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Evaluating the EPPO method for seed longevity analyses in Arabidopsis

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ABSTRACT

Seed longevity (storability) is an important seed quality trait. High seed quality is important in agriculture, for the industry, and for safeguarding biodiversity as many species are stored as seeds in genebanks. To ensure ex-situ seed survival, seeds are mostly stored at low relative humidity and low temperature. Oxidation is the main cause of seed deterioration in these dry storage conditions. The molecular mechanisms underlying dry seed survival remain poorly understood. Research on seed longevity is hampered by the lack of an experimental ageing method that mimics dry ageing well. Here, we propose the Elevated Partial Pressure of Oxygen (EPPO) method as the best available method to mimic and accelerate dry seed ageing. We have tested seed germination in *Arabidopsis thaliana* after EPPO storage at two different relative humidity (RH) conditions and confirm the large effect of oxygen and the seed moisture content on ageing during dry storage. Comparative Quantitative trait locus (QTL) analysis shows that EPPO at 55 % RH mimics dry ageing better than the commonly used Artificial Ageing and Controlled Deterioration tests at higher moisture levels.

1. Introduction

Seeds can survive conditions that would be detrimental to plants in the vegetative stage, for example prolonged periods of heat and desiccation. Desiccation tolerant seeds can have very long lifespans. A well-known example is the date palm (*Phoenix dactylifera* L.) with a recorded seed lifespan of 2000 years [1]. Seed lifespan is determined by the maternal environment in which the seeds develop, the genetic background of the seeds and the post-harvest environment [2–4]. Here, we define seed lifespan as the period from shedding from the mother plant until the seed has lost the capability to germinate (radicle protrusion). The seed lifespan curve is shaped by two physiological processes that occur during seed dry storage, seed dormancy release and a reduction in seed viability (Fig. 1). Seed dormancy is defined as the temporarily lack of germination of a viable seed under favourable conditions, due to physical and/or physiological characteristics of the seed [5]. Seed dormancy ensures the correct timing of germination (*e.g.* in the right season), while seed longevity allows the seed to overcome prolonged periods of unfavourable conditions. Both traits are established during seed maturation on the mother plant [6,7]. In Arabidopsis (*Arabidopsis thaliana*), dormancy can be released during dry storage by after-ripening (AR). In dry seeds, there is no metabolic activity [8,9]. Therefore, dormancy release by AR is caused by non-enzymatic reactions, most likely by oxidative processes [10–12]. Oxidation, among which peroxidation of lipids and the production of Reactive Oxygen Species (ROS), results in damaging alteration of molecules in the cell [13–15]. A signalling role for ROS in the release of dormancy has been proposed [16]. An example of dormancy release by AR is the inactivation of the dormancy promoting DELAY OF GERMINATION 1 (DOG1) protein. The amount of DOG1 protein in fresh seeds has a strong correlation with dormancy levels. Over time, the amount of DOG1 protein does not decrease, but the DOG1 protein is altered and dormancy is released [17]. For the commonly used Arabidopsis accessions Landsberg *erecta* (L*er*) and Colombia-0, AR takes up to a few weeks. However, in some Arabidopsis genotypes, dormancy levels can be very deep and thus take long periods of time to study, *e.g.* for over two years of AR for the accessions from the Iberian peninsula [18]. During prolonged dry storage, seed viability slowly reduces as the seeds age. One of the first phenotypes of

Abbreviations: AA, artificial ageing; AR, after-ripening; AUC, area Under the Curve; CDT, controlled deterioration test; DOG, delay of germination; EPPO, elevated partial pressure of oxygen; GAAS, germination ability after storage; LBS, laboratory bench storage; MC, moisture content; QTL, quantitative trait locus; RIL, recombinant inbred line.

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seed ageing that can be observed is a drop in the rate of germination, before the germination percentage decreases [19]. The decrease in germination rate might be caused by the extra time required to repair damage during the imbibition of the seed [20]. Ultimately, the amount of seed deterioration is greater than the capacity to repair, and then a decrease in germination percentages becomes visible [21]. Like dormancy release by AR, seed ageing during dry storage is also caused by oxidation [11,20,22–24]. In principle, redox reactions occur randomly where any molecule can be oxidized but more selective oxidation has also been reported [14,25]. The exact mechanisms underlying seed ageing remain largely unknown. In Arabidopsis, there is large phenotypic variation for seed longevity; the *GERMINATION ABILITY AFTER STORAGE* (*GAAS*) Quantitative Trait Loci (QTLs) were identified after four to seven years of laboratory bench storage (LBS, 40–60 % RH, 20−22 °C) [19]. However, after seven years of LBS the germination percentage was still high for a large number of lines (*e.g.* 45 % of the lines had more than 95 % germination). Seeds of some Arabidopsis accessions are still able to germinate after LBS for over a decade. To efficiently study the effect of dry storage on seed lifespan in Arabidopsis, the ageing has to be accelerated and mimicked. This can be done by experimental ageing using either the controlled deterioration test (CDT, ageing in high temperature and high relative humidity, where the seeds are equilibrated to the desired RH and then stored in hermetically sealed containers) or artificial ageing (AA, ageing in high temperature and high relative humidity, the seeds are placed in open tubes above an equilibrated salt solution or water) [26,27]. For Arabidopsis, ageing the L*er*/Cape Verde Islands (Cvi) Recombinant Inbred Line (RIL) population with CDT led to the identification of five loci linked to seed storability [28]. Two of these loci overlapped with the later identified *GAAS* loci (*GAAS1* and *GAAS2*) [19]. However, after CDT, the *GAAS3, 4* and *5* loci were not identified, while some QTLs identified after CDT were not present after LBS. This shows that experimental ageing by AA and CDT does not mimic dry ageing on the bench very well [29–31]. The implications of using AA and CDT for research and the prediction of seed longevity was recently reviewed, discussing the issues arising from using these methods [32]. The inability to experimentally age seeds while mimicking ageing is a problem for studying seed longevity for seed companies who rely on experimental ageing methods to predict dry seed storability. Another factor that hampers seed longevity research is its correlation with seed dormancy. Mutants with low dormant phenotypes often also displayed low longevity. This is the case for mutants that are affected in seed maturation, including *abscisic acid insensitive 3*, *leafy cotyledon 1* and *2* and *fusca 3*, but also for *dog1* [33–36], and for mutations that lead to structural changes in the seeds such as the *transparent testa* mutants [37]. In contrast, based on natural variation for seed dormancy and longevity in an Arabidopsis RIL population, the relation is negative [19]. To improve seed longevity phenotyping an additional experimental ageing method was developed, the Elevated Partial Pressure of Oxygen (EPPO) method [30]. During EPPO storage, the absolute amount of oxygen is increased by increasing the pressure (up to 20 MPa), while the relative amount of oxygen is the same as ambient air (21 %). EPPO has been successfully used to age seeds of lettuce, cabbage, soybean and barley [30,38]. Moreover, EPPO seems to mimic dry ageing better than CDT, since declines in seed viability of *Brassica oleracea* seeds during EPPO storage at 35 % RH and LBS resulted in a linear decrease in α-tocopherol (vitamin E), while no decline in this anti-oxidant was observed during CDT at 85 % RH [30]. Recently, we showed that the EPPO method can be used to accelerate and mimic seed dormancy release in Arabidopsis [12]. Here, we show that EPPO can also be used to experimentally age Arabidopsis seeds and therefore used to accelerate the entire seed lifespan. In order to investigate the accuracy of the method, we compared the EPPO longevity QTLs to those identified after ageing by LBS and CDT. Moreover, we show that ageing under different relative humidities involves partly different genetic pathways, as concluded from the different QTL profiles that are identified.

2. Material and methods

2.1. Plant material

Arabidopsis thaliana seeds from a recombinant inbred line (RIL) population of the ecotypes Landsberg *erecta* (L*er*) and Cape Verde Islands (Cvi), described in Alonso-Blanco et al. [39] were used. The L*er*/Cvi RIL population was grown on soil in the greenhouse (22 °C), under a long day regime (16 h light, 8 h dark). Three biological replicates were grown. After harvest of the seeds an aliquot for each genotype was stored at −80 °C in screw-cap tubes until the start of the EPPO storage experiments. Prior to the 35 % and 55 % RH EPPO storage experiments about 500 seeds were taken from the −80 °C aliquot (on ice) and acclimated for three days (placed in open 1.5 mL screw-cap tubes at 20 °C and 35 % RH or 55 % RH, respectively, in drying cabinets). One biological replicate was used per EPPO experiment. The biological

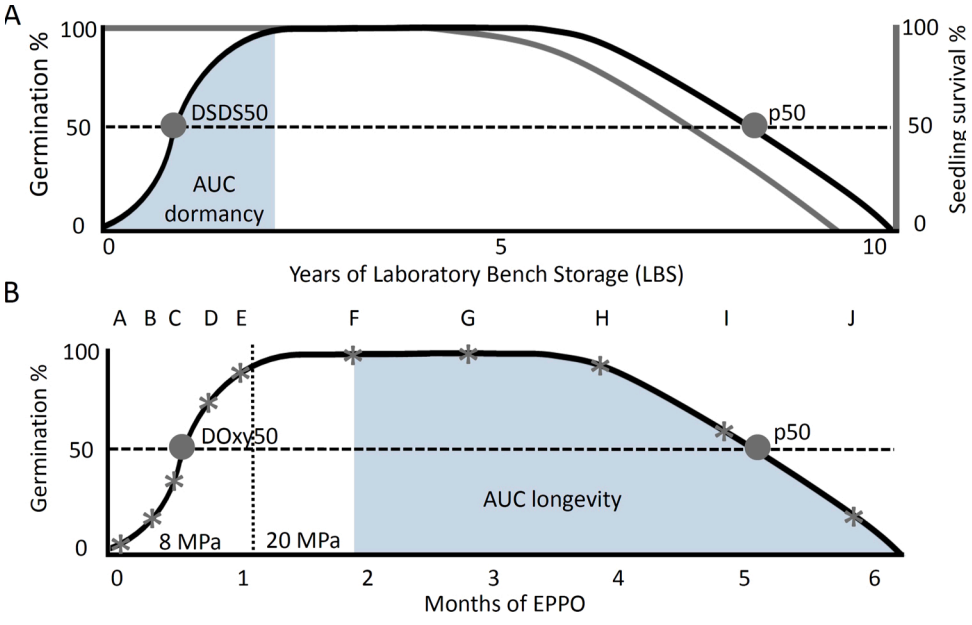

Fig. 1. Schematic representation of the germination potential of seeds throughout their lifespan. A) Germination potential during laboratory bench storage (LBS). Measures for seed dormancy (Days of Seed Dry Storage to reach 50 % of germination, DSDS50, and Area Under the Curve dormancy, AUC dormancy) and seed longevity (time to decrease to 50 % of germination, p50) are indicated. B) Germination potential during EPPO storage. Asterisks (*) indicate EPPO storage time points (A-J) at which the germination assay is performed. For dormancy release, the seeds are stored under 8 MPa air. After dormancy release, the pressure is increased to 20 MPa air (indicated by vertical dotted line) to accelerate ageing. Here, the measure for seed dormancy is the Days of EPPO storage required to reach 50 % germination (DOxy50). The Area Under the Curve (AUC longevity) is indicated by the shaded area as a measure of seed longevity. The AUC Longevity covers the germination data from storage point F (all lines 100 % of germination) until the final germination point (J).

replicate of the L*er*/Cvi RIL population used for the 35 % RH EPPO experiment comprised 154 lines, that of the 55 % RH EPPO experiment, 157 lines. For the laboratory bench storage (LBS) experiment, seeds were stored in semi-controlled (20−22 °C, 40–60 % RH) conditions after harvest. Germination data of the L*er*/Cvi RIL population after seven years of LBS was used from Nguyen et al. [19].

2.2. Germination assays

For all experiments, seeds were sown on blue germination paper (blue blotter paper; Anchor Paper Company, http://www.seedpaper.com) in trays with 48 mL demineralised water and placed in a 22 °C cabinet with continuous light. Each tray contained six germination tests of approximately 50 seeds. Seed germination was followed for five days using the Germinator system [40]. The percentage of germinated seeds and relative germination rate (t50; time in which 50 % of the maximum germination percentage is reached) were calculated.

2.3. Moisture content determination

Seed moisture content (MC) was determined by placing 100 μg of seeds (fresh weight) overnight (~16 h) in a 105 °C oven and weighed again. MC is expressed based on the dry weight basis. MC of L*er* seeds were determined after one week of 35 % and 55 % EPPO storage.

2.4. EPPO experiment

Roughly 50 seeds were placed in a screw cap tube for each line and each storage time point. The tubes were closed with a lid in which two holes were punctured and inside the lid a piece of filter paper was placed to prevent the seeds from spilling through the holes. For each storage period a separate 1.5 L tank was used (Fig. 1C). In total nine tanks were filled simultaneously with compressed air to 8 MPa at a rate of 0.4 MPa per minute as described in Groot et al. [30]. A nylon stocking with 225 g silica gel, equilibrated at 35 % and 55 % RH respectively, was added to each tank to regulate the RH in the tanks after filling with dry air, and the tanks were placed at 20 °C. After the fourth time point, the pressure of the remaining five tanks was increased to 20 MPa. The pressure release from the tanks was controlled using computer controlled flow equipment such that the relative pressure decline was at most 0.5 % per minute. The water activity of the silica gel from each tank was measured to confirm the RH in the tank.

2.5. Computational analysis of DOxy50, AUC longevity and quantitative trait loci

Dormancy levels after the 55 % RH EPPO treatment was quantified by calculating the DOxy50 (Days of seed EPPO storage to reach 50 % of germination, Fig. 1B) was calculated using the statistical program R (version 2.14, [41]) according to He et al. [3]. The area under the curve (AUC) longevity was calculated for each RIL individually with Microsoft Excel using the trapezoid area formula. The QTL analyses were performed with the MapQTL program (version 6 [42],). QTL were identified with both interval mapping and rMQM mapping according to the manual (https://www.kyazma.nl/index.php/MapQTL/Manual/).

3. Results

3.1. Acceleration of seed lifespan during EPPO storage

Earlier we have reported that EPPO storage (35 % RH, 8 MPa) can be used to accelerate and mimic dormancy release in the Landsberg *erecta* (L*er*)/Cape Verde island (Cvi) recombinant inbred line (RIL) population. This identified the seed dormancy DOG1, DOG2 and DOG6 QTLs that were previously identified by laboratory bench storage (LBS) [12]. To complete the seed lifespan curve we have continued the EPPO treatment after dormancy release (34 days of 8 MPa [12],), increasing the pressure to 20 MPa for a period of 145 days (Fig. 2A). The germination percentage of the RIL population declined gradually after the pressure increase (Fig. 2A). A proportion of the lines (19/152 lines, 13 %, including the parental Cvi accession) did not reach more than 90 % germination before germination levels started decreasing. This means that these seeds started deteriorating before reaching the full germination capacity (100 % germination). To meet the laboratory bench conditions we performed an additional EPPO storage experiment in which we used a RH of 55 % (Fig. 2B). This RH is comparable to the RH at ambient conditions in which the earlier LBS experiments were performed [19]. The effect of increased RH on the seed moisture content (MC) is less than 2 % (equilibrated at 35 % and 55 % RH the MC of the seeds is 0.054 and 0.073 g H_2O g dw^{-1}, respectively). The phenotypic differences were significant: under 55 % RH EPPO storage all lines (156) reached germination percentages above 90 % (Fig. 2B) and the seeds have a longer period in which they are non-dormant but remain viable, like reported for LBS. Moreover, seeds aged at 55 % RH have a higher longevity than seeds aged at 35 % RH. The QTL analyses for dormancy after AR and 35 % RH EPPO are described in Buijs et al. [12]. Dormancy release with the 55 % RH EPPO was quicker as reflected by the averages of the population (Fig. 2A, B) and the dormancy measure Days of EPPO storage required to reach 50 % of germination (DOxy50) (Fig. 2C). QTL analyses for dormancy release by 55 % RH EPPO led to the identification of DOG1 (Supplemental Fig. 1). The pressure in the 55 % RH storage tanks was increased to 20 MPa after 25 days, when the full germination capacity was reached. Both EPPO at 35 % and 55 % RH resulted in quicker seed deterioration compared with LBS (Fig. 2D), however seed deterioration under 55 % RH is slower than that in 35 % RH (Fig. 2D and E). Correlation analyses of the phenotypic data shows that LBS (maximum germination, GMax, after seven years) correlates best with 55 % RH EPPO (AUC longevity)(Pearson correlation coefficient of 0.5, Supplemental Fig. 2).

3.2. Genetic basis of seed longevity after dry storage and experimental ageing

To be able to compare the 35 % and 55 % RH EPPO storage with LBS [19] and CDT [28], we performed QTL analyses on the data obtained from these four different experiments. Different parameters can be used to express seed longevity, among which GMax of a single time point (Fig. 1C, E), p50 (time until seed germination decreases to 50 %, see Fig. 1A) and the AUC longevity. The p50 is a measure commonly used in longevity studies, however in this study the p50 could only be calculated for the 35 % RH EPPO storage. After the 55 % RH EPPO experiment 60 % of the lines still germinated above 50 %. To not base our results on extrapolated values we decided to use the AUC longevity instead of p50 as measure for seed longevity. The AUC longevity could be exactly calculated for both the 35 % and 50 % RH EPPO experiments. The p50 and AUC longevity of the 35 % RH EPPO correlate well, validating the AUC longevity as a parameter for seed longevity (Supplemental Fig. 2). For LBS and CDT, GMax values were used (after seven years LBS and 4 days CDT) since there were not enough separate time points to fit a curve on. For LBS only the data for the L*er*/Cvi RIL population was taken along in the analysis, not that of the other five RIL populations used in the mixed-model QTL analysis [19]. All data used in the QTL analysis is provided in Supplemental File 1. After seven years of LBS, the *GAAS1, GAAS2* and *GAAS5* loci are identified with a combined explained variance of 51 % (Fig. 3A). After 35 % RH EPPO storage we identified the *GAAS2, GAAS3* and *GAAS5* loci (Fig. 3A). The major *GAAS1* QTL, as identified after LBS, is not identified with the 35 % RH EPPO storage. The combined explained variance of the QTLs identified after 35 % RH EPPO was 45 %. The EPPO experiment at 55 % RH identified the *GAAS1* and *GAAS2* loci and a new locus on chromosome four, *EPPO1*. These loci together explained 65 % of the variance (Fig. 3A). QTL mapping for seed longevity quantified by the CDT identified the *GAAS1, GAAS2* and

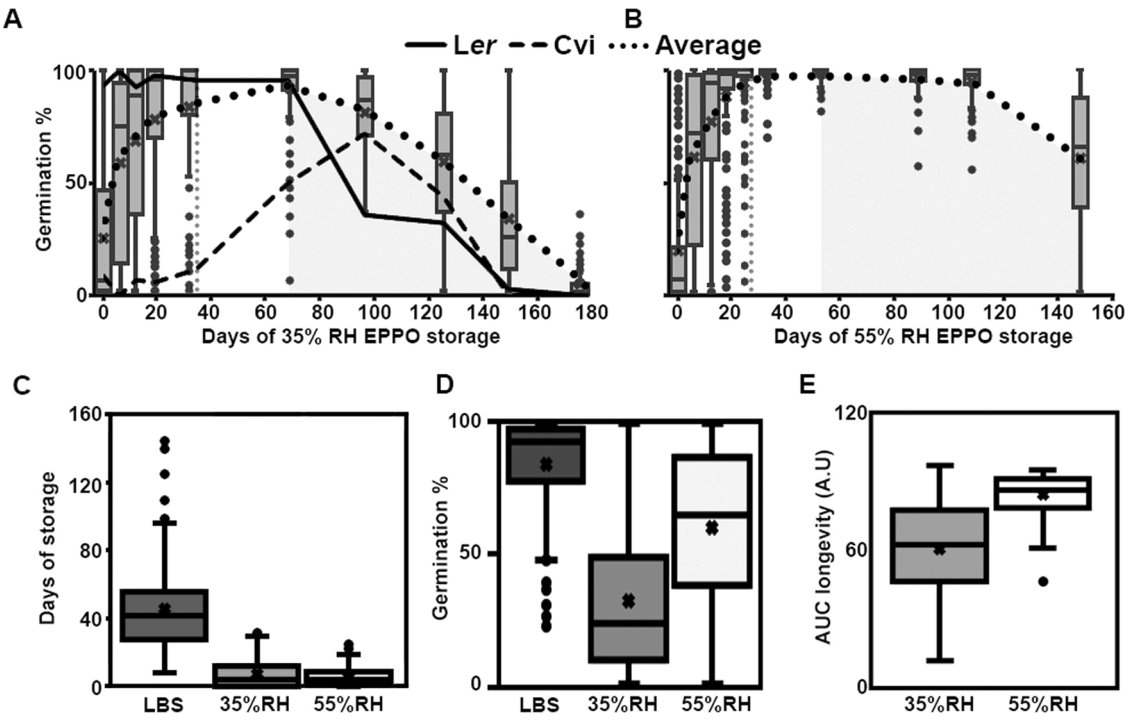

Fig. 2. Quantification of dormancy and longevity phenotypes by EPPO storage. Box and whisker plots of the germination percentages of the L*er*/Cvi RIL population at different storage periods in 35 % RH (A) and 55 % RH EPPO (B). The lines represent the lifespan curve of L*er* (–) and Cvi (–, only in A) and the average (···) for the whole population. Vertical dotted bars indicate the moment of pressure increase from 8 MPa to 20 MPa. Shaded areas indicate the AUC longevity for the average lifespan curve of the population. C) Days of storage required to reach 50 % of germination under dry conditions (LBS; DSDS50), and EPPO 8 MPa (DOxy50) at 35 % and 55 % RH. n = 129. D) Germination percentages of the L*er*/Cvi RIL population after seven years of LBS, or 149 days of the respective EPPO treatments. E) AUC longevity for EPPO 35 % and 55 % RH. n = 147. In the box and whisker plots, the outlier points are indicated with a dot and the averages with an X. In C and E, only genotypes for which a value was available in all storage treatments were included.

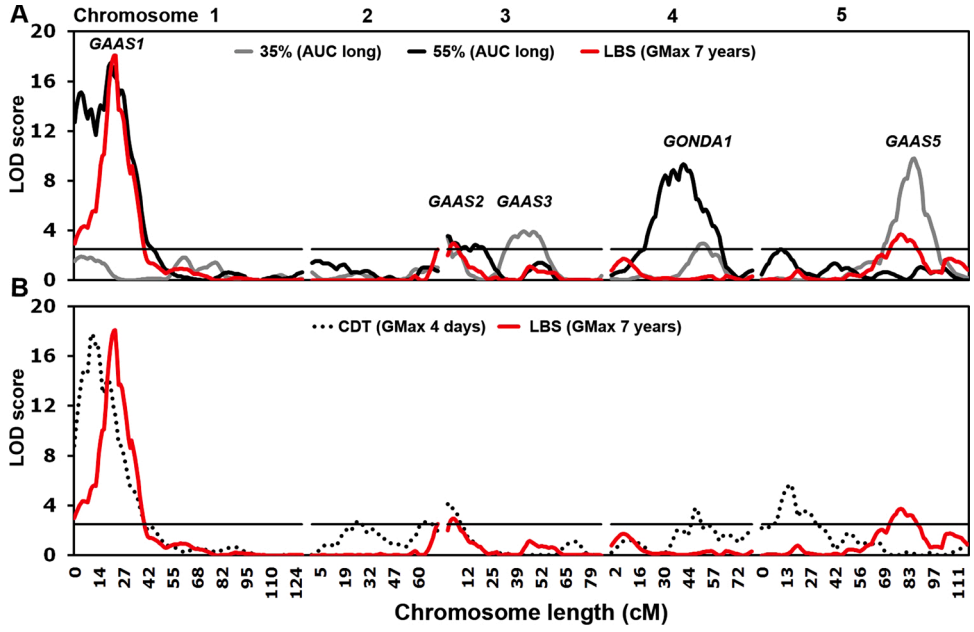

Fig. 3. QTL analyses of seed longevity. A) rMQM of seed viability after EPPO 35 % (–) and 55 % (–) RH EPPO storage (AUC longevity). B) rMQM of seed viability after LBS (maximum germination after seven years of dry storage, red line) and 4 days of CD treatment (black dotted line). The identified *GAAS* loci are indicated (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

EPPO1 loci, and three additional loci on chromosome 2 and 5. Another phenotypic measure of seed longevity is the increase of the germination rate (Δt50). QTL analyses for the Δt50 identified the *GAAS5* locus after 35 % and 55 % RH EPPO storage and the *GAAS1* after 55 % RH EPPO storage (Supplemental Fig. 3).

4. Discussion

4.1. The need for an experimental ageing method

Seeds are important for agriculture since many crops are grown from seeds. Seed companies aim to provide high quality seeds, since seed quality strongly affects seedling establishment and yield [43]. Seeds are also stored in genebanks which is important for safeguarding future breeding activities and biodiversity when areas are affected by natural disasters or war [44]. Both in genebanks and in seed companies seeds are stored dry (often around 30 % RH) [30]. The molecular and genetic mechanisms underlying seed longevity are poorly understood. This is mainly caused by the fact that studying seed longevity is time consuming since there is no experimental ageing method that mimics seed dry storage well. Another complicating factor is that seed dormancy and seed longevity are often studied independently. It is more informative to study the entire seed lifespan, since increase in germination percentage due to dormancy release and the decrease due to loss of viability during seed dry storage might not be separated in time. In this study we investigated the use of EPPO as an experimental method to mimic and accelerate the entire seed lifespan under LBS. Moreover, we tested experimental ageing by EPPO in a genetic population to be able to reveal whether experimental ageing by EPPO is associated with the same genetic loci that are identified after LBS ageing.

4.2. EPPO with 55 % RH mimics LBS seed lifespan phenotypically

Dormancy QTLs identified after 35 % RH EPPO storage did overlap with the QTLs identified for AR [12]. At 35 % RH, not all lines had completely released dormancy (*i.e.* reached 100 % of germination) before the germination reduced as a result of deterioration (*e.g.* Cvi). Thus, the lifespan curve created under 35 % RH EPPO did not very well mimic LBS lifespan as shown in Fig. 1. To overcome this we performed experimental ageing with EPPO at 55 % RH, which is more similar to the average ambient storages conditions in the former experiment [19]. The increased RH resulted in much faster dormancy release; since we did not anticipate this, we identified only little phenotypic variance during the dormancy release phase. Most lines germinated 0–10 % at the start of the experiment and then 90–100 % after five days of EPPO (Supplemental Fig. 1A). The lack of phenotypic variation is likely the reason why only the very strong *DOG1* QTL was identified in the QTL analysis for seed dormancy (Supplemental Fig. 1B). Also parameters that are based on the dormancy release curve (DOXy50 and AUC dormancy, Fig. 1B) did not improve this QTL analysis (Supplemental Fig. 1B). The low phenotypic variation observed can likely be increased by using shorter storage periods or lower partial oxygen pressure, when using 55 % RH. The fact that the dormancy phenotypes of the individual RILs after 55 % RH EPPO had a stronger correlation with AR dormancy release than 35 % RH EPPO with AR dormancy release (Pearson correlation coefficients of 0.71 and 0.65, respectively, supplemental Fig. 1C) supports that the 55 % RH EPPO is also a suitable method for dormancy release in Arabidopsis.Typically, seed dormancy release by seed dry storage is followed by a long plateau phase in which the seeds germinate around 100 % in Arabidopsis (Fig. 1A) [45]. Ultimately, when storage is continued the germination rate decreases and the germination percentage drops. With both the 35 % and 55 % RH EPPO method, seed lifespan is accelerated, although the shape of both curves is different. At 35 % RH, dormancy release (at 8 MPa) is slower, however the seeds deteriorate faster (at 20 MPa) compared with the 55 % RH EPPO treatment. The data from both the 35 % and the 55 % RH EPPO confirm that oxygen stimulates seed dormancy release and deterioration. However, seed dormancy release appears to be partly different from seed deterioration under EPPO, as they both respond differently to an increase in RH (*i.e.* dormancy release is slower but ageing is faster during 35 % RH EPPO storage compared to 55 % RH). The correlation between experimental ageing and LBS (RH varying between 40 and 60 %) is strongest after 55 % RH EPPO (0.5; Supplemental Fig. 2). The correlations with LBS (GMax after seven years) are a bit skewed since after seven years of LBS, 49 % of the lines (69 out of 140) still germinated above 95 %.

4.3. Comparative genetic analysis of LBS and the experimental ageing methods EPPO and CDT

To reveal whether experimental ageing with EPPO mimics seed ageing by LBS at the genetic level, we have compared the QTLs identified after EPPO at 35 and 55 % RH with the earlier identified *GAAS* QTLs [19]. The *GAAS1* QTL is identified after LBS and 55 % RH EPPO storage and is also associated with the germination rate during 55 % RH EPPO storage (Supplemental Fig. 3). The *GAAS1* locus identified with the 55 % RH EPPO overlapped in position with a QTL identified by LBS [46]. *GAAS2* is identified after LBS, EPPO 35 % and EPPO 55 % RH storage. *GAAS3* is only identified after 35 % RH EPPO storage and co-locates with QTLs identified after germination under stress conditions (ABA, cold, cold and heat, salt, cold and dark) [47–49] and seed size [50]. As seed dormancy was not fully released during 35 % RH EPPO storage, the *GAAS3* locus might also be partly caused by variance contributed by the *DOG6* dormancy QTL that is located at this position. The *GAAS5* locus is identified after LBS and 35 % RH EPPO storage (Fig. 3). The *DOG1* gene is the causal gene underlying the *GAAS5* locus as previously reported [19]. The *EPPO1* locus was identified after 35 % and 55 % RH EPPO. This QTL co-locates with loci that have been linked to germination under different stress conditions in the Bayreuth x Shahdara and L*er* x Shahdara RIL populations (cold, heat, cold and dark) [47–49]. Also, seed weight, seed oil and protein content QTLs are closely linked to the *EPPO1* region [51]. The co-location of the *DOG* loci with the *GAAS* loci has been described before (*GAAS1/DOG2*; *GAAS2/DOG22*; *GAAS3/-DOG6*; *GAAS5/DOG1* [19]). An overview of the known co-locating QTLs are described in Supplemental File 2. In this study we aimed at identifying which method best mimics and accelerates LBS. To complete the data set we also included CDT data that was obtained previously on the same population [28]. To be able to compare the artificial ageing methods we performed QTL analysis on the data obtained after LBS, EPPO and CDT ageing. Comparing LBS, the 35 % and 55 % RH EPPO storage methods with CDT shows that indeed CDT does not fully mimic these methods (Fig. 3B; Supplemental Fig. 2). QTL analysis after CDT identified the *GAAS1, GAAS2* and the *EPPO1* loci, and two additional loci on chromosome two and five (Fig. 2). The *GAAS3* and the major *GAAS5* loci are not identified. Another drawback of CDT is that fresh, high dormant seeds do often not respond to CDT (*i.e.* experiences learn that they deteriorate less compared with the same seed batch after a short period of AR). The CDT experiment on the L*er*/Cvi population was performed on seeds that were fully after-ripened prior to the CDT experiment [28]. Thus, here AR is combined with CDT which could influence the results as well. Another aspect that might explain differences observed between EPPO and CDT is that during CDT, the seeds are hermetically sealed and thus the ratio of the gasses in the environment might change over time [32]. Since the 55 % RH EPPO identified the major *GAAS* loci, and the correlation between 55 % RH EPPO and LBS is the strongest, we conclude that the 55 % RH EPPO is currently the most suitable tool for predicting seed longevity under LBS conditions.

4.4. The relative humidity has substantial effects on seed dormancy release and longevity during EPPO

Typically, experimental ageing in Arabidopsis seeds involves an elevated seed moisture content. In literature, storage conditions can be found that range within 75–85 % RH (reviewed in [32]). However, LBS conditions usually do not fall within this range. Therefore, results from experimental ageing might not always match with results from LBS conditions. Recently it was suggested that the reason for the difference in QTLs identified for rice seed longevity is partly due to the different RH conditions during AA experiments [32]. The difference in RH, between

the 35 % and 55 % RH EPPO treatments, has a large effect on seed lifespan. This effect is reflected in both the dormancy and longevity phenotypes as well as in the QTLs identified. During dry storage, the seeds enter a glassy state, in which there is little molecular mobility and no enzymatic activity [52–54]. The relation between the molecular reaction rate and MC is not linear [8,53,55,56]. We hypothesize that the difference in MC results in a different position in the lipid oxidation *vs* water activity graph as described by Labuza [8]. Hence, a subtle difference in MC might have quite some effect on the level of lipid oxidation and even enzyme activity. This effect might be enhanced by the increased pressure. However, it is puzzling that seed ageing is slower under 55 % RH than under 35 % RH EPPO, as this contradicts the negative relation between longevity and seed MC generally reported (*e.g.* [55,56]). A difference is that in those studies, in the CDT experiments seeds were often packed in hermetically sealed laminated foil pouches in which oxygen levels decline during the CDT. Moreover, to accelerate the deterioration, the packages with seeds were often stored at higher temperatures (*e.g.* 45 °C [56]), resulting in a further increase of the water activity [57]. The cytoplasm is still in the glass phase, for both 35 and 55 % RH at 20 °C (MC of 0.054 and 0.073 g H_2O g dw^{-1}, respectively) [53]. Nevertheless enzyme activity or mobility of metabolites at 55 % RH cannot be excluded [8]. The role of oxygen during seed storage in interplay with moisture content has been studied only to a limited extend [58–60]. Clearly more research in this area is needed to understand the processes of seed ageing in different RH environments and in gaseous environments with elevated pressure. Furthermore, it has been advised to only use very high RH in AA when the aim of the study is to investigate the effect of high RH on seed longevity (*e.g.* when mimicking tropical storage) [32]. As we showed in this study, the RH is very defining for seed longevity. Therefore, it is recommended that regardless which artificial ageing method is used, the appropriate RH has to be chosen and has to be strictly controlled.

5. Conclusion

The identification of seed longevity loci has been hampered by the lack of an experimental ageing method that mimics seed ageing under LBS conditions. The 55 % RH EPPO method is so far the best method to mimic dry seed lifespan in Arabidopsis under LBS conditions. It can be used for other species as well, albeit with adapted conditions [30,38]. This study contributes to the idea that not only the seed moisture content and temperature during ageing are important, but there are also interactions with oxygen which cannot be neglected [59,61,62]. The EPPO method is a tool to study these interactions, as it can be set at specific humidity conditions.[63–70] The identified loci require functional analysis and the EPPO method is a useful tool for this.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

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