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Quality of *Eugenia involucrata* seeds: a physiological and biochemical approach as a response to fruit maturation

Calidad de semillas de *Eugenia involucrata*: un enfoque fisiológico y bioquímico como respuesta a la maduración del fruto

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SUMMARY

Most native species in Brazil have knowledge gaps regarding seed and seedling production and ecological restoration. *Eugenia involucrata* is a species of great ecological, medicinal, and commercial value, with seeds being the primary method of propagation, despite the difficulty in selecting trees and collecting and conserving recalcitrant seeds. The objective of this study was to understand the effects of fruit maturation on seed germination and storage. Based on the classification of fruits into three maturation stages (red–yellow: RY = 7.5YR 7/10; red: R = 10R 4/10; and purple: P = 5R 3/4) characterized by the Munsell Color System, we analyzed: a) the biometrics of fruits and seeds; b) biochemical aspects (hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), and guaiacol peroxidase (POD)); c) physiological (pre-germination treatments and seed storage); and d) physical and sanitized seed quality. We found that germination was slow, occurring for approximately 60 days, and could not be improved by the pre-germination treatment. R fruits suffered fewer H_2O_2 reactions, which was related to increased SOD and POD antioxidant activity, whereas RY and P fruits were associated with higher H_2O_2 concentrations. After storage (60 d), the seed water content did not decrease significantly, regardless of fruit color; however, R fruit seeds were less affected by fungi of the genera *Aspergillus* sp. and *Cladosporium* sp. R fruits are important morphological markers for collection, extending the duration of seed usage when stored in a cold and humid chamber, whereas seeds extracted from P fruits must be sown immediately to avoid a 25 % reduction in germination in just two months of storage.

Keywords: morphological marker, biochemical aspects, antioxidant enzymes, seed storage.

RESUMEN

La mayoría de las especies nativas de Brasil presentan lagunas de conocimiento en lo que respecta a la producción de semillas y plántulas, así como a la ecología de la restauración. *Eugenia involucrata* es una especie de gran valor ecológico, medicinal y comercial, cuyas semillas constituyen el principal método de propagación, a pesar de la dificultad en la selección de árboles y la recolección y conservación de semillas recalcitrantes. El objetivo del presente estudio fue comprender los efectos de la maduración de los frutos sobre la germinación y el almacenamiento de las semillas. Basándonos en la clasificación de los frutos en tres etapas de maduración (rojo–amarillo: RY = 7.5YR 7/10; rojo: R = 10R 4/10; y púrpura: P = 5R 3/4) caracterizadas mediante el Sistema de Color Munsell, analizamos: a) la biometría de frutos y semillas; b) aspectos bioquímicos (peróxido de hidrógeno (H_2O_2), superóxido dismutasa (SOD) y guayacol peroxidasa (POD)); c) aspectos fisiológicos (tratamientos de pregerminación y almacenamiento de semillas); y d) calidad física y sanitaria de las semillas. Encontramos que la germinación fue lenta, ocurriendo durante aproximadamente 60 días, y no pudo mejorarse mediante tratamientos de pregerminación. Los frutos R presentaron menos reacciones de H_2O_2, lo que se relacionó con un aumento en la actividad antioxidante de SOD y POD, mientras que los frutos RY y P estuvieron asociados con mayores concentraciones de H_2O_2. Después del almacenamiento (60 días), el contenido de agua de las semillas no disminuyó significativamente, independientemente del color del fruto; sin embargo, las semillas de frutos R fueron menos afectadas por hongos de los géneros *Aspergillus* sp. y *Cladosporium* sp. Los frutos R son importantes marcadores morfológicos para la recolección, prolongando la duración de uso de las semillas cuando se almacenan en una cámara fría y húmeda, mientras que las semillas extraídas de frutos P deben sembrarse inmediatamente para evitar una reducción del 25 % en la germinación en tan solo dos meses de almacenamiento.

Palabras clave: marcador morfológico, aspectos bioquímicos, enzimas antioxidantes, almacenamiento de semillas.

INTRODUCTION

Forest restoration provides important environmental services, such as habitat connectivity biodiversity, species conservation and carbon sequestration (Tedesco *et al.* 2023), just as research has intensified understanding of species performance in reforestation efforts (Turchetto *et al*. 2020, Gonçalves *et al*. 2023). However, owing to the lack of incentives and knowledge of silviculture, information regarding native tree species has gaps that prevent their cultivation on a larger scale. The availability of seedlings in nurseries is restricted (Freire *et al*. 2022) and pioneer species are predominant (Turchetto *et al*. 2016). Furthermore, the seedlings used in restoration projects require genetic variability and species diversity (Sebben 2006).

Eugenia involucrata DC. (Myrtaceae) is a tree species native to Brazil, Argentina, Paraguay, and Uruguay, and plays an important ecological role in the conservation of fauna and the restoration of degraded areas. This is because they attract dispersed fauna, facilitate the arrival of propagules from different species, and promote diversity. Its fruits have commercial potential, and the leaves are rich in phenolic compounds and flavonoids with anti-inflammatory and antioxidant activities, which reduce cholesterol, increase memory, learning, and cognitive functions, and protect against cardiovascular diseases (Dametto 2014).

The difficulty in selecting a superior *E. involucrata* tree is the low density of individuals ha^{-1} and the low fruiting synchrony index associated with the rapid dispersal of fresh fruits (Tonetto *et al*. 2013). This species has recalcitrant seeds, which are characterized as sensitive to desiccation, particularly at water content levels below 45 %, when they show a considerable decline in germination (Inocente and Barbedo 2021), which is reflected in their low storage potential. In addition, *E. involucrata* seed germination is classified as hypogeal, indicating that cotyledons remain below the substrate level (Vieira and Carvalho 2023).

Studies have indicated that pre-germination treatments can accelerate and standardize germination, in addition to providing the formation of more vigorous seedlings and contributing to gaining time for seedling formation owing to faster and more uniform emergence (Griebeler *et al*. 2019). Among the treatments that can be used, gibberellins stand out, particularly gibberellic acid (GA_3) (Griebeler *et al*. 2019), biostimulants (Mendes *et al*. 2019), and pyroligneous extracts (José *et al*. 2016). However, there are no reports of their use in the germination of *E. involucrata*.

Another factor that influences the physiological quality of seeds and their germination process is the storage period, which has varying effects on the enzymatic activity of the plant's antioxidant system (Hanapiah *et al*. 2022). Under stress conditions, reactive oxygen species (ROS) levels can increase and lead to lipid peroxidation of the membranes, resulting in seed deterioration (Lima *et al*. 2021). Thus, the plant antioxidant system minimizes, degrades, or transforms these molecules into their less reactive forms (Alsherif *et al*. 2022). Therefore, it is important to evaluate the appropriate maturation time when the fruits are harvested. Different stages can influence the biochemical processes and shelf life of seeds and affect the germination percentage (Antonia 2020).

The biochemical and physiological performance of seeds and the morphology of seedlings are associated with fruit maturation and pathogen incidence. Therefore, the determination of fungal genera associated with seeds is an important tool, as the prior detection of pathogenic agents allows the use of more assertive control methods (Parisi *et al*. 2019, Aimi *et al*. 2021) to improve storage or seedling production in nurseries.

Therefore, the current study aims to analyze: a) the biometrics of fruits and seeds, b) biochemical aspects (hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), and guaiacol peroxidase (POD)), c) physiological aspects (pre-germination treatments and seed storage), and d) the physical and sanitized quality of the seeds of *Eugenia involucrata.*

METHODS

Fruit collection and seed characterization. Fruits were collected in October 2021 from four parent trees located in the municipality of Santa Maria, in the central region of Rio Grande do Sul, Brazil (29° 43' S and 53° 42' O). The regional climate, according to the Köppen classification, is humid subtropical (Cfa), with mean annual precipitation between 1,400 and 1,760 mm, well distributed throughout the year, with temperatures ranging from −3 to 30 °C (Alvares *et al*. 2013). The soil is classified as a shallow and undeveloped lithosol (Embrapa 2013), and the forest typology is characterized as a seasonal deciduous forest (Marchiori 2009).

After collection, the fruits were transported to the Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil, where they were separated according to epicarp color, hue, value, and chroma of the Munsell Color System, considering three maturation stages: red–yellow (RY = 7.5YR 7/10), red (R = 10R 4/10), and purple (P = 5R 3/4) (figure 1).

Fruit and seed volumes and biometry. Initially, three maturation stages were analyzed to determine the number of fruits per container. Using a beaker, 400 mL of fruit from the three maturation stages were filled with four replicates each. The fruits were weighed and the number of fruits and seeds contained in the predefined volume was quantified and extrapolated to liters (L).

For biometric characterization, the fruits were separated into four samples of 25 units, with a total of 100 fruits per stage. The following attributes were evaluated: length (mm), width (mm) and weight (g). Fruit length and width were measured using a digital caliper (0.01 mm precision),

Figure 1. Color of *E. involucrata* fruits according to the maturation stages. a) Red–yellow (RY), b) red (R), and c) purple (P).
Color de los frutos de *E. involucrata* según las etapas de maduración. A) Rojo–amarillo (RY), B) rojo (R) y C) púrpura (P).

and fruit weight was obtained using an analytical balance (0.0001 g).

After the biometric evaluation, the fruits were submerged in water for 24 h (the water was changed after 12 h) and the pulp was removed to extract the seeds using sieves. The seeds were biometrically analyzed using the same methodology as that used for the fruits. Subsequently, the seeds were placed on a filter paper in a ventilated environment to form the lots used in this study. The seeds were placed in packages and packed in barrels, made of Kraft paper, and stored in a cold and humid chamber (8 °C and 86 % RH) until the germination test was conducted.

Seed physical quality. The initial sample seeds were characterized by evaluating the weight of 1000 seeds, using eight samples of 100 seeds, and the moisture content via the oven method at 105 ± 3 °C for 24 h (Brasil 2009).

The moisture content was measured at two time points. The first analysis was performed immediately after seed lot preparation (time zero), considering the three maturation stages. The second analysis was conducted 60 days after collection, with only seeds from the R and P stages, owing to the unavailability of seeds from the RY fruit class.

Germination test. Germination tests were performed in transparent plastic boxes (*gerbox*), using vermiculite as the substrate (Brasil 2013). The experiment was conducted using a completely randomized design in a 2 × 4 factorial arrangement (fruit maturation stage × pregermination treatment). Factor A levels were represented by two fruit maturation stages (R and P (the RY stage was not evaluated because of the low quantity of seeds available)), and factor B by four pre-germination treatments: Test, no treatment; Bio, immersion of the seeds in a biostimulant solution (Stimulate®), 10 mL L^{-1} for 12 h; Gib, seed immersion in a GA_3 solution (ProGib 400®), 250 mg L^{-1} for 24 h; Ep, seed immersion in pyroligneous extracts (Ep do Brasil®), 0.0005 % for 2 h. Four replicates of 25 seeds each were used, totaling 100 seeds per treatment.

The seeds were superficially de-infested by immersion in ethyl alcohol (70 %) for 1 min, followed by rinsing in water and immersion in 2.5 % sodium hypochlorite for 5 min, followed by triple washing in distilled water.

The test was conducted in a mangelsdorf-type germination chamber at 25 ± 2 °C under constant light. Germination was evaluated weekly over 70 d based on the formation of normal seedlings (G %). Seedlings were considered germinated if they exhibited a primary root, epicotyl and leaf primordia (Brasil 2009).

Shoot length (LAP), root length (LRP), and total length (TL) of normal seedlings were also measured using a graduated ruler in millimeters, and the results were expressed in cm $seedling^{-1}$. Simultaneously, the shoot dry matter (SDM) and root dry matter (RDM) of normal seedlings were obtained. For this, the seedlings were dried at 65 °C for 48 h, and the weight of the samples was obtained using an analytical balance (0.001 g), with the results expressed in g $seedling^{-1}$. At the end of the germination test, the percentages of intact and dead seeds were evaluated.

Sixty days after storage, another germination test was conducted to evaluate the loss of viability in the two seed lots, considering only factor A (fruit maturation stage). For this test, we assessed only G % following the same setup methodology and evaluation standards as those used in the previous test.

Sanitary test. The sanitary test was conducted after a 60 d storage period and only for seeds from fruits at the R and P maturity stages. We used 100 seeds from each maturation stage, divided into four replicates, which were sanitized by immersing them in 100 mL of water containing five drops of neutral detergent for 5–10 min, and then washed with sterilized distilled water. We distributed 25 seeds in a *gerbox* lined with two sheets of sterilized filter paper. The boxes were placed in a biochemical oxygen demand growth chamber, with temperature controlled at 25 ± 2 °C and 12 h of photoperiod, for 7 d. Subsequently, the fungi were evaluated and identified.

Fungal structures were observed under a stereoscopic and light microscope, and the fungi were identified using an identification key (Barnett and Hunter 1999). Fungal incidence data are expressed as percentages.

Biochemical analyses. Seeds collected at the three stages of fruit maturation (RY, R, and P) were used for biochemical analyses. Furthermore, we analyzed the stored seeds (60 d) using only the R and P maturation stages.

The samples were macerated in liquid N, homogenized in a specific buffer, and subsequently analyzed. SOD activity was determined according to the spectrophotometric method described by Giannopolitis and Ries (1977) and POD activity was determined according to Zeraik *et al*. (2008). H_2O_2 content was determined as described by Loreto and Velikova (2001).

Statistical analyses. Data were subjected to normality analyses of residues and homogeneity of variances using Shapiro–Wilk and Bartlett tests, respectively ($P < 0.05$). Subsequently, analysis of variance was performed, and when a significant difference was found, the means were compared using Tukey's test ($P < 0.05$). The packages "ExpDes.pt" (Ferreira *et al*. 2021) and Metan (Olivoto, 2021) and R Studio software (R CORE TEAM 2018) were used for the analyses.

RESULTS

Biometric and physical analysis. P fruits represented the largest volume of fruits per L. Therefore, they had a smaller number of fruits and seeds per L, which differed from the other maturation stages (table 1). The highest mean fruit width and weight were observed during the P maturation stage, corroborating the volume results (table 1). The weights of 1000 seeds from the R and P fruit classes were 385.98 g and 381.75 g, respectively, which were estimated at 2,590 and 2,620 seeds kg^{-1}.

Biochemical analysis. Immediately after collection, SOD and POD activities were lower in R fruit seeds (figure 2A and 2C) ($P < 0.01$) and increased after storage (figure 2B and 2D). The H_2O_2 concentration after collection was higher in the RY fruit class ($P < 0.01$), but did not differ from the P class, and R fruits had the lowest H_2O_2 concentrations (figure 2E). For POD, we observed the highest mean values in seeds from P and R fruits (figure 2C) but with low values.

At 60 d of storage, there was no significant difference in the seed H_2O_2 content ($P > 0.05$), regardless of fruit maturation stage (0.819 µmol g^{-1} MF^{-1}) (figure 2F). For SOD and POD activities (figure 2B and 2D), greater activity was observed in R fruit seeds ($P < 0.01$), which were able to control ROS and prevent cell damage.

Germination test. Soon after collection, germination was observed on day 21 after establishing the test, which ended after 70 days (figure 3a). The germination percentage was not influenced by the study factor (fruit maturation stage × pre-germination treatment); therefore, the mean germination (74 %) was calculated, considering all treatments. The highest germination rate occurred at 57 d when all treatments were > 50 %.

Seeds subjected to storage for 60 d showed that R fruits had a higher germination percentage (68 %) ($P = 0.021976$), with an increase in germination of approximately 19 % (figure 3b) compared with P fruits. This result confirmed the positive influence of SOD and POD (figure 2C and 2D) as antioxidant enzymes capable of neutralizing biochemical and physiological stress.

For the seedling LAP (LAP: 1.75 cm $seedling^{-1}$), the highest averages were observed for those originating from fruits at the P maturity stage ($P = 0.022118$) (figure 4A). The seedling LRP was greater when seeds were collected from R fruits (LRP: 4.21 cm $seedling^{-1}$) ($P = 0.00092$) (figure 4B). The total dry matter (TDM) of the seedlings was influenced by the maturation stage of the fruits ($P = 0.021017$), with R fruits indicating more vigorous seedlings (figure 4C).

Sanitary test. In the sanitary test of seeds stored for 60 days, we observed the presence of eight fungal genera: *As-*

Table 1. Number of fruits and seeds per liter (L) and mean values of width and weight of *E. involucrata* fruits at different maturation stages.
Número de frutos y semillas por litro (L) y valores medios de ancho y peso de los frutos de *E. involucrata* en diferentes etapas de maduración.

Maturation stages	Nf	Ns	Width	Weight
	fruits L^{-1}	seeds L^{-1}	(mm)	(g)
Red-yellow – RY	141.7 a	165.82 a	16.65 b	3.14 b
Red – R	141.25 a	154.37 ab	17.44 b	3.33 b
Purple - P	116.87 b	137.5 b	18.68 a	4.34 a
CV (%)	10.00	11.35	5.23	15.16

Nf: Number of fruits per liter (fruits L^{-1}); Ns: Number of seeds per liter (seeds L^{-1}). *Different letters between treatments represent significant differences according to Tukey's test at 5 % probability.
Nf: Número de frutos por litro (frutos L^{-1}); Ns: Número de semillas por litro (semillas L^{-1}). *Letras diferentes entre tratamientos representan diferencias significativas según la prueba de Tukey al 5 % de probabilidad.

pergillus, Cladosporium, Fusarium, *Phoma*, *Phomopsis*, *Colletotrichum, Rhizopus,* and *Epicoccum.*

The genera *Aspergillus* and *Cladosporium* showed higher incidence in seeds obtained from P fruits, which was 18 % (P = 0.04375) and 17 % (P = 0.001299) higher than those observed in seeds from R fruits (figure 5A and 5B). Other fungal genera showed low degrees of infestation (< 5 %).

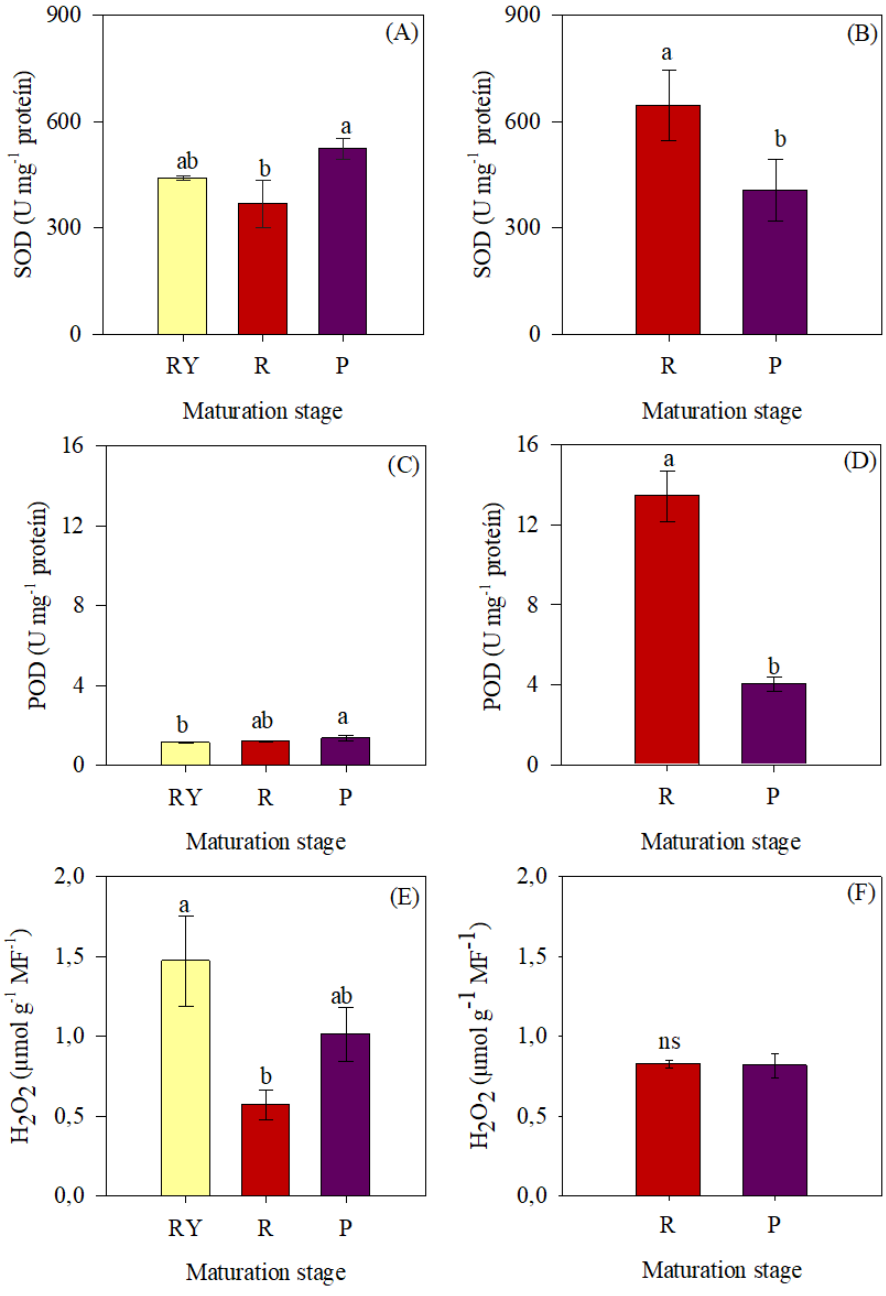

Figure 2. Concentration activity of superoxide dismutase (SOD) (A) and guaiacol peroxidase (POD) (C) immediately after collection; activity of superoxide dismutase (SOD) (B) and guaiacol peroxidase (POD) (D) after 60 d of storage, hydrogen peroxide (H_2O_2) (E) and hydrogen peroxide (H_2O_2) at 60 d (F) in *E. involucrata* seeds immediately after collection, from different fruit maturation stages. *Different letters between treatments represent statistical differences by the Tukey test at 5 % probability. Where: RY: red–yellow maturation stage; R = red maturation stage; P = purple maturation stage; ns: not significant.

Actividad de la concentración de superóxido dismutasa (SOD) (a) y peroxidasa de guayacol (POD) (c) inmediatamente después de la recolección; actividad de superóxido dismutasa (SOD) (b) y peroxidasa de guayacol (POD) (d) después de 60 días de almacenamiento, peróxido de hidrógeno (H_2O_2) (e) y peróxido de hidrógeno (H_2O_2) a los 60 días (f) en semillas de *E. involucrata* recolectadas inmediatamente después de la cosecha, en diferentes etapas de maduración del fruto. *Letras diferentes entre tratamientos representan diferencias estadísticas según la prueba de Tukey al 5% de probabilidad. Donde: RY = etapa de maduración rojo–amarillo; R = etapa de maduración rojo; P = etapa de maduración púrpura; ns = no significativo.

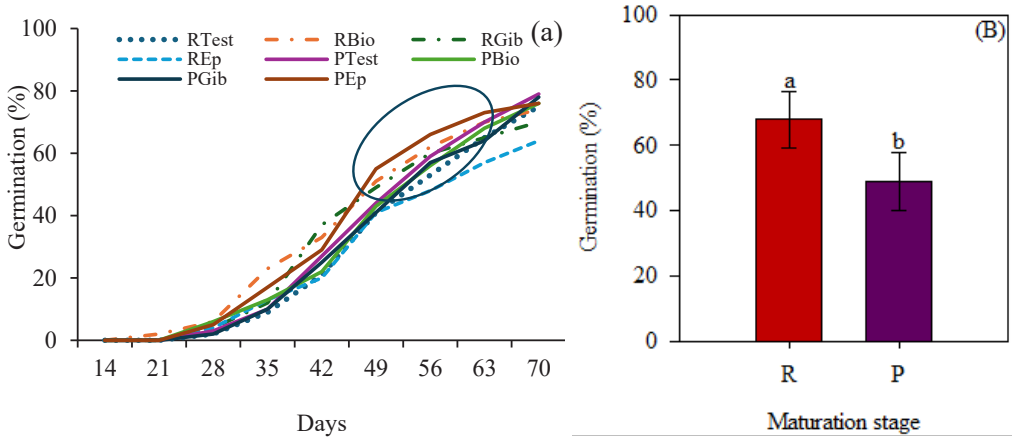

Figure 3. Accumulated germination of *E. involucrata* seeds as a function of fruit maturation stage and pre-germination treatments (A) and germination after 60 d of storage (B). Where: R = red maturation stage; P = purple maturation stage; Test = witness; Bio = biostimulant; Gib = gibberellin, and Ep = pyroligneous extracts. The blue circle represents the moment at which the seeds exhibited 50 % or more germination in the different treatments.

Germinación acumulada de semillas de E. involucrata en función de la etapa de maduración del fruto y los tratamientos de pre-germinación (A) y germinación después de 60 días de almacenamiento (B). Dónde: R = etapa de maduración rojo; P = etapa de maduración púrpura; Test = testigo; Bio = bioestimulante; Gib = giberelina; y Ep = extractos pirolignosos. El círculo azul representa el momento en el que las semillas alcanzaron el 50 % o más de germinación en los diferentes tratamientos.

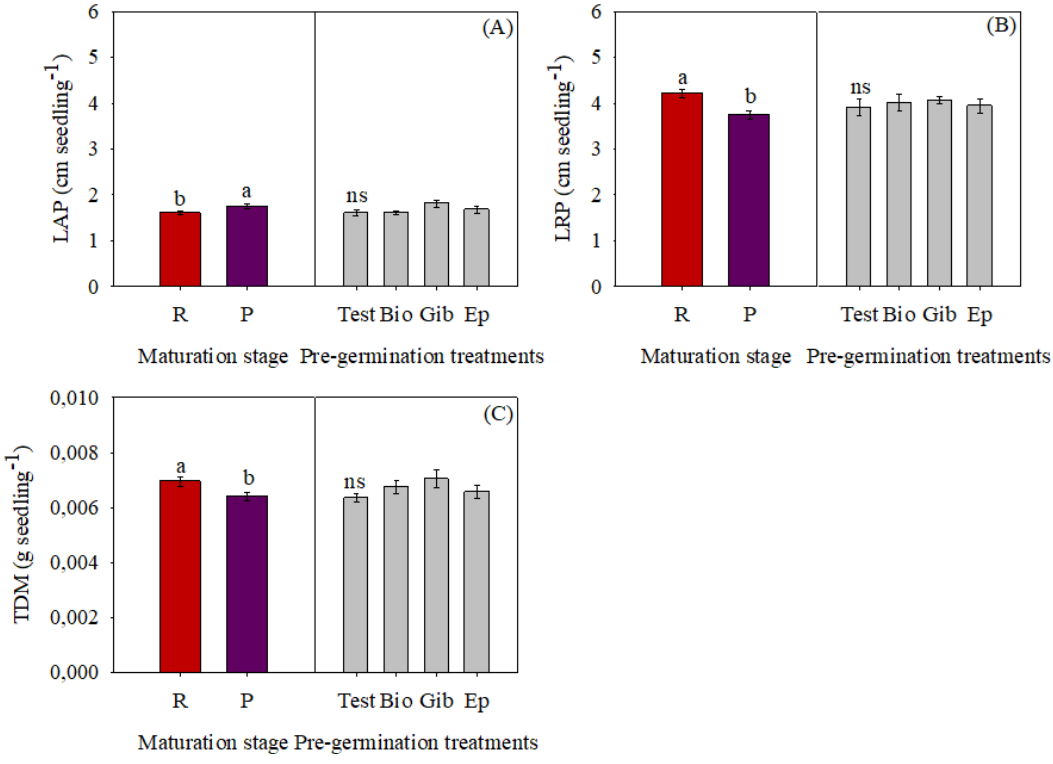

Figure 4. Shoot length (LAP) (A), radicle length (LRP) (B) total dry matter (TDM) (C). *Different letters between treatments represent statistical differences by the Tukey test at 5 % probability. Where: R = red maturation stage; P = purple maturation stage; Test - no treatment; Bio - immersion of the seeds in a biostimulant solution (Stimulate®), 10 mL L^{-1} for 12 h; Gib - seed immersion in a GA_3 solution (ProGib 400®), 250 mg L^{-1} for 24 h; Ep - seed immersion in pyroligneous extracts (Ep do Brasil®), 0.0005 % for 2 h.

Longitud de la parte aérea (LAP) (A), longitud de la radícula (LRP) (B), materia seca total (TDM) (C). *Letras diferentes entre tratamientos representan diferencias estadísticas según la prueba de Tukey al 5 % de probabilidad. Donde: R = etapa de maduración rojo; P = etapa de maduración púrpura; Test = sin tratamiento; Bio = inmersión de las semillas en una solución bioestimulante (Stimulate®), 10 mL L^{-1} durante 12 h; Gib = inmersión de las semillas en una solución de GA_3 (ProGib 400®), 250 mg L^{-1} durante 24 h; Ep = inmersión de las semillas en extractos pirolignosos (Ep do Brasil®), 0.0005 % durante 2 h.

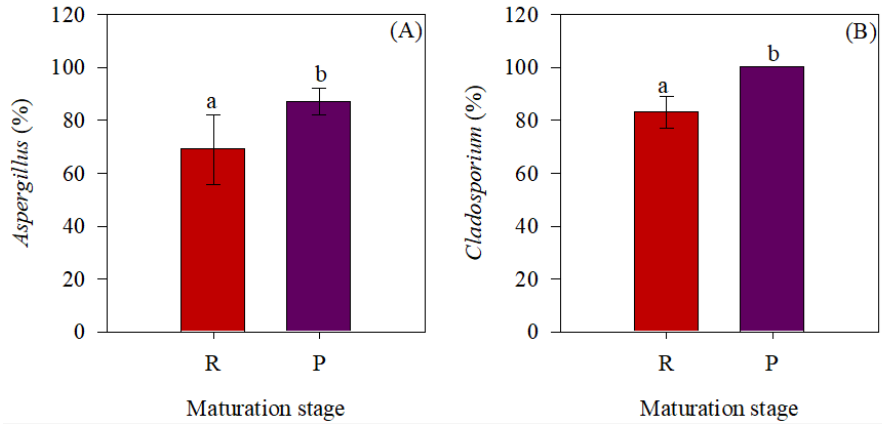

Figure 5. Incidence of *Aspergillus spp.* (A) and *Cladosporium spp.* (B) of *E. involucrata* seedlings as a function of fruit maturation stage. *Different letters between treatments represent statistical differences by the Tukey test at 5 % probability. Where: R = red maturation stage; P = purple maturation stage.

Incidencia de *Aspergillus spp*. (A) y *Cladosporium spp*. (B) en plántulas de *E. involucrata* en función de la etapa de maduración del fruto. *Letras diferentes entre tratamientos representan diferencias estadísticas según la prueba de Tukey al 5 % de probabilidad. Donde: R = etapa de maduración rojo; P = etapa de maduración púrpura.

DISCUSSION

Characteristics related to size and weight were verified in the fruits at the P maturation stage (table 1). A preliminary analysis suggested that larger fruits are associated with larger seeds, increased storage reserves, and increased vigor (Correa *et al*. 2021, Padilha *et al*. 2021). Consequently, seeds from P fruits would have a higher likelihood of successful germination, growth, and the potential to establish rapidly in the field, enduring adverse environmental conditions (Santos *et al.* 2018), which was not observed in the current study.

The seed water content (average 48.5 ± 1 %) was high and not the predominant factor capable of increasing seed weight immediately after collection, regardless of fruit maturation class. The high water content confirmed the species' recalcitrant seed behavior, sensitivity to desiccation and storage at low temperatures, and loss of viability over a short duration. *E. involucrata* seeds retain viability (53 % water content) when stored for up to 180 days inside plastic bags under cold storage (Maluf *et al.* 2003). To maintain seed viability after dispersion, water content–a parameter directly associated with physiological seed quality–combined with fruit morphological characteristics–the primary indicators of the best harvest period for some species (Garcia and Coelho 2021), are characterized as physiological maturity points.

Fruits at the R maturation stage exhibited suitable biochemical and morphological responses, reflecting the vigor of the seeds and seedlings. This stage promotes the formation of seedlings with greater DM and LRP, resulting in more vigorous individuals, as noted in the biochemical responses. Vigorous seeds can be used to produce seedlings with high growth potential in the field because of their efficient storage reserves.

Immediately after collection, the R fruits showed a lower concentration of H_2O_2, which allowed a reduction in the oxidation of organic compounds, resulting in lower production of antioxidant enzymes, such as SOD (figure 2B). RY and P fruits had higher H_2O_2 concentrations, indicative of immature seeds and those at an advanced maturation stage, respectively, which is not favorable for forming a high-quality seed lot. H_2O_2 represents ROS being released from its precursor, the superoxide free radical (O_2^-), which allows H_2O_2 to cross biological membranes. This causes metabolic disorders (Lima *et al*. 2021), and damages nucleic acids, proteins, and lipids (Smirnoff and Arnaud 2018). The consequences of ROS production in seeds include biomolecular changes, loss of seed viability during storage, reduced ATP production, lipid peroxidation, and cell membrane rupture (Kijowska-Oberc *et al*. 2021). A decrease in the expression of genes related to antioxidant systems was observed during the dehydration of recalcitrant *Araucaria angustifolia* (Bertol.) Kuntze seeds and was associated with a loss of viability (Gasparin *et al*. 2021).

Fruit maturation is a genetically programmed process that is influenced by environmental conditions and involves several physical and physiological modifications that reflect biochemical changes that occur during seed maturation(Chu-Puga *et al*. 2019). These changes were followed by increases in antioxidant enzyme activity, lipid peroxidation, and ROS release(Mondal *et al*. 2009). In the present study, we observed an increase in the antioxidant system and peroxidation at the R and P fruit stages after seed storage.

However, ROS formation is a natural process caused by respiration. ROS are the result of oxygen reduction and are present in different forms, from the most harmful, such

as the hydroxyl radical (OH^-), to those being transformed or having their levels reduced by the antioxidant system action (Bamagoos *et al*. 2022). Among the enzymes that are part of the antioxidant system, SOD, which is part of the plant's first line of defense, reduces O_2^- (superoxide anion) to H_2O_2 (Zhao *et al*. 2021). Subsequently, POD, which reduces H_2O_2 to H_2O and O_2, prevents it from being transformed into OH^-, which is one of the most powerful oxidants (Bernardy *et al*. 2020) capable of causing irreversible damage to seeds and plants.

We observed an increase in SOD levels with advancing fruit maturation (figure 2A and 2B). This trend can be explained by a possible increase in ROS levels in the seeds during fruit maturation (Ferreira *et al*. 2010), which is probably related to the beginning of the oxidation process and the high water content of the seeds. The increase in POD activity may have been due to increased SOD activity, which subsequently contributed to increased H_2O_2 levels. Therefore, POD required activation to reduce H_2O_2 levels.

Fruit maturation (R stage) had a positive influence on SOD and POD enzymatic activity after 60 days of storage (figure 2B and 2D), as the action of both enzymes contributed to reducing H_2O_2 levels during this period, preventing possible oxidative damage to seed germination. This decrease in H_2O_2 with seed storage from the R fruit stage also indicates that the plant's antioxidant system is efficient at eliminating H_2O_2. However, this reduction in H_2O_2 can also have a negative effect on germination, given that ROS is positively involved in the germination process of the genus *Eugenia* (Amorim *et al*. 2020).

We observed that recalcitrant *E. involucrata* seeds began to deteriorate in the mother plant owing to biochemical processes. However, in the analysis after collection, considerable germination occurred (74 %), although no significant difference was observed between the maturation stage and the pre-germination treatments after 60 d of storage. Other studies evaluating germination as a function of fruit maturation found values close to 90 % for *E. involucrata* and also showed greater seedling development in the advanced stages of maturation (Delgado and Barbedo 2020).

The similarity in the evaluated germination percentages between the maturation stages (R and P) indicated that seed maturation had already occurred at the time of collection, with differences observed solely for some seedling vigor variables. Similar results were reported by Lovatto *et al*. (2021), who analyzed the germination of *E. involucrata* seeds with yellow color, red spots, bright red color, burgundy color, and fruits on the ground.

After 60 d of storage, the seeds originating from R and P fruits showed a water content of 43 %, indicating a 5.5 % loss during that period compared with that of the unstored seeds. Thus, it is clear that the cold and humid chamber (8 °C and 86 % RH) contributed to maintaining seed hydration despite being considered recalcitrant seeds (Santos *et al*. 2012). The values obtained for the initial seed moisture (48.8 and 47.3 for R and P fruits, respectively) were consistent with those observed in other studies (Brüning *et al*. 2011, Carvalho *et al*. 2020). However, our study found that water loss was less damaging than high levels of H_2O_2 in seeds from P fruits, which reduced germination to 49 % after 60 d of storage. This value was lower than that of R fruit seeds (68 %). The germination potential response corresponds to important physiological indicators that can be used to confirm morphological (fruit color) and biochemical (H_2O_2, POD, and SOD) indicators. After maturity, deterioration rates progressively increase, with a decrease in germination and vigor owing to the natural aging of seeds; however, this can be minimized by adequate storage (Gasparin *et al*. 2018).

Oro *et al*. (2012) also found a higher percentage of *E. involucrata* germination in light-red fruits than in burgundy-colored or fallen fruits. Other studies have shown that a decrease in water content in recalcitrant seeds directly influences seed germination and reduces viability as a function of storage duration (Gasparin *et al*. 2020), corroborating the results of the current study. With a decrease in seed moisture content, we observed a reduction in the germination percentage, indicating a loss of natural vigor and viability for sensitive seeds.

After 60 days of storage, the improved biochemical performance observed in seeds obtained from R fruits contributed to greater germination and a reduction in pathogen development (figure 5). In this maturation class, we observed a lower incidence of fungi in the genera *Aspergillus* and *Cladosporium* than in the seeds from P fruits. The higher incidence of these genera in seeds from P fruits can be explained by the longer duration of the fruit on the tree and, consequently, greater exposure to degrading agents, such as fungi and insects (Gasparin *et al*. 2018).

The genus *Aspergillus* occurs primarily under storage conditions but superficially in the seed coat(Bewley and Black 2012). Some fungi of this genus can reduce seed germination and cause embryonic death (Cherobini *et al*. 2008). The genus *Cladosporium* is one of the most common field fungi because it infects the seeds during the final maturation stages (Lazzari 1993). It has pathogenic potential, is found in newly collected seeds, persists until storage, and can be transmitted from seeds to seedlings after sowing (Lima *et al*. 2000, Quevedo *et al*. 2020).

In this regard, we suggest further studies addressing potential treatments for pathogen control in the seeds of native species, with the aim of extending the storage duration and usability. This should be emphasized because it is difficult to increase or accelerate the germination of *E. involucrata* seeds.

Although other studies have reported the beneficial effects of seed immersion in GA_3 (Griebeler *et al*. 2019), biostimulants (Mendes *et al.* 2019), and pyroligneous extracts (José *et al*. 2016) on germination and seedling vigor

in various forest species, no promising results were found for the pre-germination treatments of *E. involucrata* seeds in this study. The effects of these substances depend on several factors, including concentration, application method, and species. Further studies are required to clarify their effects.

Thus, our study elucidated the important aspects of *E. involucrata* and identified biochemical and morphological indicators involved in seed conservation. Furthermore, we verified that the production of a small number of seedlings in a nursery (1000 units) would require 9 L of seeds, corresponding to 4.1 kg. According to the quantity of seeds per kilogram and germination percentage (74 % immediately after collection), it was necessary to collect 522 g of seeds. When the seeds are stored, the quantity increases to 6 % (553 g) extracted from R fruits and 2 % (532 g) from more advanced maturing P fruits. Finally, the availability of seedlings for forest implantation must be aligned with the seed quality. Therefore, we created a graphical abstract to facilitate the understanding of our results with *E. involucrata* seeds (figure 6).

CONCLUSIONS

P fruits (P = 5R 3/4) had higher biometric attribute values than those from the R stage, indicating greater accumulation of storage reserves. Seed germination was irregular and could take up to two months, regardless of fruit maturity (R or P), but could reach > 70 % germination when sown immediately after collection.

Morphological attributes, such as fruit color and seed biochemistry (H_2O_2, SOD, and POD), are important indicators of seed quality. Therefore, fruit color is a practical morphological marker.

Pre-germination treatments involving seed immersion in a biostimulant solution, GA_3 solution, or pyroligneous extracts were not effective in accelerating or enhancing the germination of *E. involucrata*.

The R fruits of *E. involucrata* provide seedlings with greater LRP and TDM, as well as a lower H_2O_2 concentration in response to the antioxidant system in the seeds, thus allowing a short storage duration in a cold and humid chamber. The collection of P fruits at a more advanced

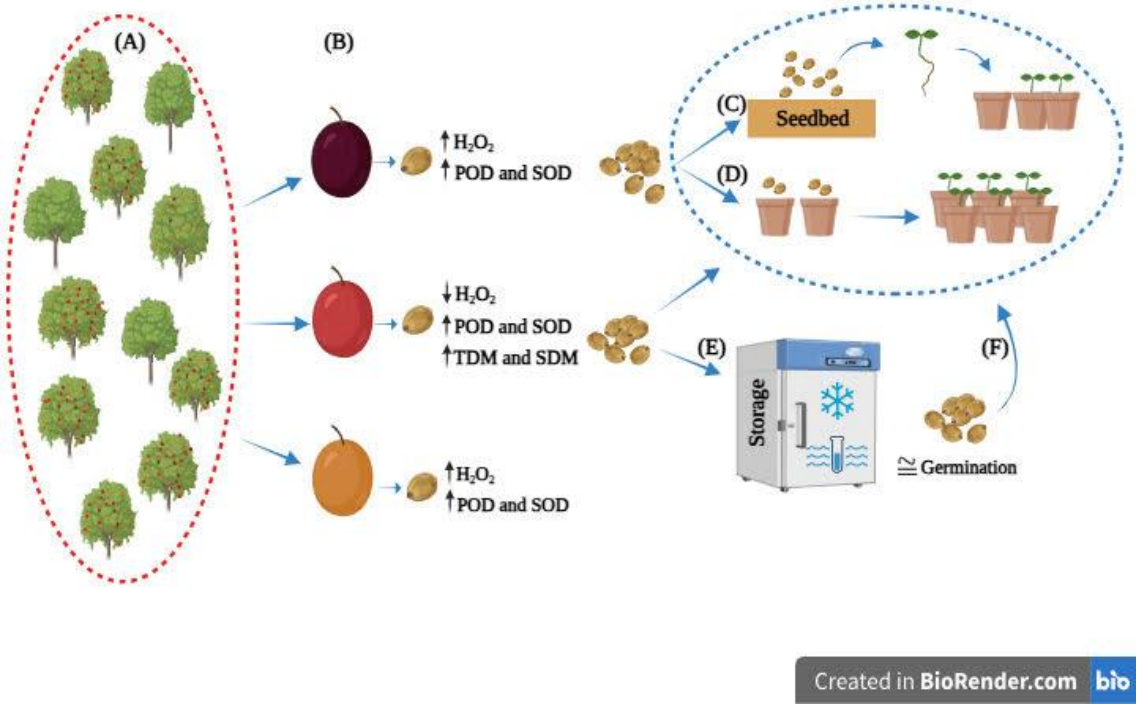

Figure 6. Schematic task results for seed management of *E. involucrata*: (A) location, selection, collection and processing of fruits, aiming to extend the time of use of *E. involucrata* seeds to produce seedlings for restoration. (B) Classification of purple (P) and red (R) fruits (higher quality), red–yellow (RY) (lower quality), and immediate use of P or R fruit to obtain quality seeds (74 % germination) followed by (C) sowing and pricking out, or (D) direct sowing, two seeds per container; (E) R seeds can also be stored in a cold and humid room for two months, maintaining 68 % germination; (F) the seeds can be used later in the same manner as in (C) and (D).

Resultados esquemáticos de las tareas de manejo de semillas de *E. involucrata*: (A) localización, selección, recolección y procesamiento de frutos, con el objetivo de extender el tiempo de uso de semillas de E. involucrata para la producción de plántulas destinadas a la restauración. (B) Clasificación de frutos púrpura (P) y rojos (R) (de mayor calidad), rojo–amarillo (RY) (de menor calidad), y uso inmediato de frutos P o R para obtener semillas de calidad (74 % de germinación), seguido de (C) siembra y repique, o (D) siembra directa, dos semillas por contenedor; (E) las semillas R también pueden almacenarse en una cámara fría y húmeda durante dos meses, manteniendo un 68 % de germinación; (F) las semillas pueden utilizarse posteriormente de la misma manera que en (C) y (D).

stage of maturation requires immediate use of the seeds because storage induces the greatest *Aspergillus* and *Cladosporium* fungal activity and a significant reduction in germination.

AUTHORS CONTRIBUTIONS

Project Idea: CC, EG, MMA, AMG. Database: CC, AMG, MVMA, ACQ. Processing: CC, AMG. Analysis: CC, AMG. Writing: CC, AMG, MVMA, GSW, CCK, HS, MPS, and ACQ. Review: CC, AMG, MVMA, GSW, CCK, HS, MPS, ACQ, EG, MMA.

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