

Physiological and Biochemical Mechanisms Underlying Viability Decline in Recalcitrant Seeds of *Eugenia uniflora* During Cold Storage

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Research Article

Keywords: Tropical seeds, moisture content, recalcitrant seed, storage metabolism, *Eugenia uniflora*

Posted Date: October 27th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7802000/v1>

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Abstract

Germination is a complex process involving active metabolism and the mobilization of reserves. Orthodox seeds, which are initially dry, begin to germinate once they absorb sufficient water. In contrast, recalcitrant seeds are intolerant to desiccation, making long-term storage and conservation efforts challenging. Unlike orthodox seeds, recalcitrant seeds are dispersed with a high water content. The mechanisms that allow these seeds to maintain viability during storage, as well as the factors influencing their storage potential, remain unclear. These factors may include active metabolism, seed aging, or a combination of both. Moreover, data on the physiological behavior of recalcitrant seeds without desiccation are scarce. This study aimed to investigate the effects of storage at $8^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 190 days on *Eugenia uniflora* L. seeds, focusing on viability, biochemical and ultrastructural characteristics, the tetrazolium test, as well as starch, carbohydrate, phenolic compounds, and flavonoid content. The results indicated a reduction in germination percentage, starch levels, phenolic compounds, and flavonoid content after 135 and 190 days of storage, despite moisture levels remaining constant after 190 days. The presence of multiple vacuoles and organelles suggested high metabolic activity in the early stages of storage. Additionally, signs of cellular deterioration and reduced germination percentages indicated seed aging throughout the storage period. The findings suggest that the loss of viability in non-desiccated *E. uniflora* seeds is a result of both seed aging and elevated metabolic activity.

INTRODUCTION

The physiological understanding and behaviour of tropical seeds are essential tools to set conservation strategies for Brazilian biomes such as the Atlantic Forest and Cerrado, important hotspots (Françoso et al. 2015; Faria et al 2023), and Amazon, which have been degraded by land use and fires. The conservation of species found in these habitats ensures the regulation of ecosystem functions and contributes as carbon storage and hydrological cycles of South America and globally (Nogueira 2019).

Eugenia uniflora (L.), belonging to the Myrtaceae family, is native to several Brazilian biomes, such as the Cerrado and the Atlantic Forest, and adapts to a wide range of vegetation types, from humid areas to semi-arid regions (Aguiar et al., 2013; Mazine et al., 2014). Its fruits are essential for the diet of frugivorous animals and are widely used in ecological restoration programs (Lorenzi, 2002; Franzon et al., 2018). Additionally, the fruit pulp holds commercial potential for Indigenous communities. The leaf extract of *E. uniflora* has proven to be a promising alternative to synthetic antioxidants, offering great therapeutic potential (de Brito et al., 2022). However, a better understanding of its physiology is necessary to ensure proper seed storage and conservation. Seeds of some *Eugenia* species have a high water content after dispersal, characteristics typical of recalcitrant seeds, making it difficult to maintain their viability for extended periods (Delgado and Barbedo, 2007; Kaiser et al., 2014).

Recalcitrant seeds such as *E. uniflora* are known to be dispersed with high water content, with no desiccation phase (Farrant et al. 1999; Lah et al., 2023), and their viability is lost as water levels decreases (Roberts 1973; Walters et al. 2013). *E. uniflora* seeds have no dormancy, are short-lived, and

dry when physiologically mature. As recalcitrant seeds are stored, respiratory rates remain high even though seed reserves are consumed (Walters et al. 2015; Bonjovani and Barbedo 2019; Dias et al 2020), which evidences permanent cellular metabolism activity, with ongoing germination process (Pammenter and Berjack 1999; Barbedo 2018). As such, the high-water content in *E. uniflora* triggers the cellular respiration process, shortly leading to the loss of their viability (Delgado and Barbedo, 2007). These traits made the seed vulnerable to drought and frost, as well as heat. For example, *E. uniflora* seeds loose viability when their moisture content goes under 15% (Delgado and Barbedo 2007) which is comparable to the fresh seed weight.

Starch and soluble sugars degradation occurs during germination or seedling development, depending on the species (Zhao et al. 2018). Losses in the content of soluble carbohydrates contribute to the decline in vigour and seed germinability (Bernal-Lugo and Leopold, 1992). The content of soluble carbohydrates decreases with seed aging, which results in low availability of respiratory substrates for germination (Aguirre 2018). In *E. uniflora* seeds starch is the compound found in greater abundance (Mello 2008) and may be associated with the intermittent germinative metabolism of recalcitrant seeds (Walters et al. 2015). The first signs of deterioration in seed tissues involve damage and loss of integrity of the membrane system, allowing a vast loss of electrolytes, sugars, and amino acids from the cytoplasmic interior (Delouche and Baskin 1973; Delouche 2002; Kijak and Ratajcsak 2020).

The cellular metabolism of orthodox seeds is slow after the period of water loss (De Vitis et al 2020). This avoids high rates of reserve consumption, cellular respiration, and the production of reactive oxygen species, for example (Aguirre 2018). In recalcitrant species there is no apparent interruption of metabolism as they do not suffer water loss as occurs in orthodox seeds (Barbedo 2018; Pammenter and Berjack 1999). During the storage of these seeds, high metabolic activity can be mistakenly interpreted as the onset of deterioration and aging (Coolbear, 2020). These processes are identified by a decline in physiological quality, loss of enzymatic activity, reduced respiration, and a decrease in germination rate (Fu et al., 2015).

Understanding the mechanisms of seed deterioration can provide valuable information for scientists and practitioners. For example, to carry out restoration plantings with native seedlings or direct seeds, a better understanding of the physiology of seed viability can increase restoration success. We present a study using a native tropical seed as a model to investigate the impact of storage on seed viability. The aim is to gather information on how storage conditions affect seed behavior and the mechanisms involved in this process. More specifically, we investigated the effect of storing *E.uniflora* seeds (at 8°C for up to 190 days) on phytochemicals (phenolic compounds, flavonoids, starch, carbohydrates), germination, and cellular ultrastructure to infer the behavior and viability of seeds. Seed viability studies typically focus on tolerance to seed dissection during storage, while ours focus on storing native seeds without dehydration and evaluating biochemical and ultrastructural modifications (through micrography) to understand better the metabolic activity behavior and seed aging during storage.

MATERIAL AND METHODS

Material source

The fresh seeds were obtained from mature fruits of *E.uniflora* plants from Atlantic Forest in Southern Brazil, Armação beach (27°44'06.7" S, 48°30'30.2" W), Florianópolis city. According to Köppen, the climatic classification is of the CFA type. Fruits were considered mature when completely coloured. The endocarp and mesocarp were manually removed to obtain the seeds, cleaned and sterilised with sodium hypochlorite (1% v/v) for 10 minutes and superficial dried for 30 minutes under laboratory conditions to remove excess of water. Immature, rotten and insect-infested seeds were removed by visual inspection.

Storage in refrigerator

The seeds were divided and placed in polyethylene pots and kept in the fridge between $8 \pm 2^{\circ}\text{C}$. They were stored for 190 days. The seeds traits were evaluated in time intervals of 0, 135, and 190 days after storage.

Moisture content

The moisture content was determined following the method described by Brasil (2009). Three replicates of ten seeds were used for each storage period (0 days, 135 days, and 190 days). The seeds were initially weighed to obtain the fresh weight, then dried at $105^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 24 hours and reweighed to determine the dry weight. The dry weight was recorded, and the moisture percentage on a wet basis was calculated.

Germination test

Germination tests were performed by placing seeds in plastic trays (33cm × 28cm × 8 cm) at 2 cm deep in moist vermiculite. The tests were performed in biochemical oxygen demand incubator (B.O.D) adjusted to 25°C and photoperiod of 12 hours, with four replications of 25 seeds (Brasil 2009). Germination was evaluated for 60 days. Germination was based on the presence of normal seedlings, showing developed primary root and shoot, with no apparent damage. Results were expressed as a percentage of total germination and germination speed index (Maguire 1962).

Tetrazolium test

The tetrazolium test was used to estimate the viability of seeds that did not germinate (0, 135 days, and 190 days of storage), using a comparative staining method adapted from Cripa (2014). The test was performed on all ungerminated seeds to ensure whether they could still be viable at the end of the estimated germination evaluation period. The seeds were soaked in water for 30 minutes. The endosperm was sectioned longitudinally to expose the cotyledons, and the embryonic axis was

immersed in a 1% solution of 2,3,5-triphenyl tetrazolium chloride at 40°C for 2 hours. The results were based on the red staining in the embryo and cotyledons

Scanning electron microscopy (SEM)

For observation by scanning electron microscope (SEM) sections of the central portion of the seeds (n = 4) were dehydrated and submitted to ascending ethanolic series. Then they reached the critical point, using the CPD-30 dryer, they were metallized with gold to perform analyses by SEM (JSM LV- Jeol Ltd., Japan).

Transmission electron microscopy (TEM)

Central portions of 1mm² of the seeds (n = 4) were fixed with 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer, pH 7.2, for 4h, at room temperature (25°C), and rinsed in the same buffer six times for 10 min. The material was postfixed in 1% sodium phosphate buffered osmium tetroxide (OsO₄) for 4h, at 4°C, and rinsed in the same buffer six times for 10 min. After dehydration in a graded acetone series, the material was embedded in Spurr's epoxy resin. Ultrathin sections were cut at a thickness of 60 to 90 nm on a ultramicrotome with a diamond knife. Sections were collected on grids and stained with uranyl acetate and lead citrate, according to Reynolds (1963) and Fotouo-M et al. (2015). Four repetitions were established, containing a pool of several seeds from the same group (0, 135 and 190 days after storage). They were observed with a transmission electron microscope (TEM), model Jeol JEM1011 (Jeol, Ltd., Japan), operating at 80kV.

Determination of total carbohydrates and starch

For analyses of soluble and starch, three replicates of thirty seeds were used. Extraction was done with MCW 12:5:3 v/v (methanol, chloroform and water) according to Shannon's (1968) method, homogenizing with pestle and heating at 80° C for 10 min. The extracts obtained were colorimetrically analysed by the phenol-sulfuric method according to Dubois et al. (1956). Sugar was measured by the Umbreit and Burris (1964) method. Absorbance was read on a UV-vis spectrophotometer (Hitachi, Model 100 – 20) at 620 nm. The total soluble sugar contents for each sample were calculated from the glucose standard curve (0 to 100 µg mL⁻¹) and the results were expressed as µg glucose per mg of dry matter. Starch extraction was performed according to McCready et al. (1950). Starch content was calculated by the method of Umbreit and Burris (1964). The absorbance was obtained by spectrophotometer at 620 nm. Starch content was calculated from a standard D-glucose curve (0 to 100 µg mL⁻¹) and expressed as µg glucose per mg of dry mass.

Determination of total phenolic compounds and flavonoids

The determination of the content of total phenolic compounds was performed according to Schiavon et al. (2012) at 750 nm on a UV-vis spectrophotometer. Total phenolic compounds were quantified from the standard gallic acid curve (50 to 800 $\mu\text{g.mL}^{-1}$ - $r^2 = 0.99$; $y = 1.254x$). The analyses were performed in three replicates and expressed in μg of gallic acid per gram of dry mass. For the determination of total flavonoids seed samples were macerating and grinding using a shredder, extracted in methanol 80% for initial extraction, adapted from the method of Costa et al. (2017). After 1 hour the sample were read at 420 nm by UV-vis spectrophotometer. Flavonoids were quantified using the standard quercetin curve (0.5 to 100 $\mu\text{g.mL}^{-1}$ - $r^2 = 0.99$; $y = 0.009x$).

Determination of starch granules total area and cell wall size using Image J software

The size of cells was analysed using ImageJ software. To measure the total area of starch grains in each treatment, eight images obtained by SEM were used and five measurements of starch grains per image were performed. For cell wall length measurements, eight images obtained by TEM were analysed and five cell wall regions per image were analysed ($n = 40$). Our protocols were adapted from methodologies established by Viana (2018) and Jin (2017).

Statistical analysis

The one-way ANOVAs was performed in order to test the effects of storing seed (fixed factor, 3 levels) on phytochemicals (phenolic compounds, flavonoids, starch, carbohydrates) and germination. When the null hypothesis was rejected ($P < 0.05$), the mean values were compared with a Newman and Keuls test performed with the R package agricolae (de Mendiburu 2015). Data analysis was performed in R 4.0.5 (R Core Team, 2021). Plots were created using the R packages ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020).

RESULTS AND DISCUSSION

Seed moisture content and germination

E. uniflora seeds had an initial water content of 53.98%. Similar results were described by Delgado and Barbedo (2007) for seeds of the *Eugenia* species. At the end of 190 days of storage, the seeds showed a water content of 57.68% (Table 1), which was not statistically significant compared to the initial moisture content. During the storage period at 8°C, the seeds maintained the initial moisture content. The results are in agreement to Kaiser et al (2014), who evaluated *E. uniflora* seeds storage in plastic bags in a refrigerator at 10°C for up to 45 days. In contrast, Barbedo et al. (2008) observed variations in the water content of *Eugenia involucreta* seeds stored for up to 120 days. Kohoma et al. (2008) found that seed moisture was preserved for 180 days in a study with *Eugenia brasiliensis*.

Even though in our study water content was preserved in the seeds storage for 190 days, germination decreased over the studied period (Fig. 1). Seed storage time affected germination and germination

rates ($P < 0.005$; Fig. 1E, F), with the initial germination capacity of 94% being reduced to 58% and 41% after 135 and 190 days of storage, respectively. The germination rate at 135 and 190 days of storage (Fig. 1F) was reduced to more than half the germination rate presented by seeds without storage (0 days), showing the loss of viability in the seeds stored during this period. High values of water content in seeds during maturity are directly associated with the sensitivity to desiccation presented by this type of seed (Pammenter and Berjack, 2009). The intolerance to desiccation is a behaviour that makes difficult to store these species (Barbedo 2018). The tetrazolium test performed on non-germinated seeds after the germination period (Table 1) is in line with the results of germination rate, showing that the viability of the seeds decreases over 190 days of storage.

Table 1
Seed moisture content and tetrazolium evaluation in fresh *Eugenia uniflora* seeds and stored for 135 and 190 days.

Days after storage (days)	Moisture content (%) (p-value 0.078)	Viability		
		Germination (%)	Non- viable seeds (%)	Viable seeds (%)
0 (fresh)	53.98 ± 4.40 a	94 ± 7.65 a	4 ± 4.40 a	2 ± 1.10 a
135	55.02 ± 4.45 a	58 ± 10.58 b	39 ± 6.55 b	3 ± 1.43 a
190	57.68 ± 6.46 a	41 ± 3.82 b	56 ± 4.40 b	3 ± 0.98 a

Means followed by standard deviation of the the treatments, 0, 135 and 190 days. Means followed by the same letter do not differ (Tukey, $p < 0.05$).

Seed carbohydrates and starch content

Our results showed that seed storage did not affect total carbohydrates content ($P = 0.1931$; Fig. 1D). However, seed storage affected starch content, with higher values after 135 days storage when compared to 190 days of storage ($P < 0.005$; Fig. 1C). Starch is one of the most common carbohydrates found in seeds, including in *Eugenia* species such as the *Eugenia stipitata* species (Mendes and Mendonça 2020) an exalbulminous seed, with cotyledon cells presenting 43% of carbohydrates in the form of starch grains. In a study with orthodox seeds, Wang et al. (2005) found a decrease in the starch content in corn seeds that passed through ultradrying; these seeds also showed a decreased of germinability. The mobilization of starch can occur during the last stages of growth of organs such as fruits before ripening and be closely associated with the respiratory demands of certain tissues (Halmer and Bewley, 1982). Likewise, the starch in seeds with a certain degree of recalcitrance, can be related to the metabolic and respiratory demand of the seed, thus occurring in the consumption of carbohydrates and deterioration of the stored seed.

Phenolic and flavonoids compounds in seeds

Seeds storage time affected the content of total phenolic compounds over the 190 days ($P < 0.005$, Fig. 1A), with significant decline in phenolic compounds through the beginning and 190 days of storage. Storing seeds also affected flavonoids contents ($P < 0.005$, Fig. 1B), with the total levels decreasing after 135 days of storage (Fig. 1B). Seeds of *E. stipitata* also showed structures containing large amounts of phenolic compounds (Mendes and Mendonça, 2020). These compounds, in addition to inhibiting the development of fungal and bacterial pathogens present in plants and fruits, are involved in antioxidant processes, restricting the amount of oxygen that reaches the embryo (Taiz and Zeiger, 2019). In a study with recalcitrant seeds of *Swartzia langsdorffii*, Vaz et al. (2016) identified phenolic compounds in radicle regions. They related the chemical composition of phenolic compounds and the morphology of the seeds of *S. langsdorffii* with abilities to maintain the seeds' viability before the rainy periods that will make possible the germination of the species. *Eugenia* species are dispersed with high water content, which favours the proliferation of fungi in these diaspores (Oliveira et al. 2011). The high amount of phenolic compounds and flavonoids found in fresh seeds in this study show strategies to protect both pathogenic fungi as well as a mechanism for reducing oxidative stress by reactive oxygen species produced during the high metabolic activity in seeds with elevated water content (Leprince et al. 1993). Pukacka and Ratajcsak (2007), investigating the action of α -tocopherol and phenolic compounds during the storage of seeds of *Fagus sylvatica* L., observed an increase in the content of these compounds associated with viability and greater germination capacity. The authors also correlate the decrease in seed viability with a reduction of the content of antioxidant compounds, such as phenolic compounds and flavonoids, and an increase in reactive oxygen species acting on seed deterioration during long-term storage. The similar patterns observed in phenolic and flavonoid compounds in freshly collected *E. uniflora* seeds (0 days) and their subsequent decrease over 190 days of storage may be linked to seed aging and deterioration during this period. During seed germination, phenolic compounds play an important role in structural growth and the protection of plantlets from abiotic and biotic stresses, in addition to exhibit high antioxidant capacity during the germination process (Wang et al. 2013). Thus indicating that the best time and high viability of *E. uniflora* seeds is after their dispersion.

Seed cell wall and starch grains area

By measuring cell wall from images obtained through TEM, we found after 190 days, a decrease in the cell wall thickness of storage cotyledonary *E. uniflora* seeds (Table 2). The starch grains presented in cotyledonary cells showed a significant decrease in surface area ($p < 0.05$) after 190 days of storage (Table 2 and Fig. 2).

Table 2
Cell wall size and starch granules area in fresh *Eugenia uniflora* seeds and stored for 190 days.

Time of stored (days)	Cell wall size - width (\varvec{\mu}\varvec{m}) (p-value $2.66.10^{-7}$)	Starch granules area (\varvec{\mu}\varvec{m}^2) (p-value $4.79.10^{-7}$)
0	1.44 ± 0.53 a	145.80 ± 70.99 a
135	1.19 ± 0.38 b	91.48 ± 46.38 b
190	0.90 ± 0.28 c	69.63 ± 28.62 b

Means followed by standard deviation of the area of the starch grains of the treatments, 0, 135 and 190 days. Means followed by the same letter in columns are not significantly different (Tukey, $p < 0.05$).

SEM ultraestrutural

The cross sections of cotyledonary seeds were observed under SEM. The surface of starch grains were smooth, most of the native starch grains had an elliptical shape, whereas some had spherical and irregular shapes. The results indicated that the fresh seeds (0 days) presented the greatest starch grains the cotyledonary cell (Fig. 2). The cell wall deterioration is notable for a long time.

TEM ultraestrutural

The images obtained by transmission electron microscopy of the cotyledon sections of the fresh seeds (Fig. 3 and Fig. 4) showed vacuolized cells with a thick cell wall, shoeing mitochondria, characteristic of tissues with high metabolic activity. Birefringence characteristics are visualized in starch grains through TEM in fresh seeds; over time, the starch grains lose this characteristic, evidencing the degradation of this material and the deterioration of the seeds during storage. After 135 days of storage, it was possible to observe a gradual decline in the thickness of the cell walls of cotyledon cells. In addition, we visualized precipitations of compounds in the cell cytoplasm, which may indicate the accumulation of phenolic compounds and other extravasated compounds from vacuoles, taking into account that the average thickness of the cell walls seems to decreased, visually, although we did not have measurements, over the storage time, which may cause the rupture of cell organelles. However, birefringence remains unchanged on both polar and equatorial sections of elongated starch grains, indicating that crystallites are extremely small and exhibit multiple orientations, which interfere during observation (Pérez et al. 2009).

CONCLUSIONS

We evaluated physiology during prolonged storage of the recalcitrant *E. uniflora* seeds without desiccation. The variations found in the proportions of starch content, total phenolic compounds, and total flavonoids in seeds, stored for 190 days at a temperature of $8 \pm 2^{\circ}\text{C}$, suggest that seed metabolism is altered during storage, affecting the viability of the seeds. However, seed moisture content remained stable and continuous during the storage period. Thus, we note that the metabolic activity in *E. uniflora* remained active at the beginning of storage and, over time, the lack of other conditions to start the germination process can lead to the consumption of reserves and deterioration of cell walls. Consequently, viability is lost, even when the seed moisture content was maintained high in *E. uniflora* seeds.

The present work sheds light on the processes by which undesiccated recalcitrant seeds pass when stored for long periods of time. Such efforts are in increasing demand as a means to conserve the biodiversity in tropical regions of the world, where most of the seeds are intolerant to dry storage. The challenge of finding effective measures, either through desiccation or not, to conserve recalcitrant seeds reinforces the need to preserve the biodiversity hotspots of the world.

Declarations

AUTHOR CONTRIBUTIONS STATEMENT

D.M, F.R, M.S, D.T.P, and A.M.R. planned the experiments and collected the data on the field; D.M and E.W.A.W analysed the data; A.M.R coordinated the study; D.M and E.W.A.W. led the writing and all authors contributed during reviewing.

ACKNOWLEDGMENTS

We thank Laboratory of Electronic Microscopy of Federal University of Santa Catarina (LCME) for help in the ultrastructure analysis. The first author thanks the program of Post-Graduation in Biology of Fungi, Algae and Plants (Federal University of Santa Catarina) for the opportunity given. We also thank Dra Marisa Santos and Dra Carmen Simioni for helping analysis of microscopy images SEM and TEM. This research was funded by CNPq (*National Council for Scientific and Technological development*) and CAPES (*Coordination for the Improvement of Higher Education Personnel*).

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Figures

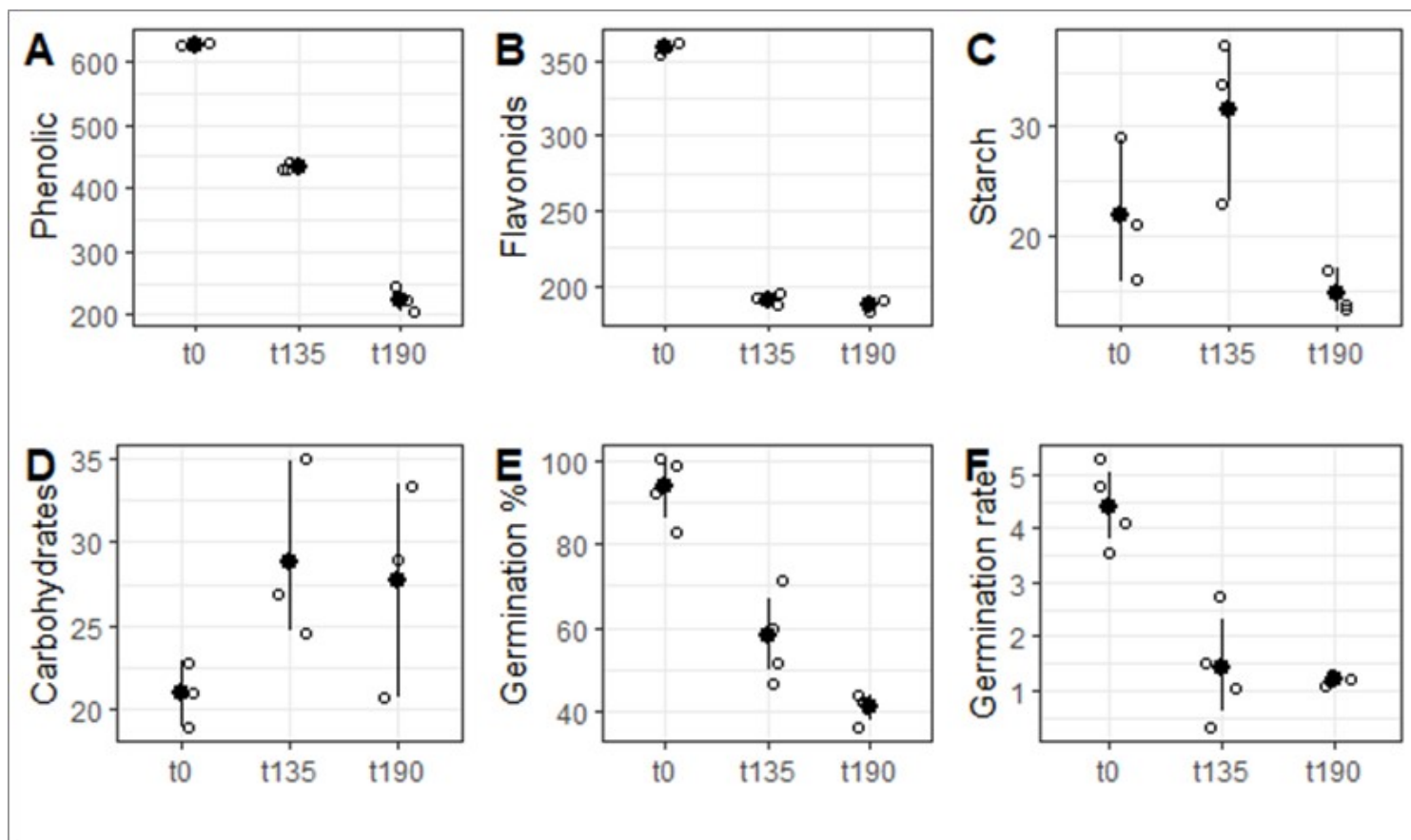


Figure 1

Effect of *Eugenia uniflora* seeds storage time (up to 190 days at $8 \pm 2^\circ\text{C}$) on total contents of phenolic compounds (A), flavonoids (B), starch (C), carbohydrates (D), and in the percentage of germination (E), and germination rate (F),

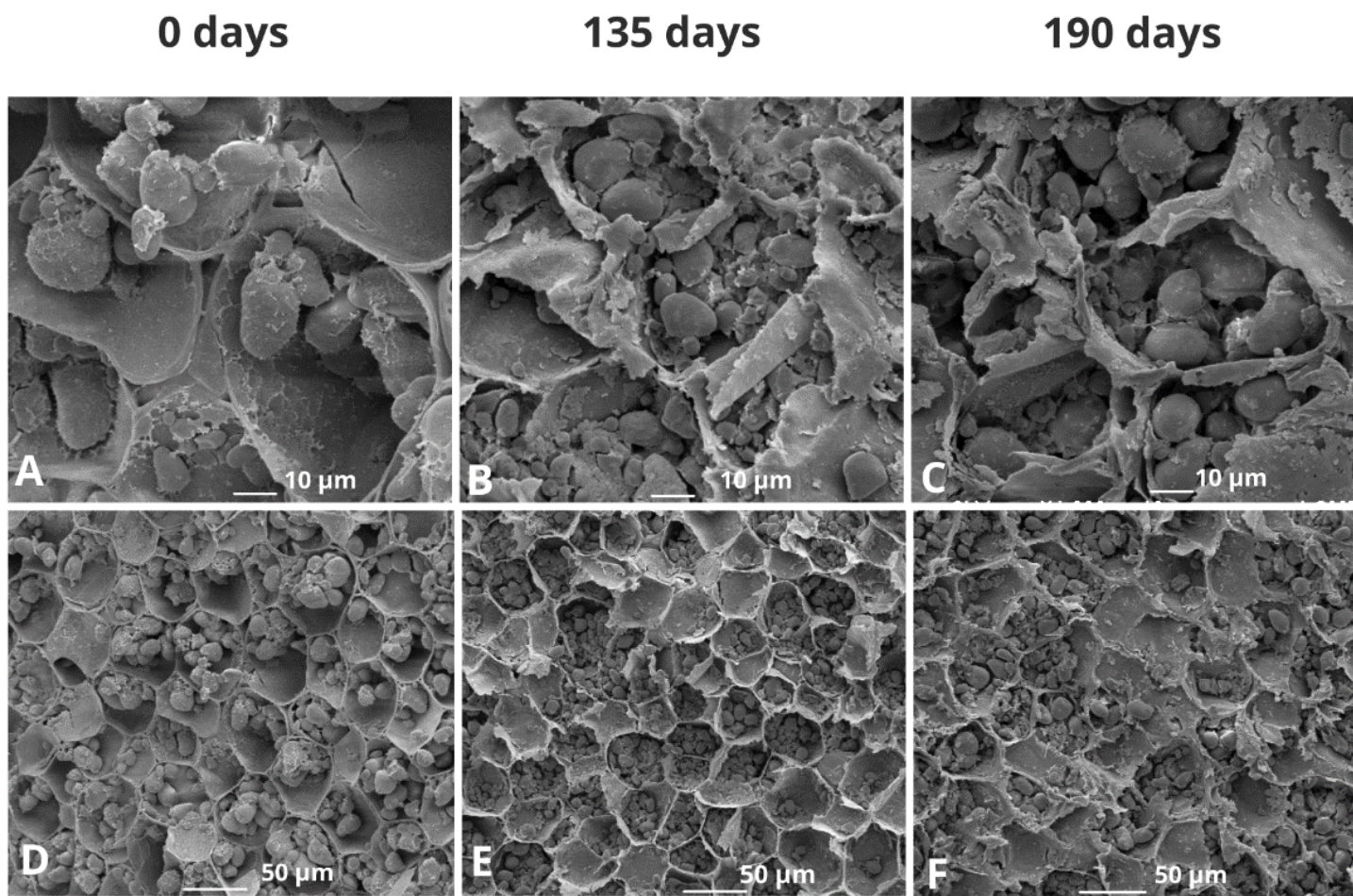


Figure 2

Scanning electron microscopic of cotyledons from *Eugenia uniflora* fresh (0 days) seeds (**A, D**), and seeds stored for 135 days (**B, E**) and 190 days (**C, F**). The white arrows indicate starch granules and amorphous materials (proteins or other biopolymers).

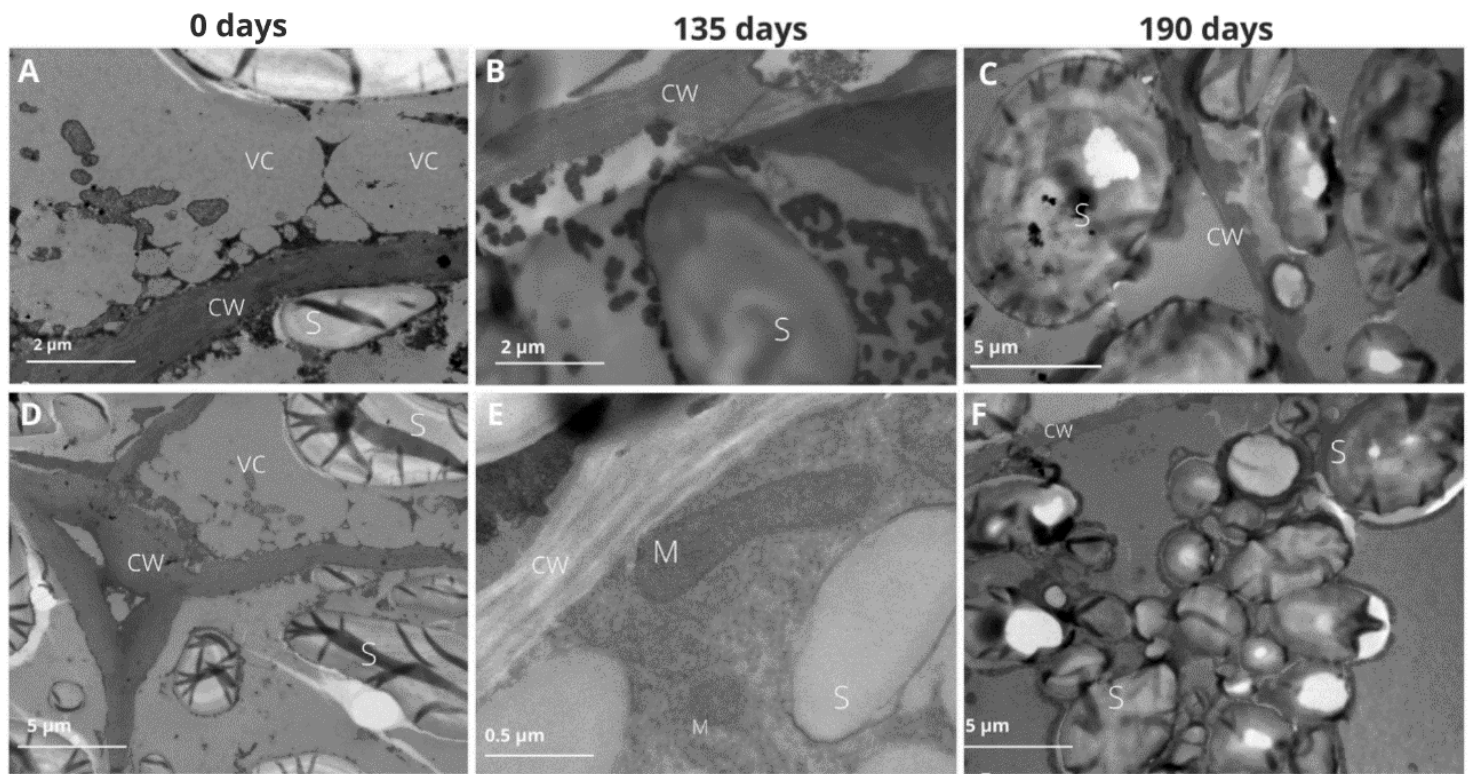


Figure 3

Micrography of transmission electron microscopy of cotyledons from *Eugenia uniflora* fresh seeds (control), and seeds stored for 135 and 190 days. Images showed starch granules and cell wall in the cells. **VC**, vacuoles; **CW**, cell wall; **S**, starch granules; **M**, mitochondria.

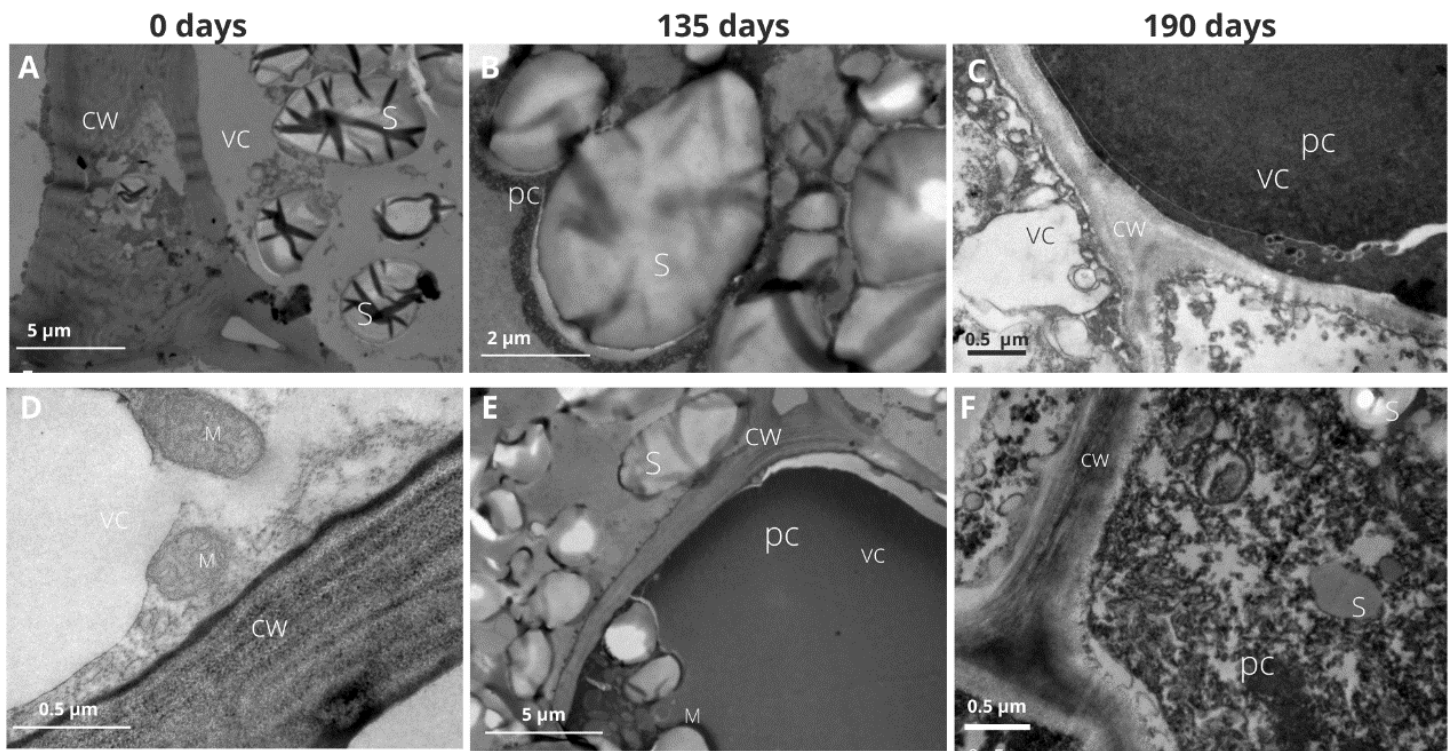


Figure 4

Micrography of transmission electron microscopy of cotyledons from *Eugenia uniflora* fresh seeds (control), and seeds stored up to 190 days. Images showed starch granules and cell wall in the cells. **VC**, vacuoles; **CW**, cell wall; **S**, starch granules; **pc**, phenolic compounds **M**, mitochondria