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Ageing kinetics of fern chlorophyllous spores during dry storage is determined by its antioxidant potential and likely induced by photosynthetic machinery

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ABSTRACT

Ageing in dry chlorophyllous propagules is leaded by photooxidation through the photosynthetic machinery, but why species differ in longevity and the ageing mechanisms of when light and oxygen are absent are unknown. We hypothesize that the cellular antioxidant capacity is key for the inter- and intra-specific differences in the ageing process. We have tested this hypothesis in chlorophyllous spores of two ferns. They were subjected to four different storage regimes resulting from light/dark and normoxia/hypoxia combinations. Lipophilic and hydrophilic antioxidants, reactive oxygen species (ROS), and photosynthetic pigments were analysed in parallel to germination and the recovery of Fv/Fm over a storage period of up to 22-months. We show that light and oxygen accelerate the ageing process, but their mechanisms (ROS, increase, antioxidant capacity decrease, loss of efficiency of the photosystem II, pigment degradation) appear the same under all conditions tested. The end of the asymptomatic phase of longevity, when a sudden drop of germination occurs, seems to be determined by a threshold in the depletion of antioxidants. Our results support the hypothesis that ageing kinetics in dry plant propagules is determined by the antioxidant system, but also suggests an active role of the photosynthetic machinery during ageing, even in darkness and hypoxia.

1. Introduction

Tissue desiccation is one of the major challenges that a living organism can deal with. Coping with severe desiccation (to water contents (WC) < 0.1 g H_2O g^{-1} dry weight (DW) or to equilibrium under relative humidity (RH) < 70%) implies the activation of a sophisticate array of protection mechanisms that allow the recovery of metabolism after the removal of almost all the cellular water without irreversible damage (Vertucci and Farrant, 1995; Leprince and Buitink, 2010; Fernández-Marín et al., 2016). Among tracheophytes, desiccation tolerance (DT) is a rare trait in photosynthetic tissues (Gaff, 1980; Alpert, 2005; Oliver et al., 2000) but is widely expressed in reproductive or dispersal structures (pollen, seeds and spores). The majority of reproductive structures can cope with drying to low WC for a short period (e.g., minutes to hours) and recover full metabolism upon rehydration (Franchi et al., 2011). Moreover, many of them can survive in the dry state for periods ranging from days to centuries, or even millennia, depending on the species and the environmental conditions during storage (Priestley et al., 1985; Shen-Miller et al., 1995; Buitink et al., 1998; Walters et al., 2005a, 2005b; Nagel and Börner, 2010; Ballesteros et al., 2019, Colville and Pritchard, 2019). Although the acquisition of DT and longevity in reproductive structures are related and are consecutively acquired during maturation, they do not relate to the same traits (Verdier et al., 2013; Pereira-Lima et al., 2017). DT implies the ability to cope with a high loss of water at the cellular level, while longevity refers to survival over time while in the dry state (Verdier et al., 2013; Walters, 2015; Pereira-Lima et al., 2017; Ballesteros et al., 2017). Longevity in the dry state requires dealing with the stress produced by initial desiccation, by that produced during deterioration over time (i.e., ageing reactions) and the subsequent stress during rehydration (Kranner et al., 2010; Farrant, 2007). Although the ageing reactions may be of similar nature amongst reproductive structures and plant species, the mechanisms that control their kinetics are diverse and dependant on the physicochemical properties of the dry cell architecture (Ballesteros et al., 2020; Farrant and

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Hilhorst, 2021).

DT plants have developed a series of strategies to face water loss and to be able to survive in a dry state (Wood, 2005). The function of these mechanisms is usually to preserve the cellular and molecular integrity, avoiding the disruption of ultrastructures, and to counteract the oxidative damage that results from the depletion of the antioxidant machinery and the presence or production of reactive oxygen species (ROS) and/or free radicals (Leprince et al., 1993; Vertucci and Farrant, 1995; Kranner et al., 2006; Dinakar et al., 2012; Walters et al., 2010; Walters, 2015; Pereira-Lima et al., 2017; Kranner and Birtic 2005; Mira et al., 2016; Nagel et al., 2016; Fleming et al., 2017; Fleming et al., 2018; Kranner et al., 2002a; Wiebach et al., 2019). In this sense, it is known that ageing reactions in dry reproductive tissues are mainly related to oxidative damage and can be expressed as lipid oxidation and hydrolysis, random RNA fragmentation, and the activation of a signalling cascade by ROS (after the decrease of the antioxidant capacity of the cell) that initiates programmed cell death (PCD) leading to inter-nucleosomal DNA fragmentation in the final (McDonald, 1999; Bailly, 2004; Kranner and Birtic 2005; Kranner et al., 2006; Mira et al., 2010, 2016; Fleming et al., 2017, 2018; Gerna et al., 2022). The pressure from ROS and oxidative stress is exacerbated in dry photosynthetic DT cells and tissues (Ballesteros et al., 2020). For example, in dry chlorophyllous seed tissues, oxidative stress has been related to lipid peroxidation in the thylakoid membranes due to photo-oxidation (Roquiero et al., 2010). This is possible because during dehydration and while in the dry state, chlorophyll molecules can still be excited by light photons and, as this energy cannot be used for photochemistry, it will generate ROS that will increase the oxidative stress on the nearby tissues (Heber and Shuvalov, 2005; Roquiero et al., 2010; Ballesteros et al., 2018). This production of ROS is the consequence of the metabolic imbalance between the received and the used light energy in the photosynthetic apparatus (Vertucci and Farrant, 1995; Verhoeven et al., 2018).

The capacity to survive in the dry state over the time, also depends on environmental factors (Walters et al., 2005; Hoekstra, 2005; Ballesteros et al., 2017, 2020). Moisture, temperature, light and gas environment (i.e. the presence of oxygen) are the more determinant factors in the ageing of DT seeds, pollen, fern spores and photosynthetic tissues in the dry state (Steiner and Ruckenbauer, 1995; Buitink et al., 1998; Walters et al., 2005; Groot et al., 2012; Telewski and Zeevaart, 2002; Gasulla et al., 2009; Roquiero et al., 2010; Fernández-Marín et al., 2013, 2019; Ballesteros et al., 2018, 2019, 2020; Gerna et al., 2022). When a DT tissue dries, its cytoplasm solidifies entering in a physical state known as amorphous solid or glass (Leprince and Buitink, 2010; Walters et al., 2010; Ballesteros et al., 2020). When a glass is formed, molecules and cell components are entrapped in a solid matrix that suppresses most molecular diffusion and mobility, hence impeding most chemical (particularly enzymatic) activities and metabolism (Leprince and Buitink, 2010; Walters et al., 2010; Fernández-Marín et al., 2013, 2016; Fleming et al., 2018; Ballesteros, et al., 2020). But glasses are not thermodynamically stable over time and tend to relax depending on the molecular mobility allowed in a process known as physical ageing (Ballesteros et al., 2020). Some vibrational and rotational mobility is still possible in atoms, bonds and groups of atoms (Ballesteros and Walters, 2011; Ballesteros, et al., 2020), as for example, the crystallization and melting of the triacylglycerols that compose the storage lipids (e.g., Mira et al., 2019; Ballesteros, et al., 2019) or the excitation of macromolecules such as chlorophyll by light and their change in redox status (Roquiero et al., 2010; Ballesteros et al., 2018). Besides, translational diffusion of gases and small solutes (such as oxygen and ROS) is still possible (Ballesteros et al., 2020). Hence, in these solid but dynamic structures, the presence of diverse types of molecular mobility among the different types of substrates and the diffusion of gases within the glass due to environmental changes will be key in the deterioration rate of the diverse dry tissues.

Since antioxidant protection in the dry (glassy) state is likely not possible by enzymatic mechanisms, ROS deactivation will mainly occur by direct interaction with free molecules. Among these non-enzymatic defences two main groups can be found: lipophilic and hydrophilic antioxidants. Among hydrophilic antioxidants glutathione (GSH) is one of the most efficient and is responsible for the removal of H_2O_2 (Kranner et al., 2006). Under non-stressed conditions, GSH is mostly found in the reduced state, able to donate an electron to several ROS (e.g.$\cdot H_2O_2$) with glutathione disulphide (GSSG) as oxidation product. The reduction of GSSG to GSH follows an enzymatic pathway by the enzyme glutathione reductase and, as commented, this is an unviable reaction when the glass is formed inside cells. In this situation, the unique direction is the oxidation of GSH pool. The decrease in GSH has been often related to various stresses (Kranner and Birtić, 2005), including seeds ageing (Kranner et al., 2006). Among lipophilic antioxidants, α-, β-, δ-, γ-tocopherol and some carotenoids seem to play a central role in ROS scavenging and membrane stability (Krinsky, 1994; Munné-Bosch and Alegre, 2002; Havaux, 1998; Fernández-Marín et al., 2013). On the one hand, the presence of tocopherols (specifically α-toc) has been correlated with seeds viability (Seal et al., 2010). Both antioxidant systems, very likely acting synergistically (Foyer and Noctor, 2005), may maintain the physiological integrity of seeds during long-term storage, needed to survival and ex-situ conservation (Fernández-Marín et al., 2017). Finally, in photosynthetic tissues, the formation of ROS by chlorophyll overexcitation can be "prevented" in the light-harvesting complexes by the dissipation as heat of the light energy absorbed by chlorophyll through specific carotenoids (Violaxanthi-Antheraxanthin-Zeaxanthin cycle) (Demmig-Adams, 1990). The reduction in the pool of all these antioxidants has been associated with injury and ageing, and therefore, loss of viability in DT tissues and seeds (Berjak et al., 2011; Illing et al., 2005) while their content positively correlates with that of chlorophyll in dry seeds (Fernández-Marín et al., 2017).

In this work we have focused on fern spores as the model study. Among ferns, two kinds of spores may be found: chlorophyllous spores (CS) which have fully developed chloroplasts after maturation and dispersion and usually short longevity, and non-chlorophyllous spores, without chloroplasts at maturity also characterised by their longer longevity (Lloyd and Klekowshy, 1970). We have previously investigated the eco-physiological diversity of DT among CS in relation to their biochemistry and biophysics (López-Pozo, et al., 2019a,b), and have found that preservation of plastid integrity plays a key role in the capacity of CS to tolerate initial drying and drying//rehydration cycles (López-Pozo et al., 2019a). We also have investigated the influence of moisture, temperature, and light in the variation of longevity of CS (Ballesteros et al., 2017, 2018, 2019), but the reasons for this variation remain unclear. Understanding what the role of the photosynthetic system is across CS species showing diverse longevity, can be key to understand what modulates DT and ageing in the dry state on these and other photosynthetic and non-photosynthetic tissues. Based on the biochemical and biophysical mechanisms exposed above and since the spores used in this work are chlorophyllous, we hypothesize that light exposure will decrease viability during storage and that this cell deterioration will be greater in the presence of oxygen. We also hypothesize that the faster deterioration by light and oxygen will be related to photooxidation and an increased production of ROS over time compared to spores stored in the dark and hypoxia. A decrease in the antioxidant machinery will be shared among storage conditions. All these biochemical changes in the redox status will ultimately shape photochemical functioning and germination. For this purpose, CS of two fern species (*M. struthiopteris* and *O. regalis*) were subjected to four different storage regimes: light + normoxia, light + hypoxia, dark + normoxia, and dark + hypoxia. We selected the CS of these two species due to their different longevity, biochemistry and biophysics in the dry state and their contrasting eco-physiology. Firstly, *M. struthiopteris* spores are at least two or three times longer lived in the dry state than those of *O. regalis* (Ballesteros et al., 2017, 2019). Secondly, *M. struthiopteris* spores contain higher initial amounts of photosynthetic antioxidants and compatible solutes (e.g., proline) than *O. regalis* spores (López-Pozo

et al., 2019b), besides the presence of certain types of tocopherols (β- δ- and γ- tocopherol) that were absent in *O regalis*. Moreover, *M. struthiopteris* spores show lower capacity to absorb water and lower water potentials during turgor loss and higher amounts of than in *O regalis* (López-Pozo et al., 2019b). Finally, both species show different phenology, maturing and germinating in very different atmospheric conditions (López-Pozo et al., 2019b). All this information suggests that the differences in longevity of the CS of *M. struthiopteris* and *O. regalis* could be modulated, at least, by their biochemical status during the dry state, particularly the antioxidant system. The use of these two contrasting species allows for a comparative study of the aging mechanisms of CS during dry storage. Lipophilic and hydrophilic antioxidants, ROS, and photosynthetic pigments were assessed in parallel to the loss of germination and the recovery of the maximum photochemical efficiency of photosystem II (PSII) over a storage period of up to 22 months. Our results support the relevance of the antioxidant system for the lifespan in the dry state, depict local damage around the photosynthetic apparatus as key factor for ageing of chlorophyllous systems, and reveal important interspecific differences for longevity of different fern species with CS.

2. Material and methods

2.1. Plant material

Fertile fronds of *Osmunda regalis* were collected in Wakehurst Place, UK, (51°03′57"N, 0°05′17"W, 120 m.a.s.l.) in June 2017. *Matteuccia struthiopteris* fertile fronds were collected at the Botanical Garden of the University of Innsbruck Austria (47°16′N, 11°23′ E, 600 m.a.s.l.) in December 2016. After collection, fronds were placed between paper sheets at room conditions in the laboratory (ca. 20 °C and 50–60% relative humidity [RH]) for 24–48 h allowing the dehiscence of the sporangia and the release of the spores. Once the spores were released, they were stored in the fridge until the beginning of the experiment in July 2017.

2.2. Experimental design

Spores were dried on open 85 mm diameter Petri dishes (with spores covering the entire surface of the petri dish) at 15% RH inside hermetic chambers that were placed at 4 ± 2 ºC for 24 h. After this first 24 h, the hermetic chambers were moved to 25 ± 2 °C and stored at four different regimes, resulting from the combination of two oxygen (normoxia and hypoxia) and two light (light and dark) conditions: "L-N": Light and normoxia | "L-H": Light and hypoxia | "D-N": Darkness and normoxia | "D-H": The storage experiment was run for up to 22 months, until germination dropped to nil, or no spores remained.

RH inside the chamber was generated with a saturated LiCl solution (Ballesteros and Walters, 2007a). When chambers were moved from 4ºC to 25ºC the RH inside the chambers decreased from 15% RH to 11% RH (Ballesteros and Walters, 2007a). Relative humidity and temperature were constantly monitored inside the chambers with Tinytag View 2 data loggers (Gemini Data Loggers, UK). In both RH/temperature combinations, spores of *O. regalis* and *M. struthiopteris* were expected to be in a glassy state (glass transition temperature, Tg, for both moisture conditions has been measured >40 °C in both species) (Ballesteros et al., 2017, 2019; López-Pozo et al., 2019b). Water content of the spores when stored at 11% RH and 25 °C remained constant for the duration of the experiment.

The photosynthetic photon flux density (PPFD) under the light conditions used was 200 μmol m^{-2} s^{-1}, with a photoperiod of 16 h. For dark conditions chambers were placed inside cover bags that totally blocked light penetration. Hypoxia was achieved by adding N_2 gas inside the chambers for 5–10 min until the O_2 levels dropped < 4%. Levels of O_2 and maintenance of hypoxia conditions was monitored for 72 h after N_2 addition with an oxygen probe. After each time the chambers were opened for sampling spores, N_2 was added until the previous hypoxia levels were achieved.

2.3. Germination assay

In order to obtain samples with viability ranging from maximum (i.e. that obtained when spores were freshly harvested) to zero, the sampling period of the experiment was adjusted according to the expected ageing rate for the species at 25ºC (e.g. Ballesteros et al., 2017, 2019).

Spores were sown into four 60 mm diameter Petri dishes filled to half depth with Dyer's culture medium for fern spores solidified with 1.3% agar and prepared with the fungicide nystatin (100 U mL^{-1}) (Ballesteros et al., 2017). Petri dishes were sealed with Parafilm (American National Can) and placed in a germination chamber set at 20 ± 2 °C with a 16 h photoperiod and a PPFD of 200 μmol m^{-2} s^{-1}. Germination was scored when the outer wall of the spore ruptured the spore wall and the rhizoid or the first chlorophyllic cell emerged (Ballesteros et al., 2017, 2019; López-Pozo et al., 2019a). This was observed using a dissecting microscope at x40 magnification. Between 100 and 200 spores were located and scored per Petri dish, and the number of germinated spores per observed spores in each dish was recorded throughout the germination period. Viability was assessed as in Ballesteros et al., (2017, 2019).

2.4. Chlorophyll fluorescence to determine photochemical efficiency of PSII

Pulse Amplitude-Modulated Fluorimeter (PAM 2500, Walz, Effeltrich, Germany) was used for chlorophyll fluorescence measurements. Spores were placed in Petri dishes with agar medium and allowed to rehydrate under the same conditions used for germination. The optical fibre was fixed off to keep the same distance to the samples in all measurements as described in López-Pozo et al., (2018, 2019a,b). The minimum chlorophyll fluorescence (Fo) was determined in dark-adapted (≥30 min) spores. The maximum chlorophyll fluorescence (Fm) was induced with a saturating pulse for 500 ms. The variable chlorophyll fluorescence (Fv) was calculated as Fm–Fo. The ratio Fv/Fm represents the maximum photochemical efficiency of photosystem II (PSII). Measurements were made at 1 h, 6 h, 12 h and 24 h after rehydration. For T_{50} calculations the recovery percentage of Fv/Fm at each point with respect to the maximum value (considering the 24 h value) was calculated and equation of linear slope was applied.

2.5. Biochemical analyses

Approximately 10 mg of spores were used for biochemical analyses. Samples were ground on ice using a pestle and mortar and due to the high resistance of the spore coat, a glass cover slide was used as an abrasive to facilitate grinding. All biochemical assays were performed in triplicate for each species, treatment and time-point storage.

2.5.1. High-performance liquid chromatography (HPLC) analysis of glutathione and glutathione disulphide

Spores were extracted in 1 mL of 0.1 M HCl with 10 mg polyvinylpolypyrrolidone (PVPP) and centrifuged at 20 min at 20,000g at 4 °C. The PVPP was imbibed in 1 mL of HCl 0.1 M the day before the extraction. The supernatant was then used to determine both GSH and GSSG as described by Bailly and Kranner (2011). Briefly, this procedure uses fluorescence labelling of thiols with monobromobimane (mBBr). GSH+GSSG were determined after reduction of disulphides by dithiothreitol (DTT). For disulphides determination, thiol groups were blocked with N-ethylmaleimide (NEM). As well as glutathione, this method can also measure the low-molecular-weight thiols cysteine, cysteinylglycine and γ-glutamyl-cysteinyl. However, only glutathione was detected in the spores. Standards of glutathione at different concentrations were prepared to construct calibration curves.

GSH and GSSG were separated by reversed-phase HPLC (Agilent 1260; Cheshire, UK) on an HiQsil RP18 column (150 ×2.1 mm i.d., 3 μm

particle size; KyaTech), and detected fluorimetrically (excitation λ: 380 nm; emission λ: 480 nm) with a gradient elution of 0.25% (v/v) acetic acid in distilled water at pH 3.9/methanol. Calculation of $E_{GSSG/2GSH}$ followed the formulas given in Schafer and Buettner (2001) and Kranner et al., (2006) using the Nernst equation:

$$E_{GSSG/2GSH}=E^{O'}- RT/nF \ln [(GSH)^2/(GSSG)]$$

Where R is the gas constant (8.314 JK^{-1} mol^{-1}); T, temperature in K; n, number of transferred electrons; F, Faraday constant (9.6485 $\times 10^4 Cmol^{-1}$); $E^{o'}$, standard half-cell reduction potential at pH 7 [$E^{o'}_{GSSG/2GSH} = -240$ mV]; [GSH] and [GSSG] are molar concentrations of GSH and GSSG, estimated using the different water contents WCs.

2.5.2. Quantification of superoxide radical

Superoxide ($O_2^{\cdot-}$) was measured as described by Bailly & Kranner (2011), with some modifications. Spores were grounded and homogenized with 2 mL of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 16,000g for 15 min at 4ºC. 1 mL of the supernatant was mixed with 1 mM hydroxylamine hydrochloride and incubated at 25 °C for 30 min. Then, 0.5 mL of 17 mM sulphanilamide and 0.5 mL of 7 mM α-naphtylamine were added to 0.5 mL of the reaction mixture and incubated at 25 °C for 30 min. After incubation and centrifugation at 13.000 g for 10 min, absorbance was measured at 540 nm. Superoxide concentrations were calculated using a calibration curve obtained with solutions of various concentrations of sodium nitrite.

2.5.3. Quantification of hydrogen peroxide

Hydrogen peroxide was measured as in Junglee et al., (2014). Spores were homogenized with 1 mL of solution containing 0.25 mL Trichloroacetic acid (TCA) 0.1% (w:v), 0.5 mL KI (1 M) and 0.25 mL potassium phosphate buffer (10 mM) at 4°C. Good care was taken to protect samples and standards from light and heat. The homogenate was centrifuged at 12,000g for 15 min at 4ºC. The absorbance was measured at 350 nm. Hydrogen peroxide content of the samples was estimated using a calibration curve obtained with solutions of H_2O_2 concentrations prepared in 0.1% TCA.

2.5.4. Determination of photosynthetic pigments and prenyl quinones

Photosynthetic pigments and tocopherols were analysed at the beginning of the experiment and during long-term storage. Samples were doubly extracted, first in acetone:water (95:5) and second in pure acetone, both buffered with $CaCO_3$. Finally, extracts were centrifuged at 16100 g for 20 min and supernatants were filtered through a 0.2 μm polytetrafluoroethylene filter (Teknokroma, Barcelona, Spain) before being analysed by HPLC. Extracts were injected (15 μL) in a reverse-phase Waters (Milford, MA, USA) HPLC system following the method of García-Plazaola and Becerril (1999) with the modifications described in García-Plazaola and Becerril (2001). Photodiode array (PDA) detector (Waters model 996) was used to measure photosynthetic pigments in the range 250–700 nm, and peaks were detected and integrated at 445 nm. Pigments were identified and quantified by the method described by García-Plazaola and Becerril (1999). Retention times and conversion factors for pigments were the same as described by García-Plazaola and Becerril (1999, 2001).

2.6. Statistical analyses

Germination data was treated as proportion data according to Crawley (2007). Germination changes over the storage time for each storage condition was fit to logistic functions using binomial error distributions to calculate P50 and P80 using IBM SPSS Statistics 19 (IBM Corp. 2010). This approach enabled us to provide error estimates and statistically compare effects of species and storage condition on spore longevity. Significance of storage time (as a covariate), species, light, and oxygen level on spore germination was tested using GLMs and a binomial error distribution available also in SPSS 19.

Changes over time in Fv/Fm measurements, as well as in the levels of photosynthetic pigments, tocopherols, and antioxidants for the different storage conditions used were fitted to linear regressions (or to non-linear/sigmoidal functions in the case of Fv/Fm measurements). Significance of storage time (as covariate), species, light, and oxygen level on these parameters were tested by the univariate GLM tool available in SPSS 19, considering that data followed a normal error distribution for these data sets.

In addition to these analyses, the differences of ROS, pigments and antioxidants measured in the spores before storage and at each sampling time were analysed by ANOVA with a Tuckey post hoc analysis, which helped us to determine a specific time point at which significant changes in these parameters could be measured.

Correspondence between germination percentage and Fv/Fm, pigments or antioxidants were tested by correlation using the Pearson coefficient (r).

3. Results

3.1. Loss of germination and photochemical efficiency of PSII

Initial germination percentages were 94 ± 1% and 99 ± 0% for *O. regalis* and *M. struthiopteris* spores, respectively. During storage at 11%RH and 25ºC, germination was lost over time following a sigmoidal pattern (Fig. 1 A, B), in which a sudden germination decrease followed a relatively steady germination phase (known as the asymptomatic phase of longevity for seeds and spores, e.g. Walters et al., 2010; Fleming et al., 2017; Ballesteros et al., 2019). The length of the asymptomatic phase of longevity was larger in spores stored in hypoxia than in spores stored in normoxia (Fig. 1 A, B) and this was quantified by significantly higher P80 values (Table 1). Germination percentage decreased significantly in all storage conditions ($P < 0.001$, Fig. 1). The loss of germination was much faster in *O. regalis* than in *M. struthiopteris* in all treatments ($P < 0.05$), as can be also observed by lower P50s for *O. regalis* for each storage condition in Table 1. Both light and oxygen level had significant effect on germination decrease over time ($P < 0.001$). Within treatments, L-N led to a significantly faster deterioration of the spores of both species ($P < 0.001$), resulting in P50 values of 154 ± 2 and 277 ± 4 days for *O. regalis* and *M. struthiopteris,* respectively (Table 1). On the other hand, D-H was the less detrimental treatment, showing P50 values of 345 ± 7 and 561 ± 7 days for *O. regalis* and *M. struthiopteris,* respectively (Table 1). Germination loss over time for treatments L-H and D-N was significantly different to that of L-N and D-H and showed an intermediate position between these two extreme treatments, however significant differences in germination loss over time between L-H and D-N were not found for *M. struthiopteris* ($P > 0.05$).

As with germination, photochemical efficiency of PSII (i.e., recovery of Fv/Fm) decreased significantly over time in a similar way to germination ($P < 0.001$), with the L-N treatment showing the steepest decrease in both species studied ($P < 0.001$). The D-H treatment showed the milder decrease in both species (Fig. 1 C, D) and was significantly different to L-N in both species ($P < 0.001$). Fv/Fm loss over time for treatments L-H and D-N showed an intermediate position between L-N and D-H, was significantly different to that of L-N ($P < 0.05$) in both species but only significantly different to that of D-H in *O. regalis* ($P < 0.05$).

In addition to the loss of Fv/Fm recovery during the course of the experiment, we also detected a reduction of the speed of photosynthesis reactivation after rehydration (Fig. 2, Suppl Figs. 1–3), which was more marked in the spores stored under the most stressful treatments (i.e. L-N). Before storage and during the first 55 days of dry storage, *O. regalis* did not show significant differences in the time to reach 50% of maximum Fv/Fm values (T_{50}) and all treatments showed $T_{50} < 5$ h. With the progression of the storage time differences among treatments became more marked and at the end of the experiment T_{50} of the spores

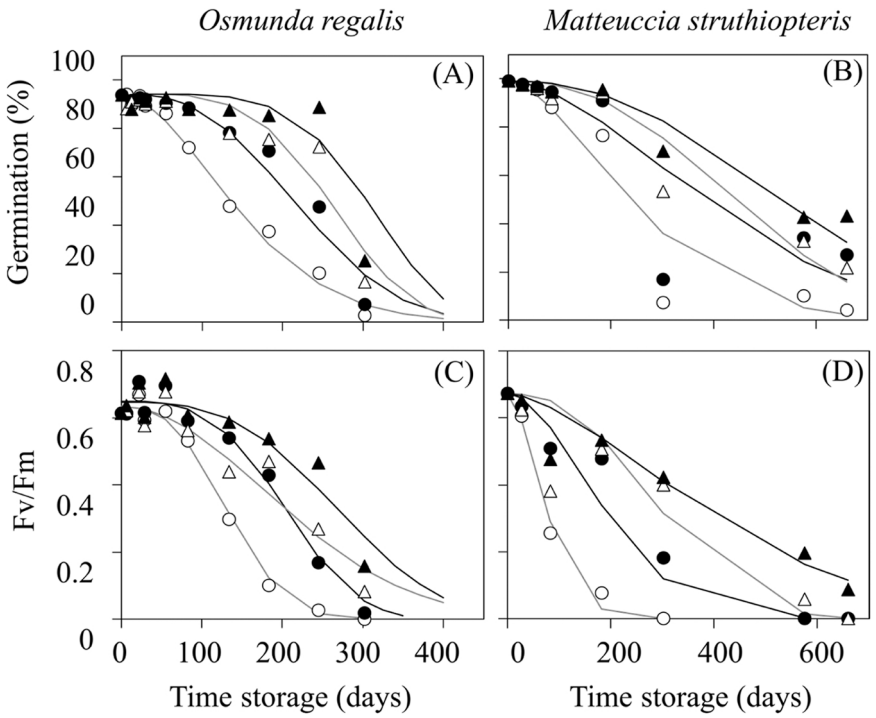

Fig. 1. Changes in germination percentage (A, B) and Fv/Fm after 24 h of rehydration (C, D) of chlorophyllous spores (CS) stored at 25ºC and 11%RH. Storage conditions are represented as follow: Dark symbols and black lines represent dark conditions. Open symbols and grey lines represent light conditions. Circles represent normoxia atmosphere. Triangles, hypoxia atmosphere. Each point represents the experimental values for the proportion of germinating spores (panels A and B) and the maximum photochemical efficiency of photosystem II (Fv/Fm) values n = 3 (panels C and D). The lines represent the fitted values obtained in the logistic functions of the GLMs applied. Note the different scale of the X-axis between species.

Table 1
Time (± standard error) required to achieve a decrease of 20% (P80) and 50% (P50) in germination and maximum photochemical efficiency of photosystem II (Fv/Fm) in chlorophyllous spores (CS) of *O. regalis* and *M. struthiopteris* under the four storage conditions used. P50s and P80s were calculated by the effective dose response option in the probit analysis for regression analysis of binomial data of Genstat version 14 (the logit transformation link was used). Letters indicate if P50s or P80s are significantly different ($P < 0.05$) for each species. P80 is a parameter often used to determine the length of the asymptomatic phase of seed longevity (Mira et al., 2016; Fleming et al., 2017).

	Germination			
	P80 (days)		**P50 (days)**	
Storage condition	***O. regalis***	***M. struthiopteris***	***O. regalis***	***M. struthiopteris***
L+N	87 ± 2 a	148 ± 4 a	154 ± 2 a	277 ± 4 a
D+N	149 ± 2 b	226 ± 6 b	228 ± 3 b	433 ± 7 b
L+H	163 ± 3c	247 ± 7 b	271 ± 4c	449 ± 6 b
D+H	216 ± 4 d	328 ± 7c	345 ± 7 d	561 ± 7c
	Fv/Fm			
	P80 (days)		**P50 (days)**	
Storage condition	***O. regalis***	***M. struthiopteris***	***O. regalis***	***M. struthiopteris***
L+N	88 ± 2 a	38 ± 3 a	134 ± 2 a	75 ± 3 a
D+N	146 ± 3c	100 ± 7 b	205 ± 4 b	184 ± 8 b
L+H	119 ± 2 b	185 ± 8c	209 ± 4 b	292 ± 7c
D+H	187 ± 4 d	185 ± 8c	271 ± 6 c	371 ± 8 d

stored in L-N was 19.5 h while it was 10.2 h for treatment D-H (Fig. 2 B). *M. struthiopteris* spores also showed a reduction on the speed of photosynthesis reactivation after rehydration (i.e. increase of T_{50}), but in a different pattern to *O. regalis*. In general, the spores of *M. struthiopteris* increased T_{50} during the first 200 days of storage, after which progressively tended to an equilibrium between 15 and 17 h (Fig. 2 A). This was clearly observed in dark-stored spores for which we were able to measure the reactivation rate of Fv/Fm at all the time-points (Fig. 2 A). *M. struthiopteris* spores have a low ability to absorb water (López-Pozo et al., 2019b) and this feature was exacerbated in spores stored in the light for more than 200 days, when germination decreased suddenly and reached values < 40% (Fig. 1 B) and Fv/Fm recovery was highly reduced or nil (Fig. 1 D). In this work, the low ability to absorb water in *M. struthiopteris* was also initially indicated by a longer reactivation time of Fv/Fm when compared to *O. regalis*. For example, in samples before storage T_{50} was 3.2 ± 0.2 h and 1.8 ± 0.2 h for *M. struthiopteris and O. regalis* respectively (Fig. 2).

3.2. ROS levels and antioxidants activity during dry storage of CS

Oxidative status of the spores of the two fern species before drying and during dry storage was measured in terms of $O_2^{\cdot-}$ and H_2O_2 production. In general, small but significant changes were observed regarding the production of these two ROS over time, that differed between the two species. Significant differences were only found between the beginning (time zero) and the end of the experiment (last two sampling times) for *M. struthiopteris* in an ANOVA/Tuckey ($P < 0.05$). These patterns suggests that ROS production was generally mild (except for the final storage times in *M. struthiopteris*), species specific and was not treatment dependent as the presence of these oxidants was independent of light or oxygen or the combination of both (Fig. 3).

The concentration of GSH in *M. struthiopteris* and *O. regalis* changed significantly over time (GLM, $P < 0.01$ and $P < 0.05$ respectively) but did not show significant differences among storage conditions despite

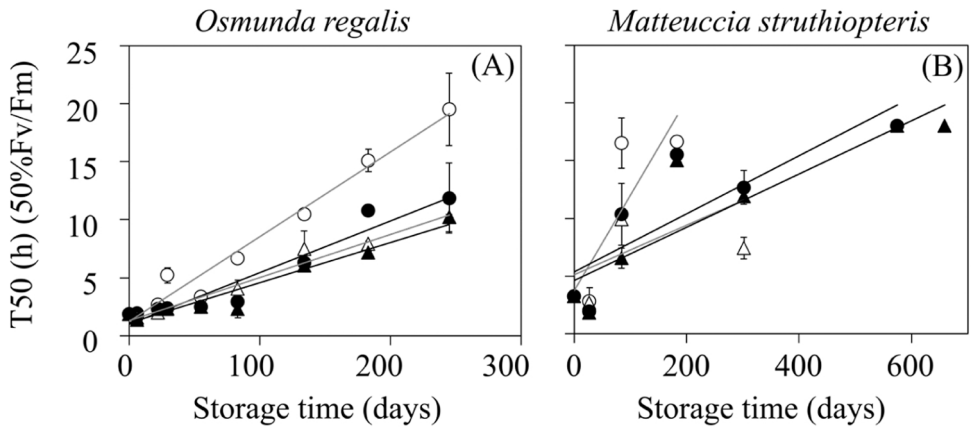

Fig. 2. Estimated time to reach 50% of maximum photochemical efficiency of photosystem II (Fv/Fm) values (T50) (hours) for chlorophyllous spores (CS) of *O. regalis* (A) and *M. struthiopteris* (B) during dry storage at 25ºC and 11%RH. Storage conditions are represented as follow: Dark symbols and black lines represent dark conditions. Open symbols and grey lines represent light conditions. Circles represent normoxia atmosphere. Triangles, hypoxia atmosphere. Each point represents the experimental values of T50 (mean±SE, n = 3). The lines represent the fitted values to linear regressions for each treatment (light and oxygen combination). Note the different scale of the X-axis between species.

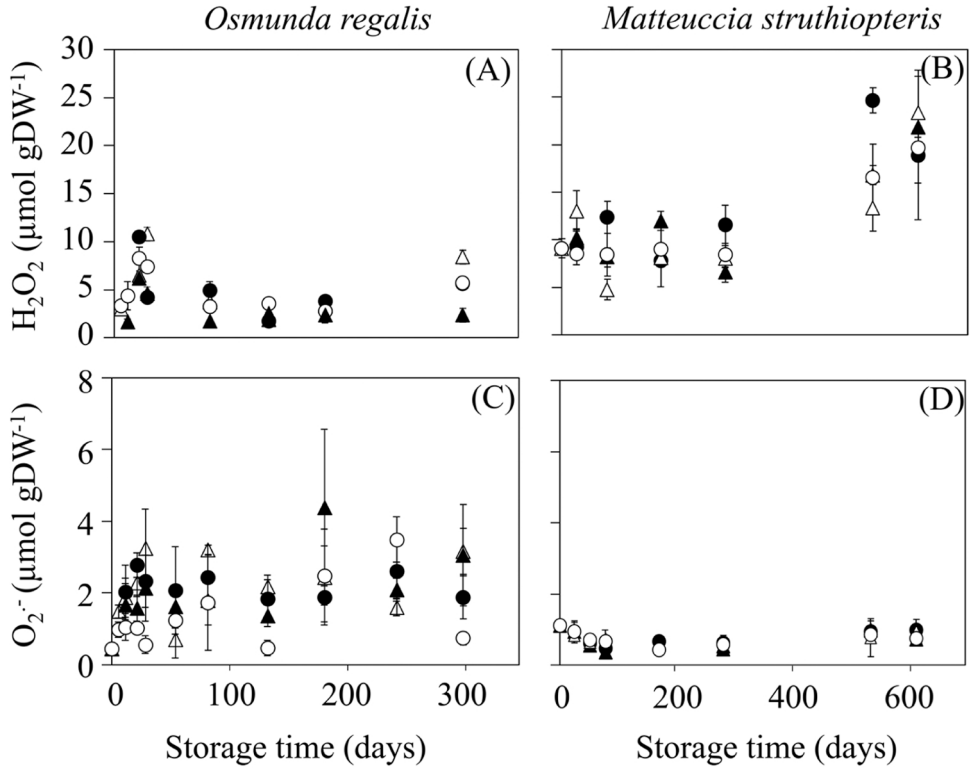

Fig. 3. Hydrogen peroxide (H_2O_2) (A, B) and superoxide ($O_2^{\cdot-}$) (C, D) levels of *M. struthiopteris* and *O. regalis* spores. Storage conditions are represented as follow: Dark symbols represent dark conditions. Open symbols represent light conditions. Circles represent normoxia atmosphere. Triangles, hypoxia atmosphere. Each point represents mean±SE (n = 3). Note the different scale of the X-axis between species.

the trend observed (Fig. 4). In the case of *O. regalis*, two trends were observed: a positive trend for spores stored in hypoxia and a negative trend for spores stored in normoxia (Fig. 4A); however, these differences among storage conditions were not significantly different. When the concentration of GSH was analysed based on the specific values of each sampling time, three-fold significantly higher values in *M. struthiopteris* spores were detected before storage compared to values at the end of the experiment (ANOVA/Tucket, $P < 0.05$), while no significant differences were observed in *O. regalis* spores.

The lipophilic pigments and antioxidants were also measured before storage (time zero) and during the course of the experiment. Initially, both species presented significant differences (ANOVA, $P < 0.001$) with respect to the concentrations of chlorophylls, some carotenoids (all except N and Lut), and tocopherols, with *M. struthiopteris* spores showing the highest proportions (Fig. 5; Suppl Fig. 4).

During dry storage, there was a general pattern of degradation of most of the lipophilic pigments and antioxidants over time in both species, with the different carotenoids analysed (A, Z, Lut, V, α-car, β-car, N), the sum of all of them (total carotenoids) and the tocopherols analysed (α, δ, γ, β) showing significant decreases over time and among treatments in both species (GLM, $P < 0.001$, Fig. 5, Suppl Fig. 4). In terms of storage conditions, light was usually the factor that better

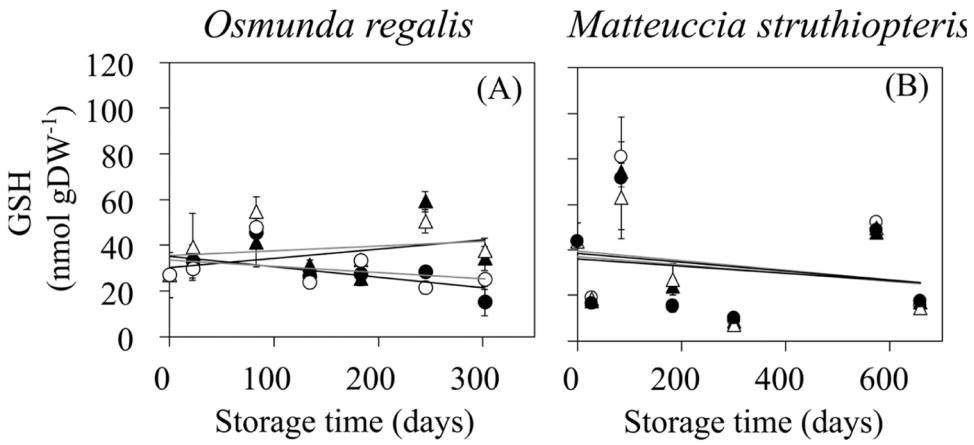

Fig. 4. Changes in GSH (nmol GSH gDW^{-1}) in *O. regalis* (A) and *M. struthiopteris* (B) during dry storage at 25ºC and 11%RH. Storage conditions are represented as follow: Dark symbols and black lines represent dark conditions. Open symbols and grey lines represent light conditions. Circles represent normoxia atmosphere. Triangles, hypoxia atmosphere. The lines represent the fitted values to linear regressions for each treatment (light and oxygen combination). Each point represent mean±SE (n = 3). Slopes for each storage condition in panels A and B were statistically significant in the GLMs applied, however statistical differences in slope among treatments were not detected in pairwise comparisons. Note the different scale of the X-axis between species.

explained the differences (also O_2 in most cases), but sometimes it was difficult to ascertain the weight of each factor as the interaction between light and oxygen was also significant. When treatments were analysed individually there were clear and significant differences between L-N and D-H in all parameters in both species (i.e., D-H was the treatment that maintained the highest proportion of pigments and antioxidants across storage time, whereas L-N showed the fastest decrease on pigments). Spores stored in D-N and L-H showed an intermediate position in degradation rate respect L-N and D-H (Fig. 5, Suppl Fig. 4, statistics for all pigments and antioxidants summarized in Suppl. Table 1). The exception to this general pattern were the amount of Chl-a, Chl-b, and the sum of both pigments (total chlorophyll) that did not significantly decrease over time in *O. regalis* for any of the different storage conditions used, while *M. strutiopteris* showed a small but significant change over time (Fig. 5, A, B; GLM, $P < 0.05$).

When comparing families of pigments and α- tocopherol, total carotenoids showed similar or slightly fastest degradation rates than α-tocopherol in *M. strutiopteris*, whereas in *O. regalis* spores α- tocopherol showed at least twice as fast degradation rates than total carotenoids (as per the slopes calculated in the GLM, Suppl. Table 2). Within carotenoids, L was the pigment that suffered the greatest decrease over storage time in all treatments for both species (as per the slopes calculated in the GLM, Suppl. Table 2) followed closer by β-car. Among the three tocopherols measured, α- tocopherol showed the largest degradation for both species (comparative data not shown).

One of the interesting findings for *M. struthiopteris* spores was that, in addition to chlorophylls, carotenoids, and the most common tocopherols (α, δ, γ, β), showed considerable amounts of another tocochromanol, the plastochromanol-8 (PC-8). PC-8 showed a significant decrease over time in all storage conditions (GLM, $P < 0.001$), with degradation rates similar to those measured for Lut (see slopes obtained in GLM, Suppl. Table 2). Also, when treatments were analysed individually there were small but significant differences between L-N and D-H. (GLM, $P < 0.001$; Fig. 5 I). Interestingly, when the changes in the amount of PC-8 were analysed based on the specific values at each sampling time, significant losses were detected for all storage conditions at sampling times immediately after the starting of the storage period (ANOVA/Tuckey, $P < 0.5$), right during the asymptomatic phase of longevity (<P80 in Table 1).

In order to determine the ageing mechanisms in CS and to detect if certain ageing reactions could be observed before germination is reduced, several relations between germination, Fv/Fm, antioxidants and pigments were analysed. Fv/Fm, correlated significantly with germination for both species independently of the storage condition ($P < 0.05$, $R^2 > 0.85$; Fig. 6 A), indicating that both parameters can be used as an indicator of viability in CS. However, as indicated above (Fig. 1), the decrease in Fv/Fm from ca.0.8 to ca. 0.5 proceeded the decrease in germination (see grey area in Fig. 6 A). Glutathione half-cell reduction potential and germination also showed a significant negative correlation, although this followed a sigmoidal trend (Fig. 6 B). The largest decrease in germination for both species was observed between − 175 and − 165 mV. Values below − 180 mV were related to healthy CS germination > 90%, while at − 140 mV germination was zero. Interestingly, it seems that *O. regalis* spores were in a greater stressed basal state when compared with *M. struthiopteris*. For example, at the beginning of the experiment, when germination for *O. regalis* was maximum (i.e. 94%), glutathione half-cell reduction potential was − 140 mV; while it was − 202 mV (100% germination) for *M. struthiopteris* (Fig. 6 B). Total carotenoids (in both species) and PC-8 (only detected in *M. struthiopteris*) also showed a positive and significant correlation with germination percentage ($P < 0.05$; $R^2 > 0.75$; Fig. 6 C, D). As with Fv/Fm, the decrease in these pigments was observed in CS before a decrease in germination, from 460 to 420 mmol mol^{-1} Chl a+b for total carotenoids and 55 to ca. 40 mmol mol^{-1} Chl a+b for PC-8 (Fig. 6C, D).

4. Discussion

The majority of seeds and spores, as reproductive and dispersal structures of land plants, should be able to persist extended periods under unfavourable conditions in a quiescent state, until the environmental conditions allow their germination and growth (Mène-Saffrané et al., 2010). The solidification of the cytoplasm during seed and spore maturation and drying increases the stability of this quiescent state by virtually stopping most metabolic reactions (Walters et al., 2010). Under these conditions it is supposed that oxidative stress, occurred during drying and while dry, is the main cause of seed and spore ageing in this quiescent and solid state, leading the slow deterioration and final death of seeds and spores (Kranner et al., 2006; Walters et al., 2010; Chen et al., 2016). In the present work, we have elucidated the deterioration of a particular subset of spores, those containing chlorophyll after maturation, mechanisms during dry storage. We have used fern spores, as a unicellular model for seeds and other complex DT organisms (Ballesteros et al., 2017, 2019; López-Pozo et al., 2018, 2019a,b), stored dry (in a glassy state) under different light and oxygen regimes. We determined that, during the asymptomatic phase of longevity, a decrease in

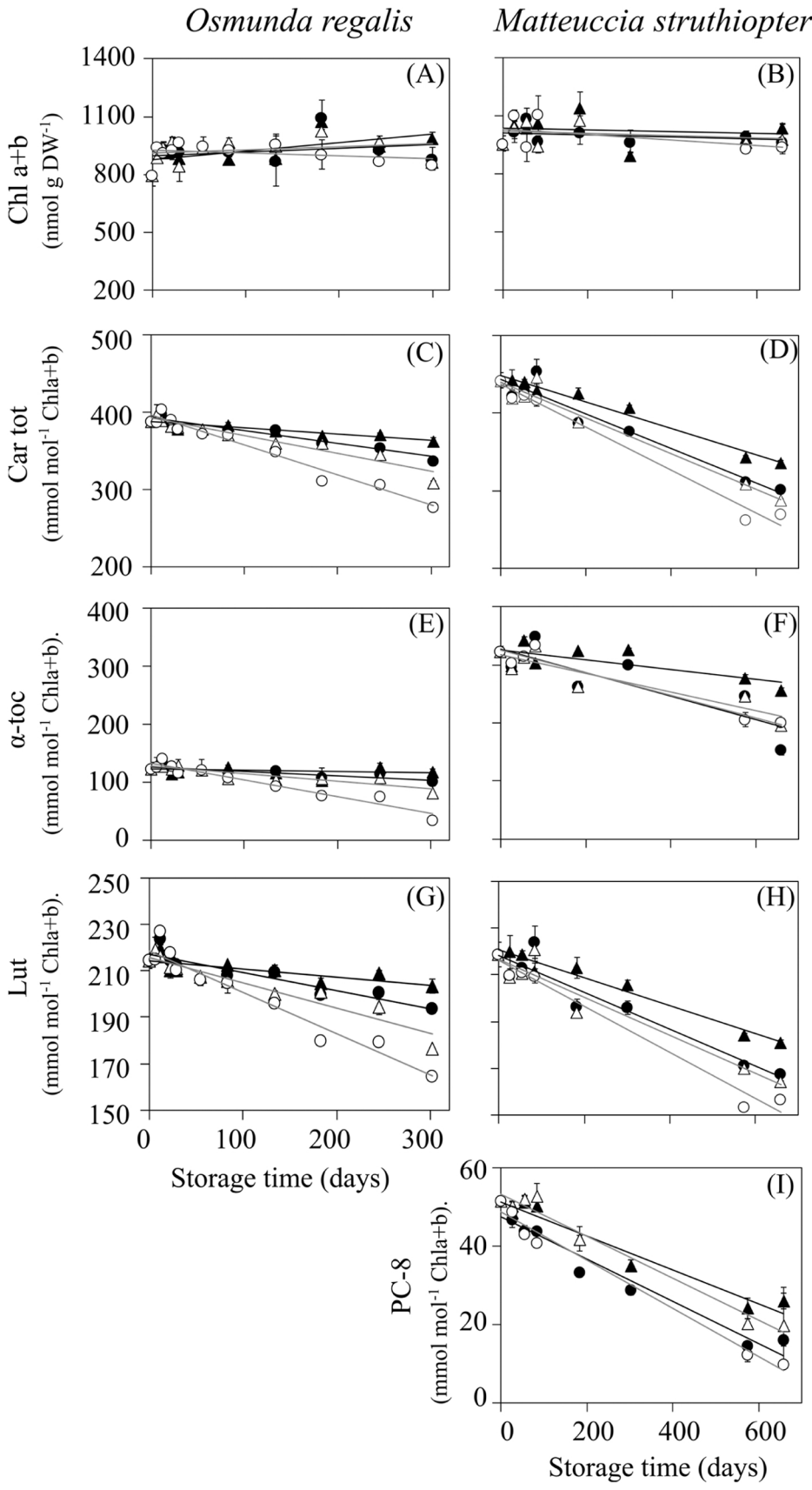

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Fig. 5. Changes in lipophilic pigments and antioxidants during storage at 25ºC and 11%RH. Chla+b (nmol g DW^{-1}) total carotenoids (car tot), lutein (Lut), α-tocopherol (α-toc) and plastochromanol-8 (PC-8) (mmol mol^{-1} Chla+b). Storage conditions are represented as follow: Dark symbols and black lines represent dark conditions. Open symbols and grey lines represent light conditions. Circles represent normoxia atmosphere. Triangles, hypoxia atmosphere. The lines represent the fitted values to linear regressions for each treatment (light and oxygen combination). Each point represent mean±SE (n = 3). Note the different scale of the X-axis between species.

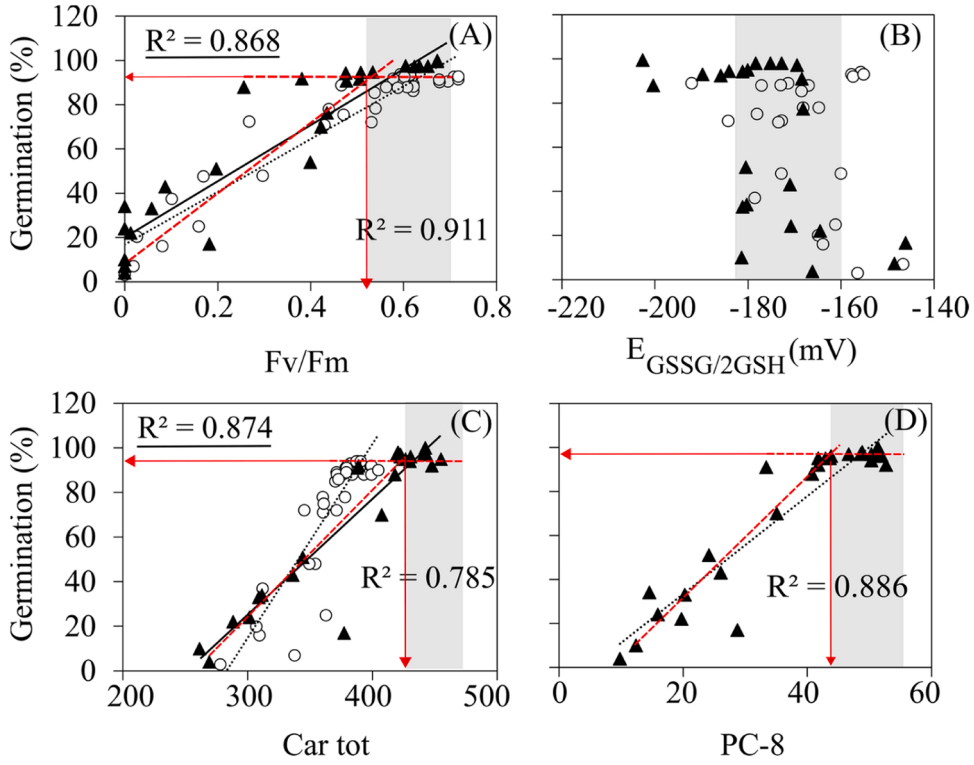

Fig. 6. Correlations between germination percentage and Fv/Fm (A), GSH potential redox (B), total carotenoids (mmol mol-1 Chl a+b) (C) or pastocromanol-8 (mmol mol^{-1} Chl a+b) (D). Each point in the correlation represents the mean values (n = 3) as plotted in Figs. 1, 4 and 5. Circles correspond to *O. regalis*. Triangles points represent *M. struthiopteris*. Correlations in (A), (C) and (D) were made using all data points (black lines) or considering two different slopes with a clear break between them (red lines). In both cases all correlations were significant at $p < 0.001$. Red arrows indicate the intersection of the two red regression lines and highlight a rage (grey shadow) in which there is a decrease in Fv/Fm, Car tot and PC-8 while germination remain constant around the maximum germination percentage. This range indicates that there are measurable biochemical changes before the physiological changes during the "asymptomatic" phase of ageing. In panel (B) the grey shaded area indicates the range of GSH potential redox in which germination is mainly lost, suggesting that GSH potential redox values below − 185 mV pinpoint to an alive spore while GSH potential redox values above − 160 mV are indicative of a dead spore.

the antioxidant machinery occurred as the recovery of the photosynthetic efficiency failed, which ultimately leads to the failure of the spore germination. Light and oxygen accelerated this deterioration mechanism for spores stored in the light and dark and in the presence and near-absence of oxygen, suggesting a common ageing mechanism for CS at a specific moisture/temperature combination independent of the light and oxygen condition. In addition, we found that the difference in longevity between species was related positively to the initial amount on antioxidants and the presence of certain type of antioxidants (e.g. PC-8).

4.1. Photooxidation of CS during light storage

Storage conditions affected CS viability to a great extent, with normoxia and light having the most deleterious effect on germination capacity of CS over time (Fig. 1). These results are similar to what has been described in other chlorophyllous propagules such as spores CS of the fern *Todea barbara* or willow (*Salix nigra*) seeds (which have chlorophyllous embryos). Propagules of both species showed a faster deterioration rate when exposed to light and normoxia, in comparison to dark (*T. barbara*) or dark and hypoxia (*S. nigra*) conditions (Roqueiro et al., 2010; Ballesteros et al., 2018). In this sense, our results support previous findings that show how photooxidation, due to the presence of photodynamic chlorophyll, is the mechanism that exacerbates the deterioration and ageing of chlorophyllous seeds and spores (Roqueiro et al., 2010; Ballesteros et al., 2018). This phenomenon has been well studied in other chlorophyllous DT organisms, where the chloroplast is one of the major sources of ROS due to the imbalance done between the energy absorbed and used by the photosynthetic apparatus when water is absent, and that these ROS are the main cause for cell deterioration in the dry state (Kranner et al., 2002, 2008; Illing et al., 2005; Heber et al., 2006; Farrant, 2007).

In the glass, different molecular mobility is allowed, including some vibration, rotation, bending and stretching of atoms, bonds, and groups of atoms (Ballesteros and Walters, 2011). Molecular mobility within the glass will allow light-induced redox changes in the double bound system of the molecules of the reaction centre of the photosystems during dry storage, such as chlorophyll, which ultimately may produce a release of electrons. In the absence of water, a double situation is found: (1) the excited chlorophyll molecule will oxidize nearby molecules in the photosystem (e.g., β-carotene and other pigments), and (2) the electrons released will oxidize other molecules within the photosystem and other nearby molecules. These molecules will include gases trapped within the glassy matrix (e.g. O_2) that will end up in the production of ROS. The ROS produced will likely oxidize nearby molecules within the

photosystem complexes or/and the thylakoidal membranes (Roqueiro et al., 2010), but also may randomly affect to other targets, as have been found in non-chlorophyllous dry seeds (e.g., RNA, DNA, storage lipids, proteins [Fleming et al., 2017; Mira et al., 2016; Rajjou et al., 2008; Smolikova et al., 2011]). The higher amount of oxygen in the system should favour a larger amount of ROS, but we did not detect significant differences respect the hypoxic storage condition in our analysis, and the only significant increase of ROS was detected in the last stages of cell deterioration (Fig. 3). Similarly, in the CS of *Todea barbara*, ROS only peaked significantly when spores were stored under the most photooxidative condition when viability was very low and the antioxidant system was highly depleted (Ballesteros et al., 2018). This lack of response could be caused by the production of a ROS different to those measured (H_2O_2 and $O_2^{\cdot-}$) or that they were quickly deactivated by nearby molecules (e.g., pigments and antioxidants) (Triantaphylidès and Havaux, 2009) so their presence was reflected by a faster deterioration (oxidation) rate of these protective molecules.

From an applied perspective, our results support that longevity of CS is optimal when they are stored in darkness and hypoxic (or anoxic) conditions. This is particularly relevant for the *ex-situ* conservation of chlorophyllous seeds and spores in germplasm banks, as these propagules tend to be extremely short lived during the dry and cold storage conditions used in these facilities (Ballesteros and Pence, 2017; Ballesteros et al., 2019).

4.2. Autooxidation of CS during dark storage

Although photooxidation promoted by the photosynthetic apparatus is the best-known mechanism of deterioration of chlorophyllous systems by light, dark and hypoxia did not stop ageing. In fact, contrarily to what we hypothesized, all treatments resulted on a similar sequence of responses, differing only in the speed of deterioration. Thus, during D-H, as well as during L-N and the other storage combinations, we found the same deterioration pattern over time: a significant decrease in germinability that was preceded by a decline in the photochemical efficiency of the PSII (measured by Fv/Fm; Figs. 1, 6) and a significant decrease in antioxidants (Figs. 5, 6). During all this process, ROS did not accumulate significantly in the spores and even the small accumulations of H_2O_2 at the end of the storage time did not differ significantly among storage conditions (Fig. 3). Similarly, Lebkuecher (1997) also found in CS of *Equisetum hyemale* stored dry (2% RH, likely in the glass as per Ballesteros et al., 2017) in the dark and normoxia, that a significant damage in the photochemical efficiency of PSII preceded the decrease in germination. In addition, differences in the levels of ROS of *T. barbara* CS stored under light and dark regimes in the glassy state were hardly detected over time (Ballesteros et al., 2018). These results and observations, when taken together, suggest that for a specific moisture/temperature combination that favours the formation of a glassy state, the same ageing mechanism occurs in CS, independently of the light and oxygen levels, that simply accelerate it. This suggestion would imply that even in the absence of light and oxygen, the photosynthetic apparatus continues releasing electrons that deteriorate nearby molecules; however, the molecular basis for the emission of electrons and the changes in the redox status in the dark are not known. Future research must address this hypothesis, perhaps by looking into dark production of ROS in PSII or electron transfer across molecules in the PSII as indicated by some authors (Vass et al., 1990; Pospíšil et al., 2007).

4.3. Mechanisms of protection against photosynthetic-related oxidation in CS

Initially, during the asymptomatic phase of longevity of CS, deterioration is primarily observed in the pigments, antioxidants and Fv/Fm whose concentrations or values decrease at a faster rate than germination loss. Our results suggest that pigments and antioxidants, through their oxidation, may be acting as a buffer for other major oxidative damage induced by ROS. However, this antioxidant defence is only effective until the level of the antioxidants has fallen below a certain threshold. For example, the coefficient between GSSG (the oxidated form of GSH) and GSH (expressed as GSSG/2GSH) was correlated with germination (Fig. 6) despite the minor change in GSH observed over time (Fig. 4). This correlation is not linear but show a clear transition zone between − 175 and − 165 mV were the germination capacity of CS appears to be lost (Fig. 6, grey area). The same transition zone has been found for diverse plant, human and fungal cells, suggesting GSSG/2GSH as a ubiquitous molecular marker for cell viability (Kranner, 2006, Seal et al., 2010). But hydrophilic antioxidants such as GSH are not the only form of protection against oxidative stress. For example, this water-soluble antioxidant is not immersed in the thylakoidal membrane (likely the main target of ageing reactions in CS, Fig. 7), its function in the stabilization of these membranes in CS may have a minor role, and other lipid-soluble antioxidants will display additional protective function in CS ageing. Examples are the carotenoids and tocopherols found in both species and the PC-8 found only in *M. struthiopteris*. In the present study we report the existence of certain thresholds in the content of these protective compounds that discriminate between highly germinating CS and CS quickly losing their germination ability (Fig. 6 grey zone suggested for total carotenoids and PC8). Thus, for *M. struthiopteris* a decrease in total carotenoids from 460 to 420 and PC-8 from 55 to ca. 40 mmol mol^{-1} Chla+b seems to mark this threshold. Similarly to pigments, antioxidants and Fv/Fm, diverse deterioration reactions during dry storage of non-chlorophyllous seeds have also been observed during the asymptomatic phase of longevity (e.g. RNA fragmentation, lipid peroxidation; Mira et al., 2016; Fleming et al., 2017), and some of these molecules (e.g. RNA) could potentially be used as a molecular marker for seed ageing (Fleming et al., 2018).

Chlorophyll may, directly or indirectly, enhance ROS production, thereby compromising viability (Roqueiro et al., 2010). In the case of CS of *M. struthiopteris* and *O. regalis*, we did not detect a degradation in this pigment during dry storage even under illumination (Fig. 1 A, B). This observation supports the idea that chlorophylls (free or in photosystems) immersed in thylakoids are sufficiently protected against photooxidation. It has been proposed that some carotenoids may quench excited pigments when these are in triplet state (Krinsky, 1994) and they seem to be particularly relevant in chlorophyllous seeds (Smolikova et al., 2011). Additionally, several carotenoids are strong antioxidants and can help in thylakoid membrane integrity when free in the membrane. In contrast to the stability of chlorophyll during storage, the concentration of total carotenoids significantly decreased in *M. struthiopteris* and *O. regalis* during storage (Fig. 5), with a high consumption of carotenoids under light and oxygen conditions. In addition, total carotenoids were positively correlated with germination (Fig. 6) in agreement with the role of carotenoids in stabilizing the photosynthetic membranes and scavenging ROS (Seal et al., 2010). Among all carotenoids, several studies have related the consumption of Lut to an increase in oxidative stress and its fundamental role as an antioxidant in seeds (Pinzino et al., 1999) as we have observed in CS of both species. Besides, Lut was also the most abundant carotenoid in CS, in agreement with the data obtained by other authors in seeds (Smolikova et al., 2011). As well as some lipophilic pigments, tocotrienols and tocopherols are also known to be indispensables to survive desiccation and long-term storage (Kranner et al., 2006). It has been demonstrated that the absence or decrease of tocochromanols increases lipid oxidation. Lipid oxidations are initiated during seed desiccation and are amplified during seed dry storage compromising its viability (Mène-Saffrané et al., 2010). In the seeds of several species, a strong decrease in α-tocopherol resulted in poor viability, concluding that it is responsible for the viability maintenance along time in dry conditions (Seal et al., 2010). Instead, other studies have shown constant level of α-tocopherol during both, desiccation and ageing in seeds. Our data showed consumption of this antioxidant during seed ageing, but antioxidants other than α-tocopherol were probably more involved in defence mechanisms against ageing.

4.4. What determine differences of longevity between CS from different species

When stored dry, CS of *O. regalis* aged more rapidly than those of *M. struthiopteris* (Fig. 1 A, B), which was in concordance with data from previous works (López-Pozo et al., 2019b, Ballesteros et al., 2017, 2018, 2019, Lloyd and Klekowski, 1970). Our results suggest two main possible reasons for these differences in longevity. Firstly, both species differed in their constitutive lipophilic pigment and antioxidant composition, as can be observed from the control data at time zero. The higher concentration of carotenoids and tocopherols found in *M. struthiopteris* CS supports a larger photoprotective status, as was previously indicated in López-Pozo et al., (2019a,b). Secondly, only CS of *M. struthiopteris*, which also showed the highest viability in the dry state, contained significant amounts of PC-8 (Fig. 5). Interestingly we observed a very strong correlation between the content of this antioxidant and germination percentage (Fig. 6). Although this tocotrienol has been found in a large number of species (Strzałka et al., 2009) only one work has established the importance of PC-8 in seed ageing and germination (Mène-Saffrané et al., 2010). PC-8 strongly inhibits lipid peroxidation (Nowicka et al., 2013) and effectively quenches and scavenges singlet oxygen that is formed under high light conditions by excitation of singlet chlorophyll (Gruszka et al., 2008). Last, the decrease in α-tocopherol and total carotenoids (especially Lut) in both species, and the decrease in PC-8 in *M. struthiopteris* support the conclusion that an effective lipophilic antioxidant system is necessary for long-time preservation of CS viability.

4.5. Conclusions

In this work, we present novel data that help us to understand the mechanism of deterioration and ageing of CS when stored in a solid, glassy, state (e.g. 11% RH, 25 °C). With our results, light and oxygen, instead than inducing a different ageing mechanism, are just accelerators of the ageing process determined by the dry physicochemical architecture of the cell. Our results support the hypothesis that an efficient antioxidant system would delay viability loss, as the depletion of the antioxidant system during the asymptomatic phase of longevity is the cause for the sudden drop of germination. In addition, we have found that the difference in longevity between species seems to be related positively to the initial amount on antioxidants and for the presence of certain type of antioxidants (e.g. PC-8). From an applied point of view, conditions of darkness and hypoxia will be necessary for CS in order to ensure the larger long-term viability of CS during dry storage.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111870.

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