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RESEARCH ARTICLE

Dry ageing: A physiologically realistic alternative to accelerated ageing for phenotyping seed longevity in soybean

A Akash[1,2#], M B Ratnaparkhe[3#], C T M Prasad[1#], B K Naik[4], M K Kuchlan[3], H R Archana[1,2], S Bhattacharjee[2], S K Lal[1], D Vijay[1] & Arun Kumar M B[1*]

[1]Indian Council of Agricultural Research-Indian Agricultural Research Institute (ICAR-IARI), New Delhi 110 012, Delhi, India
[2]Indian Council of Agricultural Research-Indian Agricultural Research Institute (ICAR-IARI), Hazaribagh 825 405, Jharkhand, India
[3]Indian Council of Agricultural Research-National Soybean Research Institute (ICAR-NSRI), Indore 452 001, Madhya Pradesh, India
[4]Indian Council of Agricultural Research-Indian Institute of Seed Science (ICAR-IISS), Regional Station, Bengaluru 560 065, Karnataka, India
# These authors contributed equally to this work.

*Correspondence email - akmbsst@gmail.com

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Abstract

Seed longevity is a vital trait that influences germplasm storability and seed quality maintenance. The accelerated ageing test (AAT) is often used to measure longevity, but its high-temperature and humidity conditions do not truly represent natural seed ageing. This study aimed to develop a dry ageing (DA) method as an alternative to the AAT for phenotyping soybean (*Glycine max* L.) seed longevity. Fifteen soybean genotypes differing in seed coat colour were evaluated under controlled dry conditions at moderate temperature and humidity (41 ± 0.3 °C and 50 % relative humidity (RH)). Germination and vigour traits, including total germination (TG %), normal seedlings (NSL %), shoot length (SL), root length (RL) and seed vigour index (SVI-I) were recorded to compare AAT and DA. Significant variations were observed among genotypes, ageing periods and their interactions. While AAT caused rapid deterioration (TG % from 97.5 to 0.3 % in 10 days), DA showed a slower and more natural decline (TG % from 97.8 to 57.7 % in 214 days). Genotypes TGX 855-32E and B 252 showed less than 30 % reduction in most of the traits. Correlation analysis revealed that DA maintained and strengthened trait relationships over time, unlike AAT, which disrupted them. Dry ageing also separated tolerant and sensitive genotypes more clearly, capturing the natural variation in seed longevity that AAT often compresses or masks. Overall, the DA method provides a simple, less stressful and more realistic alternative to accelerated ageing for screening soybean genotypes for seed longevity under conditions that better represent natural ageing.

Keywords: accelerated ageing; dry ageing; germination; seed longevity; seed vigour; soybean

Introduction

Soybean (*Glycine max* (L.) Merr.) is a globally important oilseed crop that contributes to nearly 30 % of the world's edible oil production (1). Because of its high nutritional value, containing approximately 40 -45 % protein, 20-22 % oil and 20-26 % carbohydrates (2), soybean serves as an important source of food and income in many developing regions. However, soybean seeds are naturally short-lived, especially in tropical and subtropical environments, where high temperature and humidity accelerate deterioration, leading to rapid declines in germination and vigour during storage (3, 4). This short storage life limits seed reuse in the next season, increases production costs and reduces seed distribution efficiency.

Seed longevity, defined as the ability of seeds to remain viable and vigourous during storage, is a key factor in determining seed quality and the long-term preservation of genetic resources (5). It is controlled by a combination of genetic, physiological and environmental factors (6). Longevity is achieved mainly through the structural stabilization of cell components within a highly viscous "glassy" cytoplasm, which reduces molecular movement and slows the rate of deterioration (7, 8). Certain protective molecules, such as non-reducing soluble sugars (sucrose and raffinose family oligosaccharides), late embryogenesis abundant (LEA) proteins and heat shock proteins (HSPs), play vital roles in maintaining membrane and protein stability in the dry state (9, 10). In addition, antioxidants including glutathione, tocopherols and flavonoids act as natural scavengers of reactive oxygen species (ROS), protecting lipids and proteins from oxidative damage (11, 12). Despite these mechanisms, the lipid-rich nature of soybean seeds and their relatively thin seed coat render them more susceptible to oxidative stress and lipid peroxidation during storage (13, 14).

The natural ageing of dry seeds is a slow process, which makes it difficult to study the deterioration mechanisms within a reasonable time frame. To overcome this, artificial ageing techniques, such as the accelerated ageing test (AAT) and controlled deterioration test (CDT) are widely used, where seeds are exposed to high temperature and humidity to induce damage within days or weeks. Many studies have employed these methods to assess genetic variation in soybean seed longevity (15-19). However, recent evidence has shown a weak relationship between AAT results and natural ageing, mainly due to differences in seed moisture levels and the physiological processes involved (20, 21).

Moisture content is one of the most critical factors controlling molecular mobility and reaction rates during seed storage (22). At low moisture levels (below the equilibrium relative humidity of approximately 27 %), the marked increase in matrix viscosity restricts molecular mobility, thereby slowing the rates of chemical reactions (23). In contrast, under artificial ageing, high seed moisture promotes molecular movement and accelerates these reactions, causing faster deterioration. This distinction explains the contrasting physiological and biochemical responses observed between artificial and natural ageing. For example, tocopherols, key antioxidants that protect membranes and support seed longevity in dry *Arabidopsis* seeds (12), remain relatively stable during CDT in cabbage but decline under DA at elevated oxygen pressure (24). Similarly, in the elevated partial pressure of oxygen (EPPO) method, increased lipid oxidation and volatile compounds were observed, which were negatively correlated with germination (25). Moreover, quantitative trait loci (QTLs) detected after CDT in *Arabidopsis* was not observed following natural storage (20, 24), indicating that different genetic controls operate under artificial and natural conditions.

Together, these findings suggest that existing artificial ageing tests (AATs) do not accurately reproduce natural seed deterioration, highlighting the need for methods that more closely simulate real storage behavior. Dry ageing, in which seeds are kept under low moisture and moderate temperature conditions, offers an alternative by allowing slow oxidative changes similar to those occurring during natural ageing. Therefore, this study aimed to standardize a DA method as a substitute for AAT to evaluate seed longevity in soybean. By comparing germination and vigour traits under both DA and AAT, the study aims to identify soybean genotypes with better storage performance and provide a more realistic physiology-based phenotyping approach. To our knowledge, this is the first systematic comparison of AAT and DA in soybean, providing new insights into the mechanisms of seed ageing and aiding future breeding efforts for improved storability.

Materials and Methods

Seed material

The study was conducted at the Indian Council of Agricultural Research-Indian Agricultural Research Institute (ICAR–IARI), New Delhi, during 2022-23. Soybean seeds were supplied by the ICAR–Indian Institute of Soybean Research (IISR), Indore, India. The seeds were multiplied at IISR during the 2021 Rabi season. After harvest, they were naturally dried to approximately 10 % moisture content and stored in sealed laminated aluminium foil packets at room temperature to maintain their quality until use in the ageing experiments. For both DA and AA treatments, 15 soybean accessions differing in seed coat colour (yellow to black) were selected. These included RVS 2001-18 (black), TGX 722-155 F (black), BR 15 (brown), EC 547464 (brown), TGX 854-42D (green), EC 39376 (green), EC 456566 (light green), EC 341115 (light green), MAUS 47 (yellow), ANKUR (yellow), EC 274711 (yellow), M 1052 (yellow), TGX 855-32E (yellow), ACC 1026 (yellow) and B 252 (yellow). At each storage interval, 100 seeds (two replicates of 50 seeds each) were used for germination testing.

Accelerated ageing treatment (AAT)

Seeds were subjected to accelerated ageing (AA) following the modified International Seed Testing Association (ISTA) method (26) for 2, 4, 7 and 10 days, along with an unaged control (0 days). For each ageing period, 100 soybean seeds (two replicates of 50 seeds each) were placed in mesh bags and kept inside airtight desiccators containing a saturated K_2SO_4 solution that maintained a constant relative humidity of 96 % at 40-45 °C (27). These desiccators were kept in a humidity chamber maintained at 41 ± 0.3 °C to ensure consistent temperature and humidity. The ageing conditions maintained the seed moisture content at approximately 17 % throughout the ageing period (28, 29). After each ageing duration, seed samples from all 15 genotypes were removed and tested for germination within one hr of retrieval. To determine the optimum ageing duration, the period that caused approximately 50 % reduction in germination trait was considered.

Dry ageing treatment

The same 15 soybean genotypes used in the AAT were also subjected to DA. For each ageing period, 100 seeds (two replicates of 50 seeds each) were placed in mesh bags and transferred to airtight desiccators maintained at 41 ± 0.3 °C and 50 % RH. The ageing conditions maintained the seed moisture content at approximately 8.2 % throughout the ageing period (28, 29). The seeds were aged for a total of 214 days. Samples were removed at 0, 15, 46, 89, 116, 155 and 214 days of ageing (DOA) and were immediately tested for germination traits within one hr of retrieval. This design enabled a systematic evaluation of the effects of prolonged DA on seed quality.

Germination assay

Seed germination tests were performed according to ISTA rules (26) with minor modifications. Seeds were placed between two layers of moistened blotter paper (40 cm × 33 cm) and incubated at 25 °C in the dark. The following parameters were analyzed in both treatments at the final count day i.e., on 8th day.

Total germination (TG %)

To calculate TG, both normal seedlings (NSL) and abnormal seedlings (ABSL) were considered (30).

$$TG\,\% = \frac{NSL + ABSL}{\text{Total no. of seeds subjected to germination}} \times 100$$

Normal seedlings (NSL %)

Normal seedlings were determined by calculating the ratio of the number of normal seedlings to the total number of seeds initially subjected to germination.

$$NSL\,\% = \frac{NSL}{\text{Total no. of seeds subjected to germination}} \times 100$$

Shoot and root lengths (cm)

The shoot and root lengths (SL and RL) of normal seedlings were measured separately in ten randomly selected seedlings in two replications after 8 days, at the final count day of the germination test. Their sum represents the seedling length.

Mean seedling length (cm) = Mean shoot length (cm) + Mean root length (cm)

Seedling vigour index-I (SVI-I): Seed vigour index I (SVI-I) was calculated following the standard procedure (31), using the formula:

SVI-I = NSL % × Mean seedling length (cm)

Statistical analysis

Descriptive statistics were used to evaluate trait variability using the appropriate R packages. Two-way analysis of variance (ANOVA) was performed to assess the genotype, treatment and interaction effects on all traits using OPSTAT software (32). Pearson's correlation coefficients were computed and visualized using the 'corrplot' package in R (33). Principal component analysis (PCA) was carried out using the FactoMineR package (version 2.4) and the results were graphically represented with factoextra (version 1.0.7) (34).

Results and Discussion

Seeds subjected to AA and DA exhibited distinct and contrasting trends in their germination traits over time. A detailed analysis revealed significant differences among genotypes (V), ageing intervals (A) and their interaction (V × A) for all germination traits under both conditions.

Effect of accelerated ageing on seed germination traits

Under AA, TG % dropped sharply from 97.5 % at 0 DOA to 52.9 % after 7 days and to only 0.3 % after 10 days. Some genotypes, such as EC39376, EC341115, MAUS 47, ACC 1026 and TGX 854-42D, showed more than 50 % reduction, whereas others, such as BR 15, EC 547464, ANKUR, TGX 855-32E and B 252, maintained better tolerance with less than 35 % reduction (Fig. 1). The NSL % followed a similar pattern, decreasing from 87.8 % (0 DOA) to 0.3 % (10 DOA), indicating a progressive loss of seed quality and germination ability. Shoot length showed approximately a 36 % reduction, whereas RL decreased by approximately 57 %. None of the genotypes recorded more than a 50 % reduction in SL after 7 DOA. Soybean accessions ANKUR, BR 15, M 1052 and EC 274711 exhibited comparatively higher tolerance to ageing. In contrast, most genotypes had more than 50 % reduction in RL, except EC 547464, showing that roots were more sensitive to ageing than shoots, highlighting distinct genotypic responses. The mean SVI-I decreased from 2450.3 at 0 DOA to 462.7 at 7 DOA, indicating a sharp decline in seed vigour. Overall, AA caused rapid deterioration of germination traits, confirming the strong impact of high temperature and humidity, as reported earlier in soybean and other crops (35–37). The rapid decline can be linked to several damaging physiological processes, including free-radical-induced lipid peroxidation (38), protein carbonylation and enzyme inactivation (5), membrane disruption (6), Amadori and Maillard reactions (39) and loss of the glassy state (8).

Effect of dry ageing on seed germination traits

In dry ageing, the TG % declined more gradually, from 97.8 % at 0 DOA to 57.7 % at 214 DOA and the NSL % decreased from 89.6 % to 38.9 % over the same period, with no significant change ($p > 0.05$) during the first 46 DOA (Fig. 2). A similar pattern was observed for SL, RL and SVI-I. Root length was more affected than SL, with six genotypes (EC 547464, TGX 854-42D, MAUS 47, ANKUR, EC 274711 and M 1052) showing more than 50 % reduction in RL, while B 252 maintained less than 30 % reduction, indicating better root stability. The SVI-I declined from 2903.8 to 790.3 by 214 DOA. Genotypic differences were evident, with TGX 855-32E consistently showing the highest resilience across all traits, maintaining less than 30 % loss, whereas EC 547464, EC341115, MAUS 47, ANKUR, EC 274711 and M 1052 were more sensitive, showing over 50 % reduction in most parameters.

The gradual decline in germination under DA reflects the slower deterioration that occurs in seeds stored under natural dry conditions. In contrast, the AAT and CDT methods expose seeds to high moisture and temperature, which increases molecular mobility,

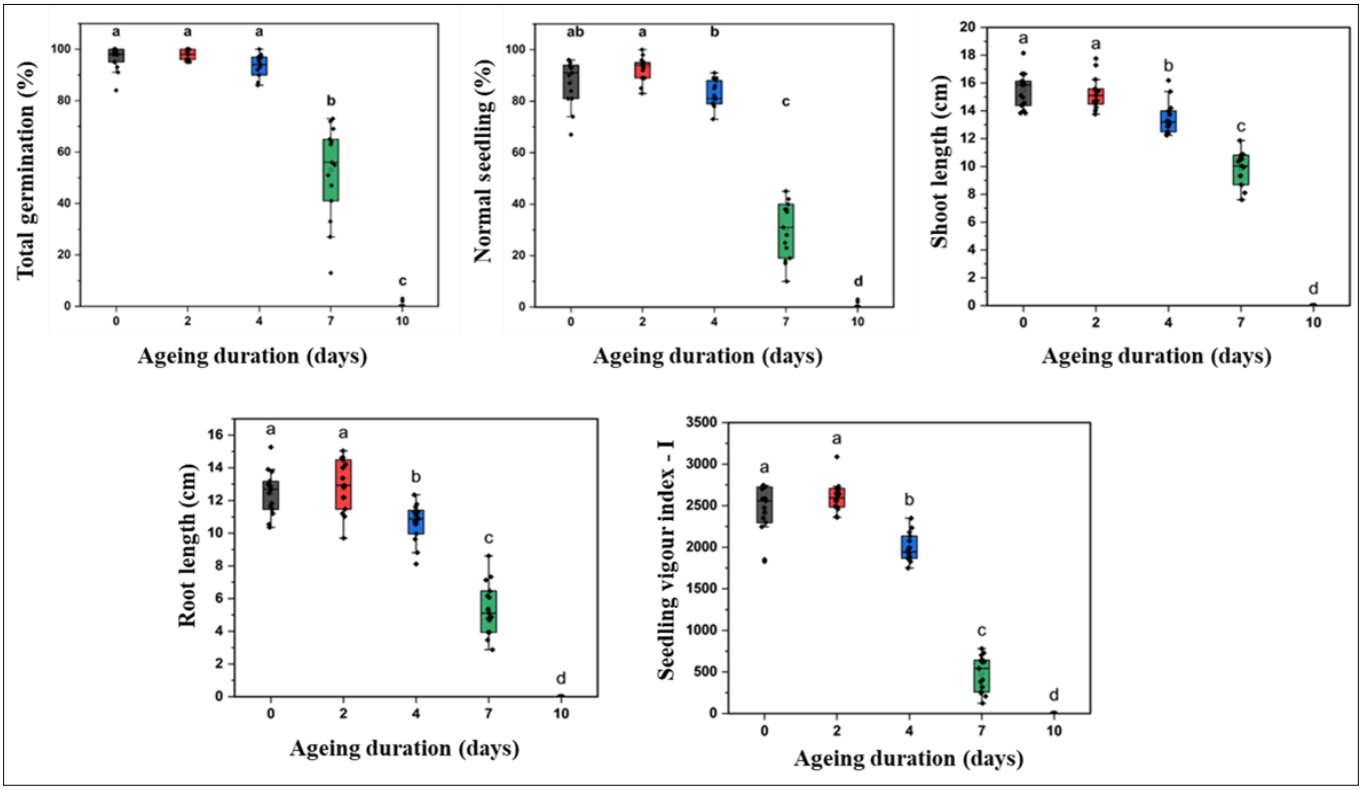

Fig. 1. Effect of AA on seed germination traits in soybean.

Values superscripted with the same letter are not significantly different from each other at $p \leq 0.05$; ageing period (A) means are denoted with lowercase letters.

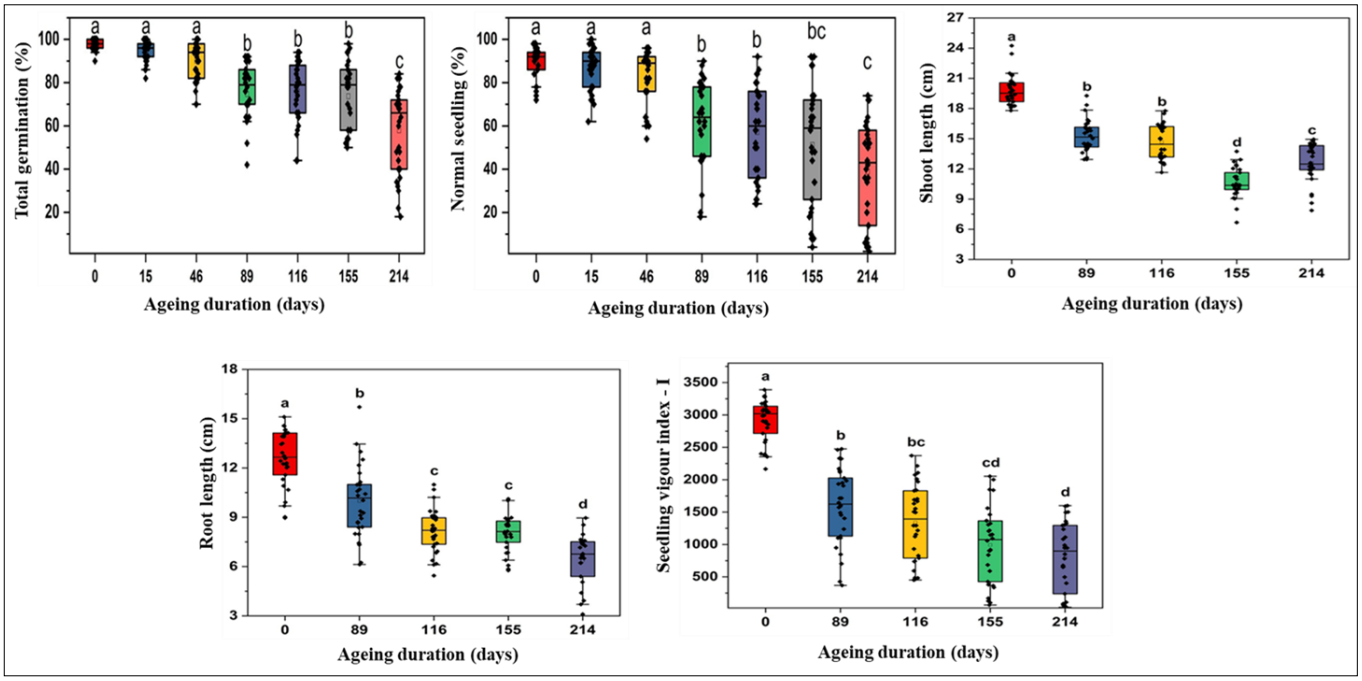

Fig. 2. Effect of DA on seed germination traits in soybean.

Values superscripted with the same letter are not significantly different from each other at $p \leq 0.05$; ageing period (A) means are denoted with lowercase letters

accelerates biochemical reactions and causes faster deterioration (8, 40). The dry ageing method used in this study maintained seeds at temperature (41 ± 0.3 °C) and relative humidity (50 %), which, as reported previously (8), kept the seeds in a glassy, metabolically inactive state throughout the ageing process. During this period, non-enzymatic oxidative damage becomes a major driver of deterioration, progressively impairing cellular structures and metabolic function. Seed lipids central to energy supply, membrane stability and signaling are highly susceptible to oxidation induced by free radicals and ROS (41). As ageing progresses, the accumulation of oxidized lipid species is largely attributed to spontaneous, non-enzymatic oxidation reactions (42). Importantly, oxidation of unsaturated lipids can proceed even when seeds are in a glassy state, depending on the relative reaction rates and moisture content, as influenced by the water activity. In this state, catalytic activity of the metal ions increases once the sufficient water is removed from their hydration cell, thereby accelerating the oxidative reactions (43). Consistent with this mechanism, studies using the EPPO DA technique have demonstrated substantially higher accumulation of oxidized lipid derivatives, which in turn show a strong negative association with seed germination (25).These findings agree with the present results and reinforce the notion that oxidation, rather than hydrolysis, dominates DA, providing a realistic simulation of natural seed deterioration.

Correlation analysis

In accelerated ageing, strong positive correlations were observed between TG % and NSL % ($r \approx 0.88$) and between NSL % and SVI-I (r = 0.96–0.98), while the correlation between SL and RL changed from strongly negative (r = –0.63) to weakly positive (r = 0.09) (Fig. 3A and B). In contrast, dry ageing produced a clear shift in RL correlations, which changed from negative (r = -0.11 with TG %, -0.13 with NSL % and –0.69 with SL) to strongly positive (r = 0.90 with TG %, 0.87 with NSL % and 0.76 with SL) (Fig. 4A and B). Accelerated ageing showed only moderate strengthening of correlations (e.g., RL with SVI-I: r = 0.17 to 0.71), whereas DA resulted in a more uniform positive association among all traits (r > 0.70). This indicates that the duration and method of ageing strongly influence the interdependence of seed traits (44), with DA promoting greater coordination among germination and vigour parameters compared to AA.

Principal component analysis

Principal component analysis provided a clear differentiation among seed traits under AA and DA conditions (Fig. 3C-D and 4C-D). In both methods, the variance was more concentrated in dimension 1, but this effect was stronger in DA (88.8 %) than in AA (70.5 %). The total variance explained by the first two dimensions was also higher in DA (95.8 %) than in AA (90.7 %), reflecting stronger overall correlations among traits. Trait contributions to each principal component varied between methods: under AA, NSL % and SVI-I dominated dimension 1, while DA produced a more balanced contribution across all traits. Similar PCA-based differentiation and stronger trait coordination under gradual or natural ageing conditions have been reported earlier, supporting the interpretation that DA promotes an integrated physiological response among germination and vigour traits (45).

Dry ageing as an alternative to accelerated ageing for phenotyping germplasm for higher longevity in soybean

A reliable phenotyping method must capture the true genetic variability of a trait. The comparison between DA and AA revealed large differences in their ability to distinguish between tolerant and sensitive genotypes. Under DA, EC 274711 was identified as the most sensitive genotype, showing a 50 % reduction in NSL % after 66.48 days, whereas TGX 855-32E maintained 84.4 % normal seedlings, the highest among all genotypes and was thus considered the most tolerant. This demonstrates that DA effectively differentiates genetic variations in seed longevity. However, under AA, EC 274711 reached a 50 % reduction in just 5.07 days. Notably, TGX 855-32E, which was tolerant to DA, showed only 59.97 % NSL at the same time point, revealing a narrower tolerance range. This compression of variability under accelerated ageing likely results from the extreme

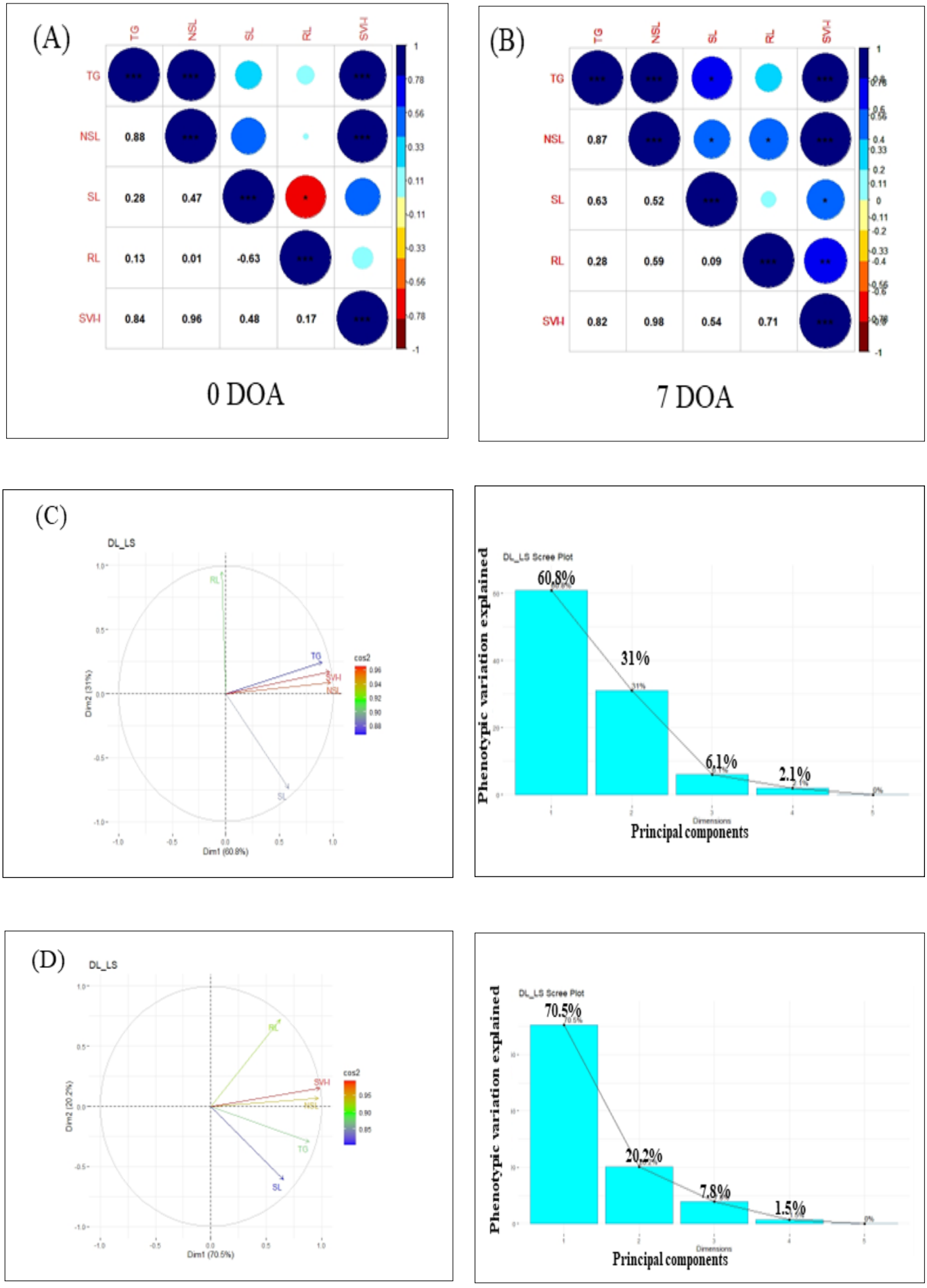

Fig. 3. A & B: Correlation among different seed germination traits at 0 and 7 days of AA; **C & D:** Principal component analysis of different seed germination parameters at 0 and after 7 days of AA. The scree plot indicates the percentage of variation explained by each principal component.

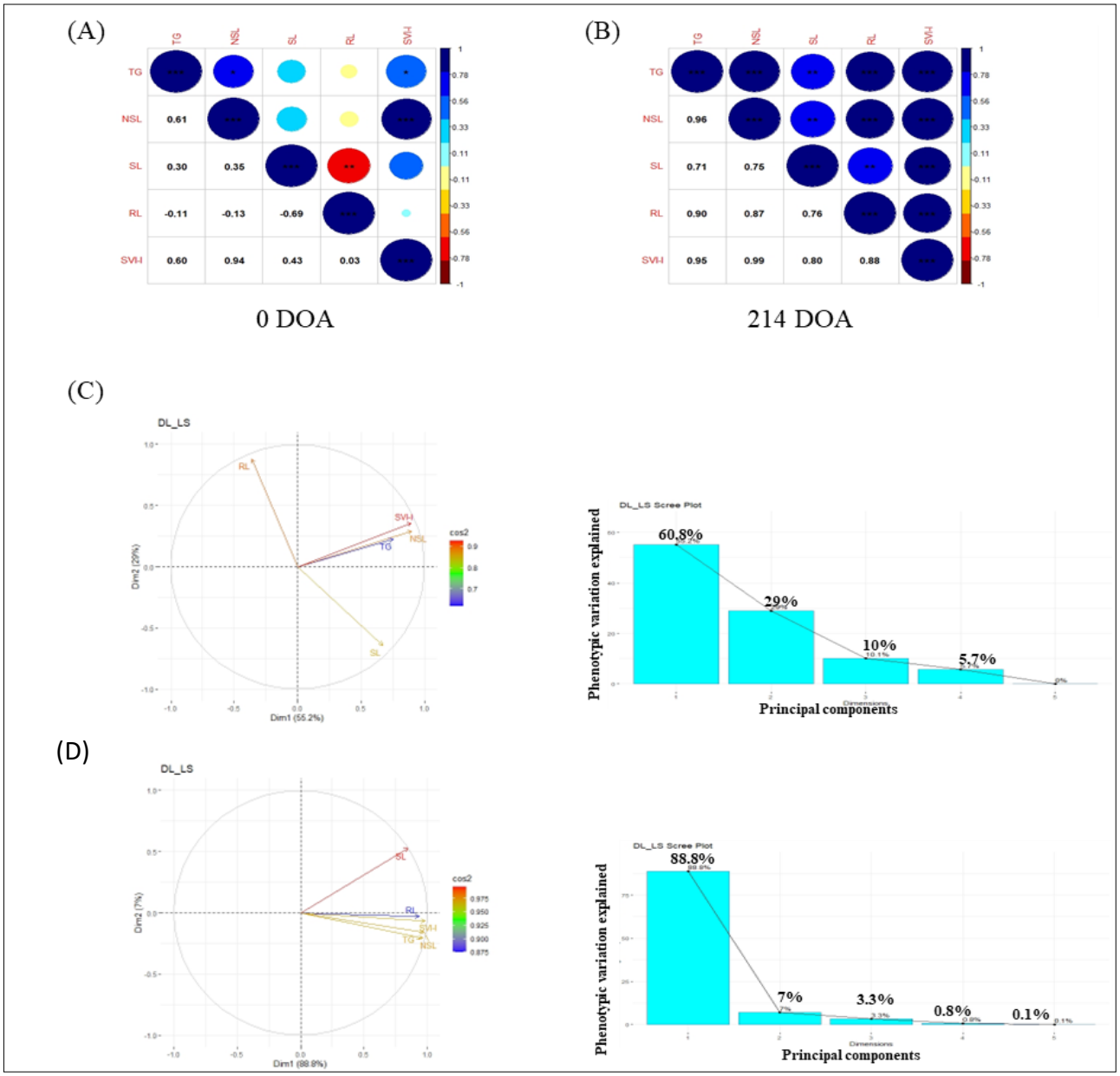

Fig. 4. A & B: Correlation among different seed germination traits at 0 and 214 days of DA; **C & D:** Principal component analysis of different seed germination parameters at 0 and 214 days of DA. The scree plot indicates the percentage of variation explained by each principal component.

temperature and humidity conditions, which not only accelerate internal deterioration but also cause physical damage and increase microbial susceptibility (46). These additional stresses may mask the true genetic potential for longevity, particularly in tolerant genotypes.

Differences in seed moisture status during ageing (AA and natural ageing) are crucial for explaining these contrasting outcomes (39). As moisture content directly affects reaction kinetics in biological and food systems, distinct physiological and genetic processes operate under each ageing method. Studies in various species support this view, demonstrating differences in free radical accumulation in soybean (38), electrical conductivity and malondialdehyde levels in *Jatropha curcas* (47), viability decline in *Vigna radiata* (39) and antioxidant enzyme activity in neem (48). In contrast, dry and natural ageing share similar low-moisture states, suggesting that DA better mimics natural seed deterioration, where non-enzymatic oxidative reactions play a central role (41). The oxidation of unsaturated lipids can proceed even in the glassy state, controlled by water activity and relative reaction rates. Under such dry conditions, metal ions (Fe^{2+}) lose part of their hydration shell, which enhances their catalytic activity and accelerates oxidation reactions (42).

Taken together, these findings indicate that DA more accurately reflects natural seed deterioration and is therefore a realistic and reliable method for phenotyping seed longevity. Hence, the present study propose DA as a superior alternative to AA for identifying genotypes and genomic regions truly associated with seed longevity mechanisms. Using DA for genetic dissection of seed longevity is expected to produce more accurate marker-trait associations and enable breeders to target authentic physiological pathways for improving storability in soybeans.

Conclusion

The results confirmed that DA effectively reproduces the typical symptoms of seed deterioration, including reductions in TG %, NSL %, SL, RL and SVI-I, similar to those observed under AA. However, based on the known relationship between moisture content and

reaction rates, it is clear that distinct physiological and genetic mechanisms govern these processes. Because DA maintains seeds in a dry, glassy state that closely mimics natural storage conditions, it is more likely to replicate the true course of natural ageing. Therefore, genetic studies based on DA can provide more realistic insights into the physiological and genetic control of seed longevity, thereby assisting breeders incorporate genuine longevity-related genes into improved soybean varieties. Thus, the present study recommends adopting the DA method as a scientifically robust and practical alternative to the conventional AA test in soybean research.

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Authors' contributions

AA, MBR and CTMP contributed equally to this work. AA, CTMP, MBR and MBAK designed this research. AA, CTMP, MBR, BKN, HRA, MKK, SKL and DV carried out the experiments. AA, MBR, CTMP, SB and MBAK analysed the data. AA, CTMP, MBR, HRA and MBAK wrote the manuscript. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of the manuscript, the authors used ChatGPT to improve the readability and grammatical errors. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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