Antioxidant depletion during seed storage under ambient conditions

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Abstract

Cumulative oxidative damage from the unavoidable formation of reactive oxygen species (ROS) contributes to seed ageing. Low-molecular-weight (LMW) antioxidants, such as water-soluble glutathione (GSH) and lipid-soluble tocochromanols, can prevent ROS from causing damage, especially when antioxidant enzymes are inactive due to desiccation. However, loss of tocochromanols does not always accompany seed ageing, such as during accelerated ageing or controlled deterioration, despite the presence of oxygen and prevalent loss of GSH. To assess relationships between total germination (TG) and antioxidant changes under storage conditions with practical relevance, commercial seeds of *Cucumis sativus*, *Daucus carota*, *Helianthus cucumerifolius*, *Latuca sativa*, *Lepidium sativum*, *Phaseolus vulgaris* and *Raphanus sativus* of the same cultivar were obtained over multiple years and stored under ambient conditions (21.9 ± 2.1°C; 36.8 ± 6.6% relative humidity). Sigmoidal fitting of TG revealed time to when 50% of seeds had lost viability, which ranged from <5 years (*D. carota*) to >15 years (*C. sativus*). Cellular redox states were quantified via the half-cell reduction potential of LMW thiol/disulphide couples. These negatively correlated with TG (i.e. cell redox states were more oxidized in lots with lower TG), with an average $R^2$ value of 0.62 for the most abundant thiol (GSH, or $\gamma$-glutamyl-cysteine in *P. vulgaris*). Concentrations of tocochromanols positively correlated with TG, with an average $R^2$ value of 0.50 for the most abundant tocochromanol ($\gamma$ or $\alpha$ in *L. sativa* and *H. cucumerifolius*). Therefore, during viability loss under ambient ageing conditions leading to the cytoplasm having a glassy state, the lipid domain in all species experienced oxidative damage.

Introduction

What lives is destined to die. Ageing is unavoidable for all life, but underlying mechanisms are still not fully understood. However, what all life forms have in common is the intertwining genetic and environmental elements that influence lifespan, and seeds are no exception. Seeds can be broadly categorized into two groups regarding their tolerance to desiccation, which predominantly influences seed longevity. Orthodox seeds, such as grains and those from most other crops, tolerate desiccation and suspend metabolism, thus have extended life-spans, whereas recalcitrant seeds, such as large-seeded temperate and tropical tree species, are desiccation intolerant and generally do not survive beyond 1 year (Ellis, 1991; Berjak and Pammenter, 2008). The extended longevity of orthodox seeds is critical for standard agricultural practices, but also enables the conservation of germplasm in seed banks. In the desiccated state, orthodox seed longevity is strictly influenced by temperature and seed moisture content (MC), with a reduction in either of these two abiotic components extending the seed life span (Ellis and Roberts, 1980). Hence, to extend longevity over decades to centuries, seed banks store seeds equilibrated to low relative humidity (RH) at low temperatures. Inversely, for investigating ageing mechanisms in shorter-term research projects, seeds are aged much faster over days to months in protocols called ‘controlled deterioration’ or ‘accelerated ageing’ (i.e. 60–100% RH and 35–50°C).

At a sufficiently low temperature and MC (≤0.1 g H₂O g⁻¹ dry weight (DW) and 20°C), the cytoplasm in seeds solidifies into a non-crystalline matrix, described as ‘glassy’. The glassy cytoplasm restricts molecular diffusion, thus extending longevity via decelerating the rates of biochemical reactions implicated in seed deterioration (Walters et al., 2005a; Buitink and Leprince, 2008; Ballesteros and Walters, 2011). At the higher MC and temperature of rapid ageing protocols, the cytoplasm is no longer glassy, and long-distance molecular mobility is enabled (Ballesteros et al., 2020). Although the cytoplasm is still highly viscous at approximately <0.3 g H₂O g⁻¹ DW and 20°C, the enzyme-dependent xanthophyll cycle occurred in lichen at 0.17 g H₂O g⁻¹ DW and 20°C (Candotto Carniel et al., 2020). Moreover, changes in primary metabolite profiles provided the evidence of aldo-keto reductase and glutamate decarboxylase activities in seeds aged at 80% RH and 45°C, in which cells had a viscous, but still fluid, cytoplasm (Gerna et al., 2022). Therefore, rapid ageing protocols may not always...
lead to the same biochemical changes that occur under conditions that the majority of seeds used in agriculture or conservation are stored. For instance, the lipid phase remains stable and without signs of oxidation in seeds rapidly aged (Lehner et al., 2008; Morscher et al., 2015; Roach et al., 2018; Schausberger et al., 2019; Gerna et al., 2022), while lipid oxidation is clearly evident during storage at lower RH and/or temperatures (Seal et al., 2010a,b; Roach et al., 2018; Wibach et al., 2020; Gerna et al., 2022; Groot et al., 2022).

The production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, is unavoidable for all aerobic organisms (Halliwell, 2006), including while desiccated (Kraner et al., 2010; Sano et al., 2016). ROS play major roles in seed physiology. On the one hand, they act in the cellular signalling pathways that promote germination, while on the other, they lead to oxidative damage and ageing, processes that hinder vigour, and contribute to viability loss (Bailly et al., 2008). Low-molecular-weight (LMW) antioxidants react with ROS, preventing oxidative damage to DNA, lipids and proteins. Crucially, in desiccated seeds, highly restricted enzyme activity results in LMW antioxidants as the only defence against ROS (Ballesteros et al., 2020; Gerna et al., 2022). Water-soluble thiols, such as glutathione (GSH) and lipid-soluble tocotrienols, are considered important LMW antioxidants that maintain longevity in orthodox seeds (Kraner et al., 2006; Mène-Saffrané et al., 2010). Oxidative attack of unsaturated lipids leads to the production of peroxyl radicals, which can be scavenged by tocotrienols (vitamin E for humans) and tocopherols, unsaturated derivatives of tocopherols also found in seeds (Falk and Munne-Bosch, 2010). Glutathione is a tripeptide, γ-glutamyl-cysteinyl-glycine, ubiquitous to all plants. In Leguminosae, the tripeptide, γ-glutamyl-cysteinyl-β-alanine, called homo-glutathione (hGSH) is also synthesized (Klapheck, 1988; Colville et al., 2015). Synthesis of GSH and hGSH occurs via the precursor γ-glutamyl-cysteine (γ-GC), which also accumulates in seeds (Birtić et al., 2011; Colville et al., 2015; Gerna et al., 2017). As part of their antioxidant activity (e.g. electron donation to ROS), LMW thiols convert to their disulphide form such as glutathione disulphide (GSSG). When the reduction of GSSG by glutathione reductase is restricted, such as in desiccated seeds, the glutathione-based cellular redox state (E_GSSG2GSH) shifts towards more oxidized values (Kraner et al., 2006).

Due to time constraints on research projects, only few studies have investigated slower ageing processes that occur in or cold-stored seed. Therefore, to improve understanding on seed deterioration under ambient conditions directly relevant to seed conservation and agriculture, we selected commercially important species with contrasting seed longevity and investigated relationships between seed viability, germination speed and changes in the LMW antioxidants such as glutathione and tocotrienols.

Materials and methods

Seeds

Seeds of Daucus carota (carrot var. Nantes 2, and var. Rotin), Helianthus cumeriferolius (sunflower), Phaseolus vulgaris (bean var. Brittle Wax), Cucumis sativus (gherkin var. Chinese Slangen), Raphanus sativus var. sativus (radish cv. Round – Half Red Half White), Raphanus sativus var. niger (black radish cv. Vienna Round Black), Lepidium sativum (cress) and Lactuca sativa (Lettuce var. crispa American Brown) were purchased from Austrosaat (Vienna, Austria), over 29 years since 2021, the latest harvest year available (Supplementary Table S1). Immediately after purchase, seeds were stored in original packets at an average room temperature of 21.9 ± 2.1°C and RH of 36.8 ± 6.6% (±SD), as recorded at hourly intervals for the whole of 2021 with a factory-calibrated Testo 175-H1 data logger (Testo, Belgium).

Germination tests and estimating time to 50% loss of total germination

For germination tests, C. sativus, R. sativus var. sativus, R. sativus var. niger, L. sativum and L. sativa were sown onto double thickness 85 mm Whatman number 1 filter papers, hydrated with 3 ml of deionized water in 90 mm Polystyrene Petri dishes, closed with Parafilm, and spaced out in five rows of ten seeds. For the bigger seeds of H. cumeriferolius and P. vulgaris, germination paper (grade 3644, Whatman) saturated with 40 ml of deionized water in closed transparent boxes was used. For each year of purchase, up to four replicates of 50 seeds were sown, each from different seed packets (Supplementary Table S2). Optimal stratification conditions and germination temperatures (Supplementary Table S1) were chosen according to the International Rules for Seed Testing (ISTA, 2010). Seeds were germinated in a growth chamber (Percival, PGC-6HO), with daytime light intensity for all temperatures of 50 μmol quanta m−2 s−1. Seed germination was scored for the first 5 days and then again on 8–12 days, 15–19 days, etc., for up to 6 weeks until no change in total germination (TG) occurred. For estimating the time to 50% loss of TG (PS0), the TG of each species from all available years was fitted with Sigmoidal Logistic function (type 1) in OriginPro 2017G (IBM), without weighting and using the highest TG recorded as the upper limit.

Seed preparation for biochemical analyses

For biochemical analyses, seeds were weighed before and after freeze-drying for 5 days (−80°C and 0.043 mbar) to determine seed fresh weight, DW and MC. For grinding all seeds, except P. vulgaris, 2 ml reaction tubes (Eppendorf) were filled one-third with freeze-dried seeds, or for H. cumeriferolius isolated embryonic axes, and depending on seed size, with either one 7 mm agate bead (larger seeds) or two 3 mm glass beads and two 5 mm agate beads (smaller seeds). Samples were ground at −80°C for 2 min at a frequency of 30 Hz in a Tissuelyser II (Qiagen). For P. vulgaris, 20 seeds were ground in 25 ml steel grinding vessels with two 10 mm metal beads for 1 min and a frequency of 30 Hz in a Mikro-Dismembrator (Satorius). When available, individual seed packets were used as replicates (Supplementary Table S2), otherwise replicates (n = 4) of different seeds were taken from the same packet.

Analysis of LMW thiols and disulphides

Thiols and disulphides were extracted, depending upon species from between 50 and 200 mg DW in 1 ml of 0.1 M HCl, in the presence of one 5 mm and two 3 mm glass beads, for 2–4 min, as required, at a frequency of 30 Hz. Initial trials were conducted with all species to see if polyvinylpolypyrrolidone (PVPP) was necessary to measure GSH, resulting in its addition, in equal amount to seed DW, during the extraction of L. sativum and both Raphanus varieties. The ground seed extract was centrifuged for 10 min at 29,000 g at 4°C, after which 700 μl of the supernatant was transferred into a 1.5 ml reaction tube (Eppendorf), which was
centrifuged for another 20 min, before analysis with an Agilent 1290 Infinity II according to the HPLC method of Bailly and Kranner (2011). Half-cell reduction potentials of thiol/disulphide redox couples (i.e. $E_{\text{GSSG/2GSH}}$) were calculated according to the Nernst equation and standard half-cell redox potentials of Birtić et al. (2011) and assuming pH of 7.3. Molar concentrations of thiols and disulphides were calculated from MC of the respective seeds.

**Analysis of tocochromanols**

For the analyses of tocochromanols, 50 mg of ground seed were placed into 2 ml reaction tubes (Eppendorf) with 800 μl of heptane and two 3 mm and one 5 mm glass bead, and shaken for 5 min at a frequency of 30 Hz. The samples were centrifuged for 20 min at 29,000 g at 4°C before injecting 20–50 μl, depending upon species, into an Agilent 1100 HPLC for separation and measurement by fluorescence (Ex: 295 nm; Ex: 325 nm) according to Schausberger et al. (2019).

**Results**

**Seed longevity under ambient storage conditions**

Overall, the TG of seeds from different years of purchase, of all species, showed a sigmoidal relationship with time, agreeing with a normal distribution in seed longevity (Fig. 1). Using the function derived from the sigmoidal fit, the P50 was calculated for each species. Only *D. carota* var. *Nantes 2* had a P50 <5 years, while *L. sativa*, *R. sativus*, *L. sativum* had P50 of 5–10 years, *H. cucumerifolius* and *P. vulgaris* had P50 of 5–15 years and *C. sativus* had P50 >15 years (Supplementary Fig. S1). In some species, such as *L. sativa*, *C. sativus* and *R. sativus*, TG decreased in seeds within the first years of storage, while for *P. vulgaris* and *L. sativum*, hardly any loss of TG occurred with the first 5–10 years of storage (Fig. 1). The gradient of slope of the sigmoidal fit, indicating the rate of viability loss, was also not uniform and much steeper for *P. vulgaris* and *L. sativum*, reflecting their initial resistance to loss of TG (Fig. 1). The most closely fitting sigmoidal relationship between TG and years of storage was found for *L. sativa* (Fig. 1E), whereas several years of deviation were observed for *R. sativus* var. *sativus* (i.e. lower than expected TG after 10 years and higher than expected after 17 years of storage) (Fig. 1C). Generally, germination speed (i.e. T20) after different durations of storage showed a positive linear relationship with TG (Fig. 1), with average $R^2$ values across species of 0.61 (Supplementary Table S3).

**Thiols and disulphides**

Glutathione makes up >90% of total amount of LMW thiol and disulphides in all species, except for *P. vulgaris* in which 92% is γ-GC and an additional 3% is hGSH (Supplementary Fig. S2).
The concentration of the most abundant LMW thiol/disulphide couple is different between seeds purchased from different years, but a consistent decline in total glutathione (GSH + GSSG) is not evident in *D. carota*, *R. sativus* var. *sativus*, *C. sativus* and *H. cucumerifolius* (Supplementary Fig. S3), as could be expected after storage and ageing. However, ageing effects are observable in seeds of all species regarding the relative concentrations of thiols and disulphides, indicative of oxidation during storage. Via the Nernst equation, molar concentrations of LMW thiols and disulphides enable the quantification of the potential of each redox couple (e.g. $E_{\text{GSSG}/2\text{GSH}}$ for glutathione), which provides an insight into cellular redox states. The range of $E_{\text{GSSG}/2\text{GSH}}$ values between seed lots with high TG and no TG was species-dependent (Fig. 2). For examples, $E_{\text{GSSG}/2\text{GSH}}$ values in non-stored seed of *P. vulgaris* are $-155$ mV, while in *L. sativa* are $-245$ mV, and in seeds with no TG are $-105$ and $-208$ mV, respectively (Fig. 2E, F). In seeds of all species, redox potentials of $E_{\gamma\text{-GCCG}/2\gamma\text{GC}}$ parallel values of $E_{\text{GSSG}/2\text{GSH}}$, although $+30$–$50$ mV higher (more oxidizing), except in *P. vulgaris* in which $E_{\gamma\text{-GCCG}/2\gamma\text{GC}}$ are the most negative (Fig. 2F). Regardless, $E_{\text{Disulphide}/2\text{Thiol}}$ of both couples values are consistently less negative (oxidative-shifted) in seeds that had been stored for longer (Fig. 2). Correlating the $E_{\text{Disulphide}/2\text{Thiol}}$ values with TG revealed a negative linear relationship (Supplementary Table S3), which is the highest for *L. sativa* ($R^2 = 0.955$) and the lowest for *H. cucumerifolius* ($R^2 = 0.321$). Across species, the average $R^2$ value is 0.619 from all linear correlation coefficients between TG and $E_{\text{Disulphide}/2\text{Thiol}}$ values (Supplementary Table S3) when considering the most abundant LMW thiol/disulphide couple (glutathione and $\gamma$-GC in *P. vulgaris*; Supplementary Fig. S2).

**Tocochromanols**

Tocochromanol compositions and amounts, on a DW basis, are different between species (Fig. 3). For example, in seed lots with >50% TG, *H. cucumerifolius* have $\alpha$-tocopherol concentrations $>0.6$ μmol g$^{-1}$ DW (Fig. 3D), while in *L. sativa* they are 0.25–0.03 μmol g$^{-1}$ DW (Fig. 3E). These two species are the only to contain $\beta$-tocopherol, which is at <10-fold lower concentration than $\alpha$-tocopherol. In *D. carota*, only tocotrienols were detected, and $\gamma$-tocotrienol is the dominant form (Fig. 3A). Notably, *P. vulgaris* possesses very low total tocochromanol levels (<0.05 μmol g$^{-1}$ DW; Fig. 3F), but like other species $\gamma$-tocopherol is the most abundant form. In *R. sativus*, *C. sativus* and *L. sativum*, $\delta$-tocopherol also makes up a minor fraction of the total tocochromanol pool (Fig. 3B, G, H). Generally, less abundant
tocochromanols weakly correlate with TG (Supplementary Table S3), whereas lower amounts of the most abundant tocochromanol are found in seeds stored for more time with low TG (Fig. 3). In *R. sativus*, *C. sativus* and *L. sativum*, this relationship is weakened by the low γ-tocopherol levels in seeds from 2021 before storage (Fig. 3B, C, G, H). Across species, the average $R^2$ value is 0.50 from all linear correlation coefficients between TG and amounts of the most abundant tocochromanol (Supplementary Table S3).

**Discussion**

The aim of this study was to investigate relationships between TG and antioxidants in seeds stored under ambient conditions from species with contrasting seed longevity. For all species, except *C. sativus*, seed lots ranged from maximum to no TG, enabling the accurate calculation of P50. Relative seed longevity between species in this study generally conform to other ageing studies (Priestley et al., 1985; Walters et al., 2005b; Nagel and Börner, 2010). One explanation for differing longevity is the composition of seed storage compounds, with lipid-rich seeds tending to possess shortened longevity (Nagel and Börner, 2010). However, all seeds contain lipids in the form of membranes, and membrane integrity is essential to organelle and cell compartmentalization, and thus viability. Seeds of Arabidopsis and tomato mutants deficient in tocochromanols show reduced longevity and suffer more lipid peroxidation than wild-type seeds (Mène-Saffrané et al., 2010; Chen et al., 2016), supporting a role for tocochromanols in enabling seed longevity. A positive linear correlation was found for all species between TG and amounts of the most abundant tocochromanol, indicating the consumption of tocochromanols occurred during seed storage, although this correlation is weak for *L. sativum* ($R^2 = 0.17$) and *R. sativus var. sativus* ($R^2 = 0.30$). In these two species, at least, low levels of γ-tocopherol were measured in seeds from 2021 without storage, contributing to this weak correlation. Temperature and soil moisture during seed development, and particularly during seed filling, affect tocochromanol concentrations (Oomah et al., 1997; Siger et al., 2015; Carrera and Seguin, 2016; Haro et al., 2020). Since seeds used in this study were harvested from different years, seasonal climatic differences during seed production would have influenced seed development and tocochromanol contents before storage. Without storage, it is not possible to say if seeds from 2021 with lowered tocochromanol amounts would have shortened longevity, but there are indications that seeds from other seasons with relatively low tocochromanol levels have shortened longevity, for example 6-year-stored *R. sativus var. sativus*.

**Fig. 3.** Tocochromanol concentrations in dry seeds stored under ambient indoor conditions (22 ± 2°C; 37 ± 7% RH) since purchase. The left Y-axis shows concentrations of α-tocopherol (filled red circle), α-tocotrienol (open red circle), β-tocopherol (upward green triangle), γ-tocopherol (filled blue square), γ-tocotrienol (open blue square), δ-tocopherol (downward filled black triangle) and δ-tocotrienol (downward open black triangle) in (A) *Daucus carota*, (B) *Lepidium sativum*, (C) *Raphanus sativus var. sativus*, (D) *Helianthus cucumerifolius*, (E) *Lactuca sativa*, (F) *Phaseolus vulgaris*, (G) *Cucumis sativus* and (H) *Raphanus sativus var. niger*, n=4 replicates ± SD. Total germination is indicated by the dotted line (right Y-axis), as shown in Fig. 1.
A decrease in tocopherol concentrations does not always occur during seed ageing, for example when rapid ageing protocols are used that lead to fluid cytoplasm (Seal et al., 2010b; Groot et al., 2012, 2022; Morscher et al., 2015; Lee et al., 2017; Roach et al., 2018; Schausberger et al., 2019). Recently, Gerna et al. (2022) showed that lipid peroxidation and the consumption of tocopherols only occur during seed ageing when the cytoplasm is glassy. Therefore, the ambient ageing conditions in the current study, leading to seeds with MC <0.05 g H₂O g⁻¹ DW and the cytoplasm being glassy-in-state (Butink and Leprince, 2008), rendered the seeds vulnerable to lipid peroxidation, which may have contributed to viability loss.

Redox poising by LMW thiol/disulphide couples is at the basis of cellular redox homeostasis (Schafer and Buettner, 2001; Foyer and Noctor, 2011), especially in seeds (Kraner et al., 2006; Colville and Kraner, 2010). Oxidative shifts of the cellular redox environment, as viewed through $E_{\text{GSSG/GSH}}$, have been closely related to losses of seed viability in seeds that age rapidly when the cytoplasm is fluid (Kraner et al., 2006; Roach et al., 2010; Birtic et al., 2011; Morscher et al., 2015; Nagel et al., 2015; Schausberger et al., 2019). In those studies, P50 associates with $E_{\text{GSSG/GSH}}$ values between −180 and −160 mV, while in this study P50 associates with $E_{\text{GSSG/GSH}}$ values between −180 and −200 mV (Fig. 2), which is a value range found in other seeds aged slower with glassy cytoplasm (Seal et al., 2010b; Gerna et al., 2022). An exception is P. vulgaris, in which $E_{\text{GSSG/GSH}}$ values were much higher due to very low GSH concentrations. Although P. vulgaris seeds are known to possess more lGSH than GSH (Klapheck, 1988), the main LMW thiol/disulphide couple in this Brittle Wax var. of P. vulgaris was γ-GC. P. vulgaris is known to produce γ-glutamyl dipeptides (Giada et al., 1998), but γ-GC was, so far, never found as the dominant LMW thiol/disulphide couple in seeds of 73 investigated species of Leguminosae (Colville et al., 2015). Although considered more as a GSH precursor than antioxidant, γ-GC has a γ-glutamyl moiety coupled to cysteine, enabling this dipeptide to be a substrate to glutathione peroxidase and glutathione transferase (Quintana-Cabrera et al., 2012; Muraoka et al., 2021). Evidently in all species, $E_{\text{GCCG/GYG}}$ parallels $E_{\text{GSSG/GSH}}$, indicating that both are equally responding to the given seed redox environment and buffering ROS production. Indeed, the average $R^2$ values between TG and $E_{\text{GSSG/GSH}}$ or $E_{\text{GCCG/GYG}}$ for all species are 0.59 and 0.58, respectively (Supplementary Table S1). Thus, the main difference leading to the lower redox potential of these two couples is not a different redox activity, but the concentration of thiol form, for which GSH is much higher than γ-GC in all species except P. vulgaris.

In conclusion, viability loss under the ambient conditions of this study, leading to a glassy cytoplasm, associated with oxidative damage to the lipid and aqueous parts of the seed, regardless of species-specific seed longevity. This is in contrast to rapid seed ageing tests often used in research projects, which places the cytoplasm into a fluid state and leaves the lipid phase mostly undamaged during ageing. Furthermore, a lesser thiol/disulphide oxidative shift occurred in seeds aged with glassy cytoplasm, relative to when the cytoplasm is fluid, also highlighting that different ageing mechanisms occur when seeds are aged with glassy or fluid cytoplasm.

**Supplementary material.** To view supplementary material for this article, please visit: https://doi.org/10.1017/S0960258522000101.

**Financial support.** This work was supported by the Austrian Science Foundation (FWF P 32599).

**Conflicts of interest.** The author(s) declare none.

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