RH was chosen as being sufficiently within the range, while ensuring an acceleration of the ageing process, such that it would be possible to collect useful data (i.e. observe a decline in germination) within a reasonable length of time (weeks to months, rather than years). For similar reasons, the ageing temperature of 45°C was chosen, being within established limits to orthodox seed storage behaviour (a predictable decrease in longevity with increase in temperature; Dickie et al., 1990), while also accelerating the ageing process. To ensure that seeds were at the target moisture level when they were placed at the ageing temperature of 45°C, the seeds were initially allowed to take up moisture at 47% RH and 20°C (Fig. 1A).

In discussing the measure of seed longevity to use to compare seeds of different species, it was agreed that the germination data from the storage experiments would be analyzed, as was customary, using probit analysis (e.g. Mead and Gray, 1999), essentially fitting the viability equation,

$$v = K_i - \frac{p}{\sigma}, \quad (1)$$

where v is the viability in probits of the seeds (germination tested upon removal from the ageing conditions) after p days, K_i is the estimated initial viability in probits and σ is the time for viability to fall by one probit (Ellis and Roberts, 1980a). The probit analysis, therefore, provides estimates of K_i and σ , both of which could be considered indicators of seed lot longevity. K_i is a seed lot trait, that is a seed lot of any species could have any reasonable value of K_i , while σ varies between species but, at that time, was expected to be constant for all seed lots within a species stored in the same ageing environment (Ellis and Roberts, 1980b). Thus, we considered σ to be a better measure of relative longevity of seeds of different species than K_i . Nevertheless, without an understanding of the viability equations or probit analysis, σ as

a measure of longevity is rather abstract. Hence, in our discussions, we concluded that it would be better to use p_{50} , the time for viability to fall to 50% during storage (or the storage/ageing period after which 50% of the seeds would germinate upon removal from storage/ageing *sensu stricto*), as a more understandable measure, accepting that, since p_{50} is directly related to σ through the equation,

$$p_{50} = K_i \times \sigma, \quad (2)$$

if we made sure that all the seed lots used for the comparative longevity experiments had similar, high germination in their last routine seed bank monitoring test, the p_{50} values that were determined would reflect σ .

Probert et al. (2009) published estimates of p_{50} for seeds of 195 diverse species from the MSB collection, all aged using the MSB comparative longevity protocol, and were able to explore relationships between p_{50} and climate variables and seed traits. Seeds of some species were found to be particularly long-lived when stored according to the standard MSB protocol; for these species, seeds were placed at 60% RH and 60°C (Probert et al., 2009). The standard seed storage environment was also used by MSB partners, for example, to understand the relative longevity of seeds of the Australian flora, where long-lived seeds are common and hence ageing at the higher temperature was used for a number of species (Merritt et al., 2014), or of the alpine flora (Mondoni et al., 2011), and by others (Long et al., 2008; Börner et al., 2018). A revised version of the protocol requiring fewer seeds was published by Davies et al. (2016), to identify species producing seeds with a short lifespan.

Adapting the MSB protocol for hermetic storage

It is well known that the availability of oxygen in the environment (i.e. hermetic vs. open storage) can influence seed longevity (e.g. Ibrahim and Roberts, 1983; Ellis and Hong, 2007; Schwember and Bradford, 2011). What has not yet been established, is the differential effect of oxygen across different species, which clearly might be expected to vary depending on seed composition. In order for our experimental storage conditions to somewhat better reflect those of genebank storage, in considering the relative longevity of different rice accessions and seed lots at the T.T. Chang Genetic Resources Center of the International Rice Research Institute (IRRI), we decided to use hermetic storage, raising the moisture content of the seeds at a lower temperature than the ageing temperature before hermetically sealing inside aluminium foil laminated packets (the same material as those used by the genebank for medium-term storage and for safety duplicate samples) and transferring them to the ageing temperature (Fig. 1B; Hay et al., 2019; Lee et al., 2019, 2020). We assumed that the oil content would be consistent across seed lots (since we were focusing only on rice) and that the equilibrium moisture content–RH relationship would therefore be similar for all the seed lots. Thus, all the seed samples were expected to have similar moisture content and eRH during ageing at the higher temperature. In a genome-wide association study for seed longevity among diverse *Indica* rice accessions using this hermetic storage protocol, we considered three 'longevity variables': K_i , σ and p_{50} (equations 1 and 2; Lee et al., 2019). Interestingly, there was considerable variation in σ and different quantitative trait loci could be found for the three parameters.

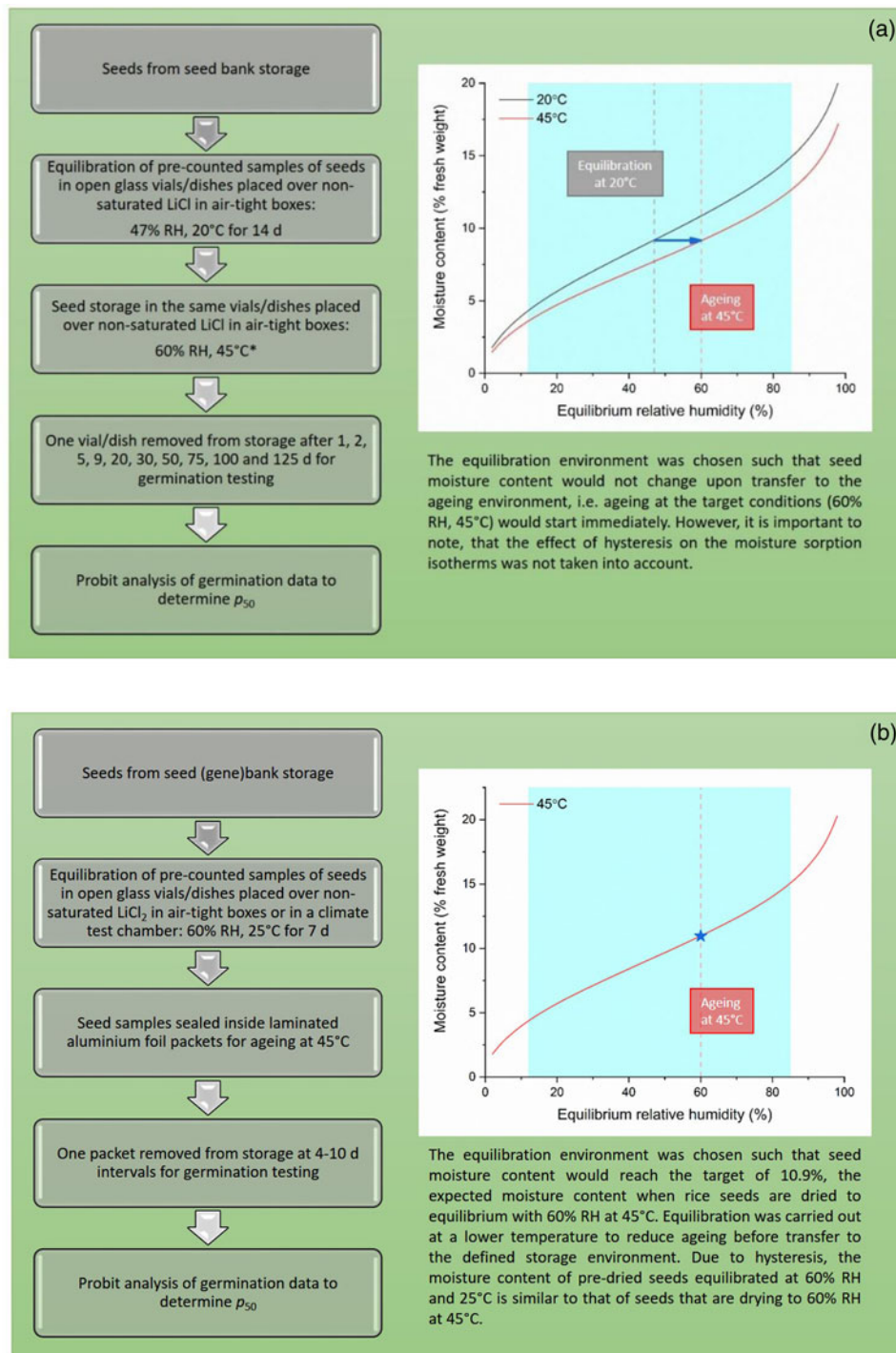


Fig. 1. Outline of the standard seed storage experiment protocols used at (a) the MSB (Newton et al., 2009, 2014) and (b) at IRRI (Hay et al., 2019; Lee et al., 2019, 2020). The isotherms showing the relationship between seed moisture content and equilibrium relative humidity were estimated using Cromarty's equation (Cromarty et al., 1982). In (a) which is for illustration only, an oil content of 20% was used in the equation; in (b), the isotherm was determined for rice (2.2% oil content). The turquoise region of the isotherm is intended to reflect region II of the isotherm (Bewley et al., 2013), where there is a linear relationship (within limits) between seed moisture content and longevity (Roberts and Ellis, 1989). *Reference samples of a control species were included to make sure the ageing environment was consistent across 'runs' which typically involved a number of species. For species' seeds that were found to be long-lived, with little or no viability loss over the 125 d, ageing was carried out at 60°C (Probert et al., 2009; Merritt et al., 2014). A number of species were then aged at both ageing temperatures to determine a correction factor that would allow comparison of the data across species aged at the different temperatures.

We discussed seed longevity phenotyping in relation to rice in Hay et al. (2019), pointing out, that not only did ageing conditions vary considerably between studies, but also the measure of longevity, with the latter often being simply the decline in

germination percentage after a period of storage or even, the germination percentage after storage. Given the sigmoidal shape of the survival curve (Fig. 2), it is clear that the germination after storage/ageing is highly dependent on the initial physiological

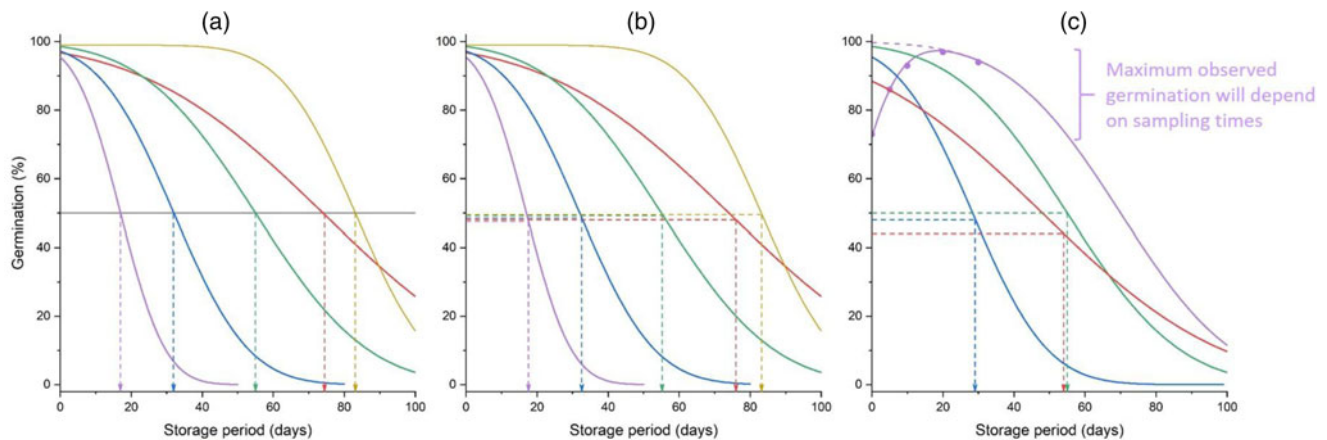


Fig. 2. Illustration of how different definitions of p_{50} would influence the estimate of seed longevity. In (a), the horizontal line is at 50% and p_{50} is estimated as the time when the respective survival curves cross this line. It is also possible to see in (a), how p_{50} would separate seed lots more than, for example, p_{85} (time for viability to fall to 85%). Note, the yellow survival curve is an example where there is a prolonged initial plateau phase before germination percentage declines. For data where there is a plateau phase (not necessarily as prolonged as this), probit analysis should be applied from the last observation with maximum germination (see also Hay et al., 2014). The length of the plateau phase is then indicated by the magnitude of K_i (equation 1). In (b) the survival curves are the same, but the respective dashed lines and arrows, show how the estimate of ' p_{50} ' would vary if defined as the time to half-initial viability. Since the initial viability is similar for the five curves, the p_{50} estimates are not very different from those estimated in (a). However, if there is greater variation in initial viability, or if the seeds show some dormancy which is released during storage (c), the estimates of ' p_{50} ' if defined as the time to half-initial viability would vary more. It can also be seen in (c) how, if there is a dormant seed lot and ' p_{50} ' is defined as the time to half-maximum viability, the time when a sample is removed from storage (circles) could influence the estimated value.

quality of the seeds (i.e. K_i), which as stated above, is a seed lot trait that is highly influenced by the 'history' of the seeds during seed development and after harvest. Thus, neither the change in germination nor the germination after storage are acceptable measures of seed longevity. As well as commenting on seed longevity parameters, we also made some recommendations for seed longevity experiments, including to consider whether the results are intended to get a better understanding of relative seed longevity in, for example, a genebank or a warehouse, or even under local farmer storage conditions (Hay et al., 2019). Interestingly, the moisture content of the rice seeds in our seed ageing protocol was lower than might be the typical storage moisture content of rice seed lots intended for sowing in many tropical countries, illustrating that it is perhaps more benign than some 'real' storage environments, certainly with respect to moisture (see below).

More on p_{50}

As described above, in a lot of the literature where the MSB comparative longevity protocol has been used, the time for viability to fall to 50% (p_{50} ; equation 2) has been used as the measure of seed longevity (Probert et al., 2009; Mondoni et al., 2011; Merritt et al., 2014). Even when post-storage germination data is analysed using other methods, p_{50} , again, usually defined as the time when germination upon removal from storage/ageing environment has fallen to 50%, has also been determined (e.g. Walters et al., 2005). Indeed, some of the earlier literature modelling seed longevity, used the 'mean viability period', defined as 'the point on the time scale at which the survival curve intersects the 50% level of germination', as the measure of seed longevity (Roberts, 1973).

However, some authors have used a p_{50} defined as the time when viability (post-storage germination) is half that of the initial or maximum germination (Fig. 2; e.g. Mira et al., 2011; Tausch et al., 2019). Indeed, a viability threshold that is a proportion of the initial germination is referred to in the FAO Genebank Standards for Plant Genetic Resources for Food and Agriculture

(FAO, 2014). As discussed elsewhere (Hay and Whitehouse, 2017), from a genebank management perspective, this is problematic for a number of reasons, including the fact that the initial germination is only ever an estimate of the viability of a seed lot and germination is often seen to increase due to dormancy loss in the initial stages of storage, before decreasing (Chau et al., 2019; Hay et al., 2021). Another reason is because it means a seed lot may have quite low viability (e.g. 76.5% in the case of a seed lot with an initial germination of 90%) and already have reached the part of the survival curve where there is a faster decline in percentage germination before regeneration is triggered, meaning that there is a greater risk of losing alleles from the accession due to low seed lot viability.

A measure that is a proportion, for example, 0.5 (50%) of the maximum observed germination, which would perhaps be better indicated as, for example, $p_{\max/2}$ or $p_{0.5\max}$, is also not ideal, since maximum germination may not occur at the start of storage, and hence may be lower than the maximum viability of the seeds, in effect extending the time when ' p_{50} ' is observed (Fig. 2C). Tausch et al. (2019) justified their use of p_{50} defined as time to half-initial viability since Walters et al. (2005) used this definition "For the few species with initial germination percentages <70% ...". However, as stated above, defining p_{50} as the time when viability is halved is questionable since it is so dependent on the initial germination result (Fig. 2C), which can be affected by so many factors that it is difficult to control them all and have seed lots that are worth comparing. It is also not a 'half-life', in the sense that, due to the shape of most fitted survival curves, the expected time taken for viability to fall from, for example, 80 to 40% is not the same as the time for viability to fall from 40 to 20%.

Substituting any probit-viability value for v in equation 1, estimates of longevity, p_x , as the time to reach percentage viability, x , could be estimated (and ideally in the statistical software, so that the error for the estimates is also calculated based on all the germination data). For example, for p_{50} , 50% is equivalent to 0 probits (equation 2); for p_{85} , 85% is equivalent to 1.036 probits; for

p_{25} , 25% is equivalent to -0.674 probits, etc. When a number of seed lots are being compared, for example, from different species, using p_{50} as the measure of seed longevity is likely to give greater separation of seed lots than p_{85} . Using percentages $<50\%$ may be more relevant when the initial viability of one or more seed lots being compared is so low, that the estimate for p_{50} would be negative and therefore difficult to use in further analyses. This may be more relevant when comparing seed lots from within a species for which variation in σ is relatively small, for example, when comparing the effect of harvesting at different times or adopting different post-harvest protocols (Hay et al., 2015; Whitehouse et al., 2015), than for comparing species, where we would still recommend using seed lots with similar high germination.

Relating the results of seed comparative longevity experiments to ‘real-life’ storage conditions

A related question to ‘how should we measure seed longevity’ that is often posed, is ‘are the results meaningful in understanding longevity in real-life or “natural” storage environments?’ Indeed, perhaps this corollary question is partly why the original question has not been resolved: on paper, ageing seeds at 45°C and 60% RH seems very different from storage under genebank conditions, or in warehouse storage, or even to the conditions seeds experience in the soil, where ‘persistence’ is the ecological equivalent of longevity, and dependent on other factors than simply water, temperature and gaseous environment (Long et al., 2008; Carta et al., 2018). As described above, the conditions chosen for the protocols already described are within the limits where we expect the effects of changes in seed storage moisture content (or eRH) or temperature to be continuous (Ellis and Roberts, 1980a; Dickie et al., 1990). That is, we expect a seed lot with greater longevity according to the results of protocols described in Fig. 1, to also have greater longevity in genebank or controlled environment/temperate climate warehouse storage. Nonetheless, we will not be certain that the results from laboratory storage experiments are dependable until we do the experiments where samples of genebank seed lots are subjected to a storage experiment before the bulk is put into genebank storage (Bradford and Bello, 2022). The method for such pre-tests could be as described in Fig. 1B.

We would not expect the results from comparative longevity experiments at 60% RH to necessarily correlate well with storage in an environment with very high humidity (i.e. $>80\%$ RH), where the seeds are above the high moisture content/eRH limit to continuous relations (Roberts and Ellis, 1989) and therefore, if oxygen is available, repair and recovery from ageing-induced damage might occur. This is also elaborated on in Hay et al. (2019). The same reasoning can be applied in relation to soil seed bank persistence, that is we would not expect longevity in the soil seed bank to correlate with longevity based on comparative longevity storage experiments, though Long et al. (2008) did find a weakly significant correlation between longevity determined using the MSB comparative longevity protocol and persistence.

A related concern, is whether p_{50} is the best measure of longevity for real-life situations, not least since both genebanks and seed companies are likely to have a very much higher quality standard than 50% viability. As stated above, using time to 50% germination as the measure of longevity is likely to provide greater differentiation of seed lots, also since the standard error associated with estimates of p_{50} is lower than it is for other percentage values.

This is even more important when the amount of data available to fit the survival curve is low – which is often the case for ‘real’ genebank data from routine viability monitoring (Hay et al., 2021).

It should be emphasized, that the p_{50} (or other p_x) from a seed storage experiment cannot be directly used to predict longevity in a different storage environment (e.g. genebank storage) unless the viability equation parameters have been solved (in which case, we can only consider hermetic storage) and are constant within a species, which is not what we found among diverse Indica rice accessions (the estimate of σ in equation 1 varied even though the seeds were stored in the same ageing environment; Lee et al., 2019). Nonetheless, estimates of p_{50} from storage experiments carried out on samples of seeds at the same time as when the bulk seed lot is put into storage can be used to adjust monitoring intervals, such that they are shorter for seed lots with lower estimates of p_{50} , or even postponed for seed lots with high estimates of p_{50} until those with low estimates have reached the viability threshold.

Conclusions

At the recent meeting of the ISSS (August 2021), the need for a seed traits database was discussed, and seed traits related to the conservation and restoration of species were previously included as an aspect of ‘a research agenda for seed-trait functional ecology’ (Saatkamp et al., 2019). Thus, there is increasing urgency for a ‘standard measure of longevity’ that is consistent across studies, as much as possible. This will require again, consideration of the two main aspects that were considered when the MSB comparative longevity protocol was being developed: the storage environment and the measure of seed longevity. Perhaps it is more likely that there will be more than one ‘standard’ protocol, not least since the ranking of seed lot longevity can change in different storage environments (Hay et al., 2019 and references therein). Thus, our proposal in the first instance, is for wider adoption of the two protocols illustrated in Fig. 1, with open storage (Fig. 1A) to reflect the ‘real-life’ situation of storage conditions in commercial warehouses, farmer-stores and national stores of seed reserves, and hermetic storage (Fig. 1B) to reflect the needs of primarily, seed/genebanks. This distinction is also important in terms of understanding ageing mechanisms. In the case of hermetic storage, the equilibration environment may need to be adjusted for seeds of different species, to reflect differences in the effect of changes in temperature on the eRH–moisture content relationship, to ensure ageing seeds still have the same eRH. Regardless of seed storage/ageing environment, p_{50} is a useful measure of longevity, but it is important that the definition used for p_{50} is consistent across studies and meaningful from a scientific perspective. In terms of populating a seed traits database, as well as recording the observed p_{50} value, it is important that all the germination data, as well as relevant experimental details, are also entered, so that researchers in the future can reanalyse data from different laboratories to further understand the environmental and taxonomic basis of orthodox seed longevity. Reaching wider consensus on adoption of these conditions may require further discussion, and could perhaps be an objective for a future ISSS meeting.

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Conflicts of interest. The authors declare none.

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