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Redox imbalance accompanies loss of viability in seeds of two cacti species buried *in situ*

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Research Paper

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Abstract

This work provides insights into the deterioration of cacti seeds of *Escontria chiotilla* (F.A.C. Weber ex K. Schum) and *Stenocereus pruinosus* (Otto ex Pfeiff.) Buxbaum stored *ex situ* at 25 °C, under dry and dark conditions or buried *in situ* conditions in a xerophytic shrubland. Viability, germination speed, electrolyte leakage and indicators of the redox balance including glutathione content, glutathione half-cell reduction potential ($E_{GSSG/2GSH}$) and malondialdehyde (MDA), oxidized protein content, together with water-soluble antioxidant enzyme activity were assessed. Over a period of two years of storage, viability was maintained when seeds were stored *ex situ* at 7–9% water content compared to seeds buried in the soil. A second burial experiment showed that seeds of *E. chiotilla* maintained viability during a year of storage that included a rainy season followed by a dry season. Thereafter, they died rapidly during the second rainy season. In contrast, those of *S. pruinosus* started to lose viability after 6 months of burial at the end of the rainy season and were mostly dead at the end of the dry season. This difference in persistence between species was related to a difference in the glutathione content and antioxidant enzyme activities. In both storage experiments, the loss of viability of both species was associated to a $E_{GSSG/2GSH}$ shift to a more oxidative state during burial. Yet, contents in MDA and oxidized soluble proteins were not related to redox imbalance and loss of viability, indicating that these compounds are not good markers of oxidative stress in cacti seeds during storage.

Introduction

Seed persistence refers to the survival of seeds in the environment after they reach maturity on the mother plant (Long et al., 2015). Long after the death of the mother plant, seeds can persist *in situ* in soil seed banks, or *ex situ* in gene banks (Reed et al., 2011; Long et al., 2015). The formation of soil seed banks is a reproductive strategy for Spermatophytes to allow plant populations to withstand adverse periods and to persist in time and space. This occurs particularly in unpredictable environments such as those prevailing in arid and semi-arid zones (Cano-Salgado et al., 2012; Álvarez-Espino et al., 2014). Soil seed banks can be transient, short- or long-term persistent (Walck et al., 2005). In transient seed banks, seeds lose viability in less than one year, whereas in short-term persistent banks, they maintain their viability between one and five years. Long-term persistent seed banks consist of seeds remaining viable for more than five years (Walck et al., 2005). Seed gene banks aim to preserve viable seeds *ex situ* for the longest period possible in dry and cold conditions (Hay and Whitehouse, 2017).

Although seeds should maintain their viability to fulfil their different purposes both in *ex situ* and *in situ* conditions, viability progressively decreases albeit at different rates based on the storage conditions (Hay and Probert, 2013; Nascimento and Meiado, 2017). Seed ageing or deterioration is commonly described as the loss of viability and vigour during storage (Jyoti and Malik, 2013). Ageing depends on the seed maturity state, genetic composition and storage conditions, namely, temperature, seed moisture content and oxygen concentration (Roach et al., 2018; Zhou et al., 2020; Gerna et al., 2022). In soil banks, buried seeds can be exposed to various stress factors, both biotic (predators and pathogenic microorganisms) and abiotic (temperature, humidity, pH, salinity). Furthermore, they experience cycles of imbibition and desiccation (Munné-Bosch et al., 2011; Garcia et al., 2012).

The causes of seed deterioration leading to death are not yet fully understood since ageing is a complex and multifactorial trait involving a network of molecular, biochemical and physiological processes (Fu et al., 2015; Ebone et al., 2019; Kurek et al., 2019; Lee et al., 2019; Zhou et al., 2020). The mechanisms related to seed ageing are associated with production and accumulation of reactive oxygen species (ROS) during storage. ROS are highly reactive and toxic molecules, with the potential to cause oxidative damage to membrane lipids (lipid

peroxidation), proteins and nucleic acids, causing irreversible damage to cellular systems and ultimately leading to cell death. This has been documented indirectly by measuring ROS induced damage in *Arabidopsis thaliana* (Rajjou et al., 2008) and in crops (*Avena sativa, Cucumis sativus, Daucus carota*, *Helianthus cucumerifolius*, *Jatropha curcas*, *Lactuca sativa*, *Lepidium sativum*, *Phaseolus vulgaris*, *Raphanus sativus*, *Triticum sp* and *Zea mays*) (Xin et al., 2011; Xia et al., 2015; Suresh et al., 2019; Tian et al., 2019; Stegner et al., 2022). For example, the comparison of oxylipid profiles of 15 year-old seeds stored in ambient and cold conditions revealed that lipid oxidation processes in aged seeds was enhanced under warm conditions, a treatment associated with reduction in viability (Riewe et al., 2017). Oxidative shifts of the cellular redox environment, which can be assessed by half-cell reduction potential ($E_{GSSG/2GSH}$) have been related to loss of seed viability in seeds that age rapidly during storage (Kranner et al., 2006; Seal et al., 2010). Malondialdehyde (MDA) is one of the down-products of lipid peroxidation and widely used as a marker of oxidative stress because it can be easily assessed using its binding to thiobarbituric acid (TBA) that leads to the MDA-(TBA)2 adduct of reddish colour with an absorbance maximum at 532 nm. However, other plants compounds can react with TBA, such as sugars, which can lead to misinterpretation (Morales and Munné-Bosch, 2019). MDA alters cell membrane fluidity and permeability, leading to changes in their structure and function, as well as increased solute leakage (Morales and Munné-Bosch, 2019). In addition, free radicals derived from lipid oxidation can also damage other biomolecules and cellular organelles such as mitochondria (Roach et al., 2018). To cope with oxidative stress, seeds have enzymatic antioxidant systems, such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidases (POD; EC 1.11.1.7); and molecular antioxidant such as glutathione (GSH), ascorbate, phenolic compounds, tocopherols and tocotrienols (Ratajczak et al., 2015; Xia et al., 2015; Roach et al., 2018).

In cacti seeds such as *Escontria chiotilla* (F.A.C. Weber ex K. Schum.) and *Stenocereus pruinosus* (Otto ex Pfeiff.) Buxbaum, seed persistence and the mechanisms of deterioration during *ex situ* and *in situ* storage are poorly understood. *E. chiotilla* and *S. pruinosus* are columnar cacti endemic to Mexico, particularly from arid and semi-arid regions, such as those located in the Balsas River basin and Tehuacán–Cuicatlán Valley (Arellano and Casas, 2003; García-Cruz et al., 2013). Both species are ecologically important in their growing regions due to their interactions with pollinators and dispersers. In addition, they produce edible fruits that are of economic, nutritional and cultural interest (Arellano and Casas, 2003; García-Cruz et al., 2013; Sandate-Flores et al., 2020).

Our research group has studied the viability of *E. chiotilla* and *S. pruinosus* seeds preserved in *ex situ* and *in situ* conditions. Seeds of both species stored in *ex situ* conditions (in rubber sealed glass jars at 25 ± 3 °C and darkness) maintain viability for at least eight years. On the other hand, viability of seeds stored in *in situ* conditions decreased from 90% to 30% after 12–15 months of burial, possibly as a consequence of temperature and humidity changes experienced by the seeds in the soil. Based on this evidence, this study tested whether seeds buried *in situ* showed increased symptoms of oxidative damage such as increased MDA, oxidized proteins and/or decrease in antioxidant enzymes activity (SOD, CAT and POD), and a shift of the $E_{GSSG/2GSH}$ to an oxidative state, compared to seeds stored in *ex situ* conditions, that could be associated with the loss of seed viability and vigour.

Materials and methods

Fruit collection and seed extraction

For this study, 150 *E. chiotilla* and 60 *S. pruinosus* fruits, respectively were collected in Tehuacán–Cuicatlán Valley region, Mexico (18°12′41″ N, 97°23′58″ W), in May 2016, 2017 and 2018. Seeds were extracted from the fruits without pericarp and washed with running water until pulp removal. An *E. chiotilla* fruit contains between 400 and 900 seeds, while an *S. pruinosus* fruit has between 600 and 2000 seeds. For each harvest year the viability of seeds freshly extracted from the fruits was determined, and in each case, germination percentages were ≥90%. After extraction, seeds were dried at room temperature (25 ± 3 °C) on blotting paper for 48 h until reaching water content (WC) of 7–9%. Seed WC was assessed gravimetrically before and after drying at 103 °C for 17 h (ISTA, 2020) and expressed on a fresh weight basis.

Ex situ and in situ conservation

For *ex situ* storage, seeds were kept in rubber sealed glass jars at 25 ± 3 °C under dark conditions. Seed burial was carried out in the soil of a xerophytic shrubland located in the town of Trinidad Huaxtepec, Municipality of Santiago Chazumba, Oaxaca, Mexico (18°16′24″ N, 97°70′93″ W), a region that is part of the Tehuacán–Cuicatlán Valley. The climate is classified as hot semi-arid (García, 2004), with a mean annual temperature of 19.7 °C. May is the warmest month (14.3–32.8 °C) and January the coldest (6.5–14.2 °C). Average annual precipitation is 532 mm. Rainfall occurs mostly during August (74–181 mm) and September (52–178 mm). Dry season begins between October–November and ends between March–April. Climate data for the region for the years of 2016, 2017 and 2018 were obtained from climate stations at Acatepec (18°13′ 36.97″ N, 97°34′44.03″ W), Caltepec (18°12′ 14.18″ N, 97°27′12.62″ W) and Tonahuixtla (18°11′ 13.51″ N, 97°55′16.36″ W) (https://smn.conagua.gob.mx/es/). No climatic anomalies were registered during the study period (See Supplementary Figure S1).

Two experiments were conducted at this site, which are described below.

Experiment 1

The purpose of this experiment was to assess physiological and biochemical changes in seeds buried *in situ* for one and two years compared to seeds that were preserved *ex situ*. One thousand seeds of *E. chiotilla* and *S. pruinosus* were placed in five polyethylene mesh envelopes (6 × 4 cm) in May 2016 and 2017 and buried 5–10 cm deep, which is the depth at which cacti seed banks naturally form (Holland and Molina-Freaner, 2013; Álvarez-Espino et al., 2014). In May 2018, buried seeds were exhumed and viability, vigour, glutathione contents (GSH and GSSG), redox state ($E_{GSSG/2GSH}$), MDA content, soluble proteins and oxidized proteins, as well as the activity of antioxidant enzymes (SOD, CAT and POD) were determined. These parameters were evaluated simultaneously in seeds stored in *ex situ* conditions, as well as in freshly harvested seeds collected in 2018.

Experiment 2

The purpose of this experiment was to determine if the loss of *E. chiotilla* and *S. pruinosus* seed viability during burial was related to the oxidative damage in response to the weather pattern. One

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thousand seeds of both species collected in 2018 were buried as described above and exhumed at 6, 12, 14 and 16 months after burial. In each exhumation, five seed envelopes of each species were recovered for physiological and biochemical analyses, as described below.

Seed viability and vigour

Seed viability was determined by germination. Three replicates of 50 seeds were sown in Petri dishes with 1% (w/v) bacteriological agar and incubated in a germinator (Precision®, Dual Program Illuminated Incubator) at 25 ± 3 °C and a 12 h light (15.25 μmoles m^{-2} s^{-1}) photoperiod. Germination was scored based on the radicle emergence during 21 days after sowing. In order to verify seed viability, a tetrazolium test (ISTA, 2020) was performed. Three replicates of 10 seeds were cut longitudinally and placed in a 1% (w/v) Tetrazolium red solution at 40 °C for 1 h. Seeds, in which the embryonic axis was completely stained reddish, were considered as viable.

Vigour was determined as the time necessary to reach 50% of the final germination percentages based on the equation reported by Farooq et al. (2005):

$$T_{50} = t_i + \frac{(N+1)/2 - n_i}{(n_j - n_i)}(t_j - t_i)$$

where N is the final number of germinated seeds and n_i and n_j the cumulative number of seeds germinated by adjacent counts at times t_i and t_j when $n_i < (N+1)/2 < n_j$.

Solute leakage was determined by measuring electrical conductivity (EC) according to Silva et al. (2020). Hundred mg of seeds was placed in 20 mL of deionized H_2O for 24 h at 25 ± 3 °C. EC was measured with a conductivity meter (OAKTON® CON 700) and results were expressed as μS cm^{-1} g^{-1} DW.

Lipid peroxidation

Lipid peroxidation was determined by assessing TBA-reactive substances using the Lipid Peroxidation (MDA) Assay Kit® (ABCAM), according to the manufacturer's instructions. Two hundred mg of seeds were frozen in liquid nitrogen and homogenized with 300 μL of lysis solution, 3 μL of butylated hydroxytoluene (BHT) solution and 303 μL of 2 N $HClO_4$. The homogenate was centrifuged at 13,000 *g* for 10 min at 4 °C. Thereafter, 200 μL of supernatant or 200 μL of the MDA standards provided by the manufacturer were mixed in 600 μL of TBA. The mixture was incubated at 95 °C for 60 min and then cooled in an ice bath for 10 min. The absorbance was immediately measured at 532 nm (ScanReady, Microplate photometer P-800, Life Real®). MDA concentration was derived from the calibration curve using 0–5 nmol of MDA standard.

Soluble protein extraction and quantification

Soluble proteins were extracted according to Reyes de la Cruz et al. (2004). Two hundred mg of seeds were frozen in liquid nitrogen and homogenized with 2 mL of extraction buffer (pH 7.5) containing, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, 20 mM NaF, 4 mM ethylenediaminetetraacetic acid (EDTA), 20 mM ethylene glycol tetraacetic acid (EGTA), 200 mM mannitol and 1% (w/v) polyvinylpolypyrrolidone (PVPP). To 200 mL of extraction buffer, tablet of protease inhibitors COMPLETE® (Roche) was added. The homogenate was centrifuged at 16,000 *g* at 4 °C for 25 min and the supernatant was recovered and stored at −80 °C until use.

Soluble protein concentration was determined using the Bioassay Dye Reagent Concentrate (Bio-Rad) according to the manufacturer's instructions. Oxidized proteins were determined by quantifying carbonyl groups according to Hernández-Arciga et al. (2020). Twenty μL of 50 mM phosphate buffer (pH 7.2) or 20 μL of sample were mixed with 20 μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH, dissolved in 0.5 M H_3PO_4) and 10 μL of 6 M NaOH. Samples were placed on an ELISA plate and shaken for 10 min. Absorbance was measured immediately at 450 nm.

Glutathione content and redox state

Reduced and oxidized glutathione content was determined using the Quantification kit for oxidized and reduced glutathione (Sigma-Aldrich), according to the manufacturer's instructions. Two hundred mg of seeds were frozen in liquid nitrogen and homogenized with 1.5 mL of an aqueous solution containing 0.5% (w/v) of 5-sulfosalicylic acid. The homogenate was centrifuged at 8,000 *g* for 10 min at 4 °C. The absorbance of the supernatant was measured at 405 nm. GSH and GSSG were assessed using a calibration curve with 0–25 μM GSSG and 0–50 μM GSH standard solutions. $E_{GSSG/2GSH}$ was calculated as described by Kranner et al. (2010) using the Nernst equation that was corrected for the seed WC.

$$E_{GSSG/2GSH} = E^{0'} - \frac{RT}{nF} ln \frac{[GSH]^2}{[GSSG]}$$

where R is the ideal gas constant; T is the temperature in K; n, the number of electrons transferred; F, the Faraday constant; $E^{0'}$, the standard half-cell reduction potential at pH 7 [$E^{0'}_{GSSG/2GSH}$ = −240 mV].

Antioxidant enzyme activity

SOD activity was determined according to Parrilla-Taylor and Zenteno-Savín (2011). Twenty-five μL of potassium phosphate buffer (0.1 M, pH 7.5) or protein extract was added to 1.45 mL of assay solution buffer (pH 9.5) containing, 50 mM Na_2CO_3, 0.1 mM xanthine, 25 μM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 25 μL of Xanthine oxidase (XO) solution (0.1 U of XO in 1 mL of 2 M ammonium sulfate). Absorbance was measured at 560 nm every 30 s for 3 min at 25 °C. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate.

CAT activity was determined according to Roshan et al. (2018). An assay mixture was prepared with 600 μL of 100 mM phosphate buffer (pH 7) and 200 μL of 10 mM H_2O_2. The reaction was started by adding 200 μL of protein sample to the mixture. The decomposition of H_2O_2 was determined by measuring the absorbance at 240 nm every minute for 3 min at 25 °C. One unit of CAT activity was defined as the amount of enzyme needed to catalyse the decomposition of 1 μmol of H_2O_2 per minute.

POD activity was determined according to Corona-Carrillo et al. (2014). An assay mixture was prepared with 870 μL of

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50 mM phosphate buffer (pH 6.8), 10 µL of the protein sample and 10 µL of 1 mM guaiacol. The reaction was started by adding 10 µL of 3% (v/v) H_2O_2 to the mixture. Absorbance was measured at 475 nm every minute for 3 min at 25 °C. One unit of POD activity was defined as the amount of enzyme needed to catalyse the peroxidation of 1 mmol of guaiacol per minute.

Statistical analysis

All physiological and biochemical tests were performed in triplicate and results are expressed as mean ± standard deviation. Normal distribution of the data was confirmed using a Shapiro–Wilk test. Data were statistically analysed by one-way ANOVA followed by a Tukey's post-hoc test at $p < 0.05$ as significance level using the statistical packages SPSS® (ver. 15.0) and NCSS® for Windows.

Results

Water content of dried freshly harvested seeds of *E. chiotilla* and *S. pruinosus* was 8.6 ± 0.5% and 7.2 ± 0.3%, respectively. After *ex situ* storage and *in situ* burial, seed WC did not show significant difference with freshly harvested seeds. However, WC of buried seeds of *E. chiotilla* and *S. pruinosus* increased to 28.6 ± 4.7% and 28.1 ± 3.7% after one day of rainfall, and then decreased to 8–9% and 7–8%, respectively, 24 h after rainfall.

Experiment 1: comparison between ex situ and in situ storage

Effect of ex situ storage and in situ burial on the seed viability and vigour

Germination of both *E. chiotilla* and *S. pruinosus* seeds remained above 90% after two years of *ex situ* storage (Fig. 1A, B). After one year of burial, seeds of both species germinated at *ca.* 35%. After two years of burial seeds of *E. chiotilla* germinated to 23 ± 2.8%, whereas those of *S. pruinosus* were dead (verified by tetrazolium chloride test) (Fig. 1A, B). After one year of storage, T_{50} values of both species were respectively 1.2 and 1.6 days higher compared to the T_{50} values in stored *ex situ* seeds (Fig. 1C, D). In *E. chiotilla*, T_{50} values of two-year old seeds were also significantly higher for buried seeds. Solute leakage from seeds of both species buried *in situ* for one and two years was more than twice that of seeds stored *ex situ* (Fig. 1E, F).

Effect of ex situ storage and in situ burial on markers of oxidative stress

Before storage, the MDA contents in seeds harvested in 2018 were *ca.* 16 nmol g^{-1} DW for both species. Seeds harvested in 2017 and buried *in situ* for one year exhibited a MDA content that was 2.4-fold higher than seeds stored *ex situ*. In contrast, the MDA content in seeds harvested in 2016 and buried for two years was low and not significantly different from those stored *ex situ* (Fig. 2A, B).

The soluble protein content in freshly harvested seeds of *E. chiotilla* and *S. pruinosus* was 118.8 and 164.8 mg g^{-1} DW, respectively. Compared to seeds stored *ex situ*, it was significantly lower in buried seeds of both species, particularly after two years of storage (Fig. 2C, D). The content of carbonyl groups from freshly harvested *E. chiotilla* and *S. pruinosus* seeds was 0.022 and 0.010 µmol mg^{-1} protein, respectively. Both species showed similar contents in seeds harvested in 2017 and stored *ex situ* or buried *in situ* for one year. However, for the seeds harvested in 2016 and buried for two years, the content of carbonyl groups *in E. chiotilla* and *S. pruinosus* seeds was respectively 5.3-fold higher and 28.4-fold higher, compared to those seeds stored *ex situ* (Fig. 2E, F).

The GSH and GSSG contents in freshly harvested seeds of *E. chiotilla* and *S. pruinosus* were 277.2 and 178.7 nmol g^{-1} DW, and 304.7 and 175.5 nmol g^{-1} DW, respectively (Fig. 3). Seeds of both species stored *ex situ* showed significantly higher GSH and lower GSSG contents than seeds buried *in situ*. As a result, the glutathione half-cell redox potentials were significantly higher in seeds buried *in situ* compared to seeds stored *ex situ,* indicating a difference in the oxidative response according to the storage environment (Fig. 3).

Activities of antioxidant enzymes exhibited similar values in seeds that were harvested in 2018 before storage and in seeds that were harvested in 2017 and stored *ex situ* and *in situ* for one year. However, in seeds of *E. chiotilla* harvested in 2016 and buried for two years, the activities of SOD, CAT and POD were 1.6, 1.8 and 2.9-fold lower respectively than in seeds stored *ex situ* (Fig. 4A, C, E). Likewise, in seeds of *S. pruinosus* buried for two years, the activity of SOD was 4.2-fold lower, and activities of CAT and POD were not detectable in comparison with seeds stored *ex situ* (Fig. 4B, D, F).

Experiment 2

In experiment 1, it is unclear whether time and conditions of storage, year of harvest or the combination of both was responsible for the differences found between seeds buried *in situ* for one and two years. For this reason, the oxidative markers and loss of viability were monitored in seeds harvested in 2018 and buried *in situ* for shorter time intervals (6, 12, 14 and 16 months). In addition, rainy periods during burial were monitored as a proxy to assess imbibition/desiccation cycles of buried seeds (see Supplementary Fig. S1).

In *E. chiotilla* seeds, viability was maintained during the first rainy season and dry season, then decreased abruptly from 83.5% at 12 months of burial (at the beginning of the second rainy season to 10% after 14 months (Fig. 5A). In *S. pruinosus* seeds, a different pattern was observed. Loss of viability started during the first rainy season but was accelerated during the dry season between 6 and 12 months after burial (Fig. 5B). Time of 50% germination in the remaining living seed lots increased progressively during burial of both species, resulting in 1.7-fold increase after 14 months in *E. chiotilla* seeds and 1.4-fold increase after 12 months in *S. pruinosus* seeds (Fig. 5C, D). Symptoms of deterioration were observed in the EC profiles during storage, exhibiting a similar pattern as loss of viability. In *E. chiotilla* seeds, EC slightly increased 1.3-fold during the first 12 months of burial then 1.7-fold between 12 and 14 months (Fig. 5E). Likewise, EC in *S. pruinosus* seeds increased 1.7-fold between 6 and 12 months of burial, and 1.6-fold between 12 and 14 months of storage while all seeds were already dead (Fig. 5F).

Changes in markers of oxidative stress during burial

The MDA contents in *E. chiotilla* seeds increased progressively 2.6-fold during 12 months of burial before the dramatic loss of viability. Thereafter, they decreased 4.4-fold after 14 months of burial that lead to the largest increase in dead seeds, compared to freshly harvested seeds (Fig. 6A). A similar pattern was also observed in *S. pruinosus* seeds, with a 2-fold increase in MDA

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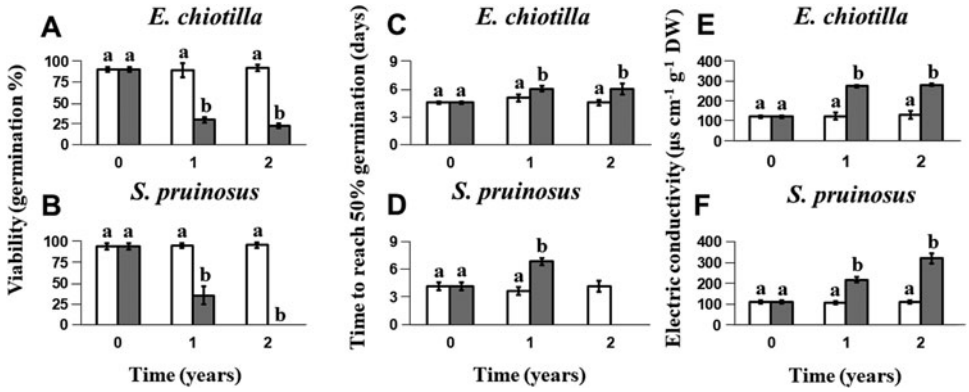

Figure 1. Effect of *ex situ* storage (white bars) and *in situ* burial (grey bars) for 0, 1 and 2 years on viability (A, B), T_{50} (C, D) and electric conductivity (E, F) of *E. chiotilla* and *S. pruinosus* seeds. Data show mean ± standard deviation ($n = 3$). Comparison of means was carried out between seeds stored *ex situ* and buried *in situ* at the same time.

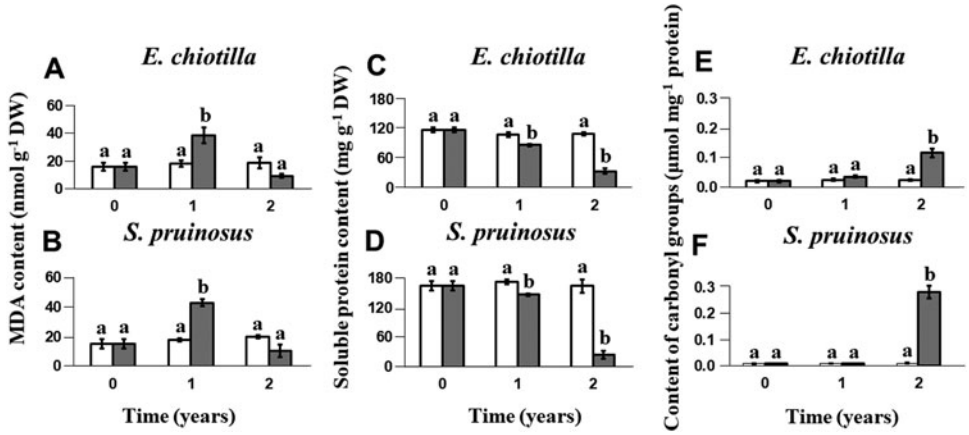

Figure 2. Effect of *ex situ* storage (white bars) and *in situ* burial (grey bars) for 0, 1 and 2 years on the content of MDA (A, B), soluble proteins (C, D) and oxidized proteins (E, F) of *E. chiotilla* and *S. pruinosus* seeds. Data show mean ± standard deviation ($n = 3$). Comparison of means was carried out between seeds stored *ex situ* and buried *in situ* at the same time.

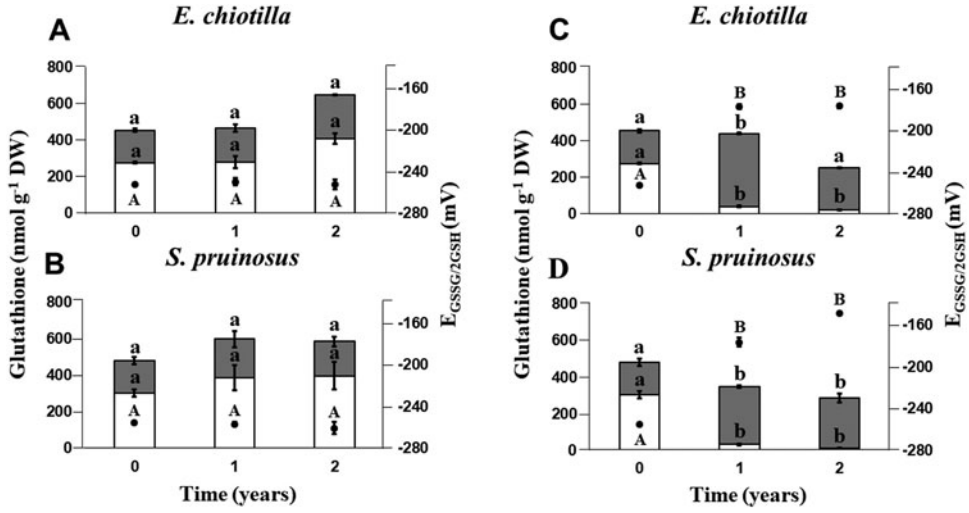

Figure 3. Effect of *ex situ* storage (A, B) and *in situ* burial (C, D) for 0, 1 and 2 years on glutathione content and redox state of *E. chiotilla* and *S. pruinosus* seeds. GSH (white bars), GSSG (grey bars) and $E_{GSSG/2GSH}$ (black circles, right Y-axis). Data show mean ± standard deviation ($n = 3$). Lowercase letters indicate the comparison of GSG and GSSH, while uppercase letters indicate the comparison of $E_{GSSG/2GSH}$ between seeds stored *ex situ* and buried *in situ* at the same time.

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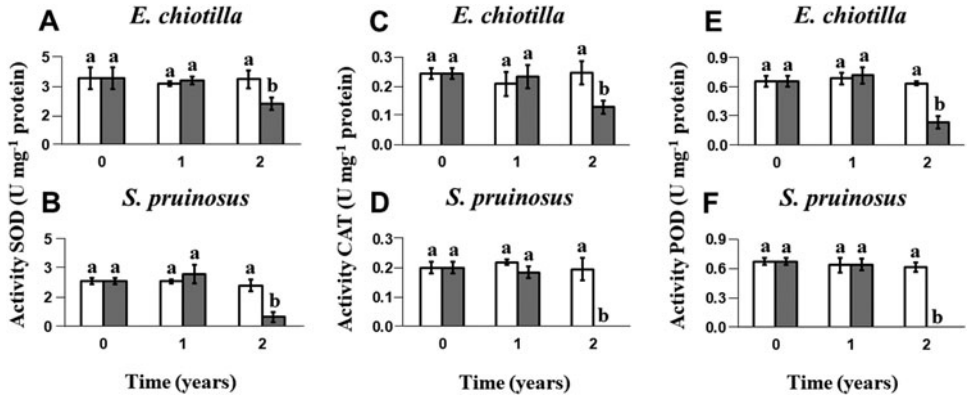

Figure 4. Effect of *ex situ* storage (white bars) and *in situ* burial (grey bars) for 0, 1 and 2 years on the activity of SOD (A, B), CAT (C, D) and POD (E, F) of *E. chiotilla* and *S. pruinosus* seeds. Data show mean ± standard deviation ($n = 3$). Comparison of means was carried out between seeds stored *ex situ* and buried *in situ* at the same time.

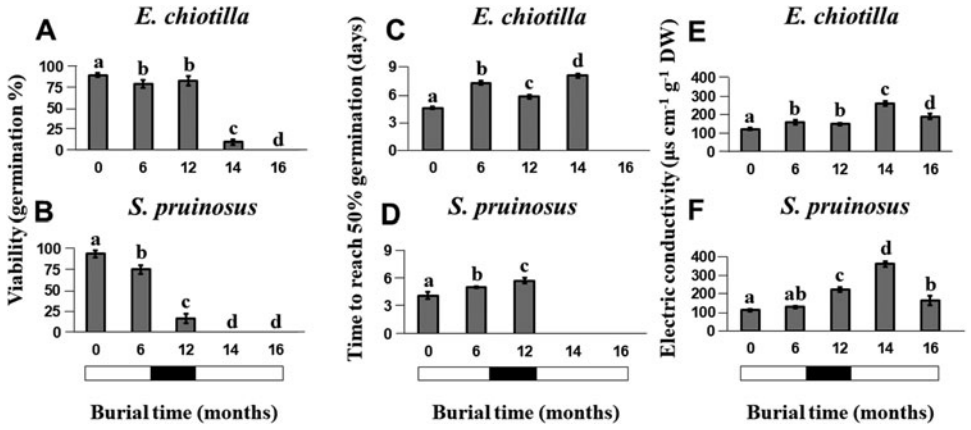

Figure 5. Viability (A, B), T_{50} (C, D) and electric conductivity (E, F) of *E. chiotilla* and *S. pruinosus* seeds buried *in situ* for 0, 6, 12, 14 and 16 months. Data show mean ± standard deviation ($n = 3$). Seeds buried *in situ* were exposed to two rainy periods (white bars) and one dry period (black bar).

content at 6 months and a 3.6-fold decrease at 12 months (Fig. 6B).

In *E. chiotilla* seeds, content of soluble proteins decreased slightly during burial when seeds remained alive, then decreased 3-fold after 14 months of burial when most seeds were found dead (Fig. 6C). In contrast, the soluble proteins content of *S. pruinosus* slightly decreased during storage leading to the decline of viability at 12 months of burial, thereafter they decreased by

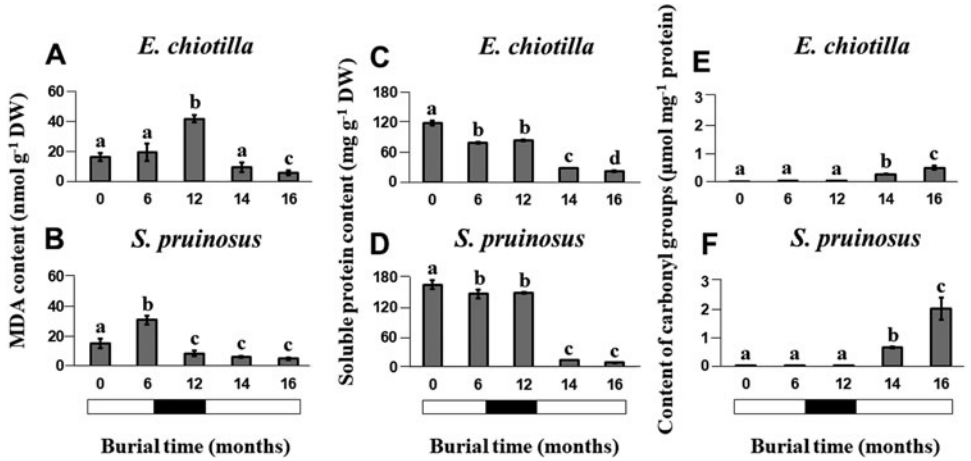

Figure 6. MDA (A, B), soluble proteins (C, D) and oxidized protein contents (E, F) of *E. chiotilla* and *S. pruinosus* seeds buried *in situ* for 0, 6, 12, 14 and 16 months. Data show mean ± standard deviation ($n = 3$). Seeds buried *in situ* were exposed to two rainy periods (white bars) and one dry period (black bar).

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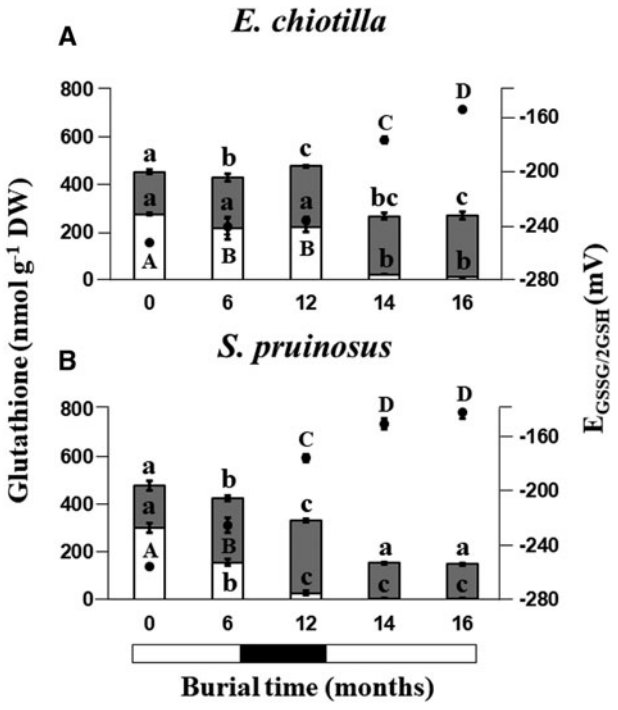

Figure 7. Glutathione content and redox state of *E. chiotilla* (A) and *S. pruinosus* (B) seeds buried *in situ* for 0, 6, 12, 14 and 16 months. GSH (white bars), GSSG (grey bars) and $E_{GSSG/2GSH}$ (black circles, right Y-axis). Data show mean ± standard deviation (n = 3). Seeds buried *in situ* were exposed to two rainy periods (white bars) and one dry period (black bar). Lowercase letters indicate the comparison of GSG and GSSH, while uppercase letters indicate the comparison of $E_{GSSG/2GSH}$.

10.6-fold in dead seeds after 14 months (Fig. 6D). Contents of carbonyl groups in seeds of both species did not change significantly during the first 12 months of burial. An important increase was only observed concomitantly with the increase in dead seeds of both species at 14 and 16 months (Fig. 6E, F).

In *E. chiotilla* seeds, GSH content mostly decreased between 12 and at 14 months corresponding to the loss of viability, while GSSG did not change significantly during that period (Fig. 7). This resulted in an oxidative shift in $E_{GSSG/2GSH}$ from −236.2 at 12 months to −176.8 mV at 14 months. This value continued to increase, reaching −154.6 mV in dead seeds after 16 months of burial (Fig. 7A). A similar pattern was observed in *S. pruinosus* seeds during the 12 months of burial. Within that period, the $E_{GSSG/2GSH}$ values shifted from −255.4 to −175.6 mV and reached −150.1 mV at 14 months in dead seeds (Fig. 7B).

A high antioxidant activity was observed during the first rainy season, but then decreased during the loss of viability in buried seeds of both species, respectively between 12 and 14 months in *E. chiotilla* and between 6 and 12 months of burial in seeds of *S. pruinosus*, a period in which more than 50% of antioxidant activity was lost (Fig. 8). Altogether, this suggests that seeds death during burial is accompanied by an oxidative shift.

Discussion

Under *in situ* conditions, seeds of both species form a short-term persistent soil seed bank (Fig. 1A, B and 5A, B). Similar results have been reported for other species of cacti, such as *Opuntia rastrera* (Montiel and Montaña, 2003), *Arthrocereus glaziovii*, *Arthrocereus melanurus* (Cheib and García, 2012), *Harrisia fragrans* (Goodman et al., 2012), *Stenocereus stellatus* (Álvarez-Espino et al., 2014), *Discocactus bahiensis* (Nascimento and Meiado, 2017) and *Echinopsis* and *Gymnocalycium* spp (Lindow-López et al., 2018).

In experiment 1, *E. chiotilla* and *S. pruinosus* seeds stored *ex situ* deteriorated slower than those buried *in situ* (Fig. 1–4). During *ex situ* storage, the dry condition at room temperature (25 ± 3 °C) maintained a low WC in seeds between 7–9%. A plausible explanation is that seeds would have formed a glassy state leading to restricted molecular diffusion, thereby slowing the rates of biochemical reactions involved in seed deterioration (Walters et al., 2005; Ballesteros and Walters, 2011). However, a biophysical study of water properties as a function of temperature is needed to ascertain such explanation. In contrast, seeds of both species harvested in 2017 and 2016, and buried *in situ* for one and two years, respectively, were exposed to different rainy periods with fluctuant temperatures in contrast with seeds stored *ex situ* (see Supplementary Fig. S1). Throughout the burial, seeds stored for one or two years experienced one or two precipitation cycle with warm temperature between 25 °C and 33 °C, leading to increased WC up to 28% after rainfall. These conditions are similar to accelerated ageing conditions because the seeds are no longer in a glassy state and are submitted to a deranged

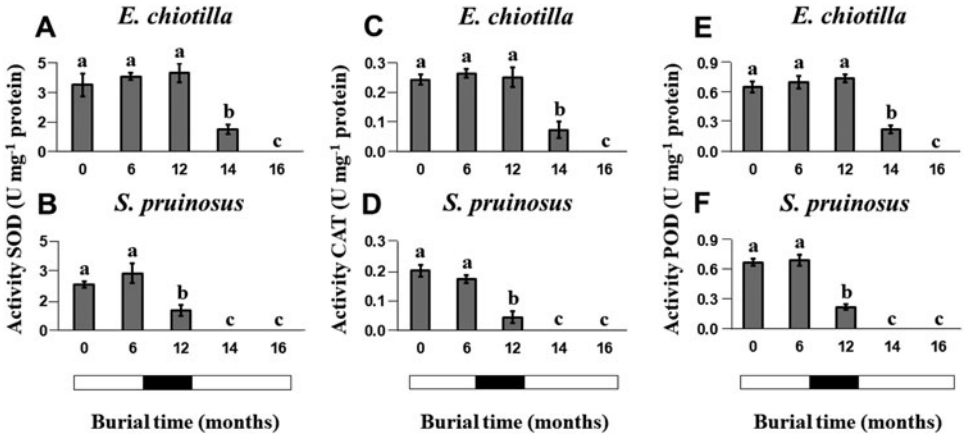

Figure 8. Activity of SOD (A, B), CAT (C, D) and POD (E, F) of *E. chiotilla* and *S. pruinosus* seeds buried *in situ* for 0, 6, 12, 14 and 16 months. Data show mean ± standard deviation (n = 3). Seeds buried *in situ* were exposed to two rainy periods (white bars) and one dry period (black bar).

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metabolism (Rajjou et al., 2008; Ballesteros and Walters, 2011; Zhou et al., 2020).

A limiting factor in experiment 1 was the use of seeds harvested in different years from which the maternal environment was not monitored. Indeed, seed longevity can be altered by the maternal environment during seed formation and maturation (Long et al., 2015; Leprince et al., 2017). Furthermore, the storage conditions of buried seeds for one and two years were different: seeds stored for two years experienced two rainy seasons in contrast to seeds stored for one year. Therefore, in order to clarify if the loss of viability was related with redox imbalance as a consequence of *in situ* burial, experiment 2 used seeds of the same harvest to avoid the complication of a putative impact of the maternal environment on seed longevity and to better take into account the rehydration/dehydration cycle. Interestingly, seeds of *E. chiotilla* were able to maintain a high viability throughout the first year of burial, during which they were exposed to high and dry storage conditions. This could be explained by a high antioxidant activity throughout this period. In contrast, the moist soil conditions during the first six months were already deleterious for *S. pruinosus* as viability significantly declined. Loss of viability was accelerated rated during the dry season.

This study showed no correlation between the content of carbonyl groups and MDA as oxidative markers and longevity during storage. The increase in carbonyl groups occurred when the seeds were mostly or completely dead (Fig. 5A, B). Changes in MDA contents during loss of viability did not match the patterns of redox imbalance. In experiment 2, 14 months of burial in *E. chiotilla* and 12 months in *S. pruinosus* seeds lead to a strong decrease in antioxidant activity. If we assume that such decrease would lead to an increase in oxidation as the $E_{GSSH/GGH}$ suggest, we should have expected a major increase in MDA, which was not the case. Therefore, our data argues against the use of TBA as a proxy to assess MDA as a marker of oxidative damage during storage in cacti seeds. There are other examples suggesting that great care should be taken in interpretation in the data using the TBA assay (Morales and Munné-Bosch, 2019). Similar results were reported in aged seeds of *Glycine max* (Priestley and Leopold, 1979; Priestley et al., 1980).

The decrease in GSH content and increase in GSSG in the seeds of the species studied resulted in an increase in $E_{GSSG/2GSH}$ values during *in situ* burial (Fig. 7). In buried seeds of *E. chiotilla* and *S. pruinosus*, $E_{GSSG/2GSH}$ values of −176.8 and −175.6 mV were recorded at 14 and 12 months, respectively, corresponding to the times when seed viability loss was recorded (Fig. 5A, B). At later times, when most seeds were dead, $E_{GSSG/2GSH}$ values were recorded at *ca.* −150 mV. According to Kranner et al. (2010), values of $_{EGSSG/2GSH}$ between −180 and −160 mV show a loss in viability, while values higher than −160 mV indicate that the seeds have died. Similar results to those observed in this study have been reported in *S. maritima* seeds, which lost viability at $E_{GSSG/2GSH}$ values between −180 and −160 mV (Seal et al., 2010). In seeds of other species such as *Hordeum vulgare*, *D. carota*, *C. sativus*, *R. sativus* and *L. sativa*, viability loss has been reported to occur at $E_{GSSG/2GSH}$ ranges between −200 and −180 mV (Roach et al., 2018; Stegner et al., 2022). Altogether, our data confirm that the redox imbalance accompanies loss of viability in seeds of *E. chiotilla* and *S. pruinosus* buried *in situ*. Long et al., 2011 suggested that wet-dry cycles play a key role for seed persistence in soil through a mechanism that may involve the recovery of the glutathione antioxidant system. At the species level, our data do not support this hypothesis for cactus seeds. A year-long wet-dry cycle did not affect GSH content in *E. chiotilla* whereas the moist conditions during the first six months of storage led to a significant decrease in GSH content that was exacerbated by the dry season in *S. pruinosus* seeds. For both species, the antioxidant activities remained high, suggesting that the GSH homoeostasis is regulated differently between both species. This warrants further work investigating the response of GSH synthesis and antioxidant responses to short- and long-term wet-dry cycles.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0960258524000011.

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Author contributions. D.A.G-H. performed the experiments, collected and analysed the data, and drafted the manuscript; C.B-M., J.M.V-H. and L.J.P-F. conceived the project, contributed to the experimental design, data analysis and interpretation, and critical revision of the manuscript. All authors reviewed and approved the final manuscript.

Competing interest. The authors declare no competing financial interest.

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