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Storage of recalcitrant seeds of *Eugenia brasiliensis* Lam. under control of water availability

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ABSTRACT: The reduction in humidity and temperature makes it possible to prolong the storage of seeds, except for those sensitive to desiccation (recalcitrant), which therefore cannot be included in *ex situ* conservation banks. One way to control the metabolic activity and the movement of water into or out of the seed is to use osmotically active chemicals, such as polyethylene glycol (PEG). In this study, the effect of storage of recalcitrant seeds of *Eugenia brasiliensis* with water movement control in osmotic medium was evaluated. The results showed that the maintenance of these seeds at water potentials between -1 and -2 MPa allowed increasing the capacity for conserving viability in storage. They also demonstrated that the expansion of this conservation, unlike what occurs with the reduction of temperature, is not necessarily associated with the reduction of metabolic rates.

Index terms: deterioration, PEG, recalcitrance, tropical species.

RESUMO: A redução da umidade e da temperatura permite prolongar o armazenamento de sementes, exceto para aquelas sensíveis à dessecação (recalcitrantes), o que dificulta a inclusão destas em bancos de conservação *ex situ*. Uma forma de controlar a atividade metabólica e a entrada ou saída de água na semente é o uso de substâncias químicas osmoticamente ativas, como o polietilenoglicol (PEG). Neste estudo avaliou-se o efeito do armazenamento de sementes recalcitrantes de *Eugenia brasiliensis* com controle hídrico em meio osmótico. Os resultados demonstraram que a manutenção dessas sementes em potencial hídrico entre -1 e -2 MPa permitiu ampliar a capacidade de conservação da viabilidade em armazenamento. Demonstraram, também, que a ampliação dessa conservação, diferentemente do que ocorre com a redução da temperatura, não necessariamente está associada à redução das taxas metabólicas.

Termos de indexação: deterioração, PEG, recalcitrância, espécies tropicais.

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INTRODUCTION

Recalcitrant seeds (sensitive to desiccation) usually show intense metabolic activity even after their dispersion and may even germinate during storage. The high water content and this intense metabolic activity, when germination is not completed, create favorable conditions for rapid deterioration (Castro et al., 2004; Barbedo et al., 2013; Amorim et al., 2020). Thus, the conservation of viability during the storage of recalcitrant seeds remains one of the greatest challenges for seed science.

The reduction in water content for orthodox seeds (tolerant to desiccation) and reduction of temperature are the main tools to control the deterioration during seed storage. Besides being sensitive to desiccation, consequently not being able to be dried to adequate water levels for storage, recalcitrant seeds cannot benefit from the reduction of temperature up to freezing levels. Therefore, there is no way to include species with recalcitrant seeds in *ex situ* conservation banks (Barbedo, 2018; Srivstava et al., 2022).

In recent years, many studies have been carried out on the behavior of seeds of different species; however, few advances have been made regarding the extension of the viability period of recalcitrant seeds (Barbedo, 2018). Currently, despite being a more economical method than keeping plants growing, cryopreservation of embryonic axes is more efficient to preserve species with recalcitrant seeds (Sershen et al., 2012; Hamilton et al., 2013; Walters et al., 2013). However, this is a method that requires specific protocols, which do not yet exist. Developing alternative methods for the storage of seeds intolerant to desiccation, which reduce this intense and disordered metabolism, is extremely important (Andréo et al., 2006; Umarani et al., 2015); however, for this, it is necessary to know the processes involved in the rapid rates of deterioration.

One way to control metabolic activity and the entry or exit of water into or out of the seed is to use osmotically active chemicals (Santos et al., 2008), from imbibition in moist substrate or immersion in osmotic solutions with known water potential (Andréo et al., 2006). Studies were carried out using the regulation of water mobilization in embryos of *Inga vera* Wild. subsp. *affinis* to control their metabolism and succeeded in extending storage, reaching germination four times higher at 90 days of storage (Andréo et al., 2006; Pereira et al., 2020). One of the most used solutions for this type of treatment is polyethylene glycol (PEG), a high-molecular-weight polymer with no signs of toxicity to seeds (Andréo et al., 2006). However, there are still no studies demonstrating the relationship between the expansion of storage capacity, by this technique, and the reduction of metabolic rates of seeds.

Eugenia brasiliensis Lam., known in Portuguese as *grumixama*, is a tree of occurrence in the Semideciduous Seasonal Forest, in the Rainforest, in the Mixed Ombrophilous Forest and in the Restinga, from Bahia to Santa Catarina States, Brazil. (Mazine et al., 2022), produces fruits of pleasant flavor that can be consumed fresh or in the form of preserves and sweets (Lamarca et al., 2013; 2020), and have seeds sensitive to desiccation (Delgado and Barbedo, 2012). These seeds lose viability with water content close to 45-50% (Delgado and Barbedo, 2007) and, according to Kohama et al. (2006), can be stored for up to 180 days, with water content close to 50%, in a cold chamber at 7 °C.

The aim of this study was to evaluate the effects of conventional storage in cold chamber and storage with water control in osmotic medium on the longevity of recalcitrant seeds of *Eugenia brasiliensis* Lam.

MATERIAL AND METHODS

Ripe fruits of *E. brasiliensis* were collected from trees of the *Parque Estadual das Fontes do Ipiranga*, São Paulo, SP, Brazil, and sent to the laboratory of the Seed Research Center of the *Instituto de Pesquisas Ambientais* for seed extraction. After being manually extracted, the seeds were washed in running water over a sieve and left to dry superficially in trays. After the samples were collected for physical and physiological determinations, the remaining seeds were stored in a semipermeable plastic bag, perforated with a needle, in a refrigerator at 8 ± 2 °C until the beginning of the experiments (3 to 5 days).

Respiratory rates of seeds under different thermal and water regimes

To analyze the respiratory activity of the seeds at different temperatures, they were incubated in respirometers and kept at 8, 15, 25 and 35 °C. At temperatures of 25 and 35 °C, they were incubated for 24 hours and at 8 and 15 °C, for 360 hours. At the end of the periods, air samples from the respirometers were taken and analyzed, as described above. To analyze the respiratory activity of the seeds under different levels of water availability, the propagules were sown in rolls of germination paper, previously moistened with water or with solutions of polyethylene glycol 6000 (PEG) with water potentials of -2 and -8 MPa (Villela et al., 1991). Seeds in rolls with water or PEG solutions were incubated in respirometers at 8 °C for 360 hours. Seeking to simulate the conditions normally used for storing these seeds, seeds that were not placed in paper rolls, remaining loose inside the respirometers, were also incubated under the same conditions as the previous treatments. After this period, air samples were taken from the respirometers and analyzed as described above.

Storage of seeds under different water and thermal conditions

In a first experiment, seeds were stored in two packages (perforated plastic bags and glass jars with airtight closure) and at two temperatures (8 and 25 °C) for 60 and 120 days. At the end of each period, samples were taken to evaluate germination, as described above.

In a second experiment, seeds were stored in perforated plastic bags, loose or in a roll of germination paper moistened with PEG solutions of different water potentials (-1, -2, -3, -7, -8 and -9 MPa), for 60, 180, 360 and 540 days. At the end of each storage period, samples were taken to evaluate water content, water potential and germination, as described above.

The experiments were carried out in three replications of 15 seeds each. For the seed storage experiments, a 2 x 2 factorial scheme (temperature x package) and single factor (seven storage environments) were used, within each storage period, as described below.

Water content and dry mass content were determined gravimetrically by the oven method at 103 °C for 17 hours, and the results were presented in percentage, on a wet basis, for water content and in $g.seed^{-1}$ for dry mass (Brasil, 2009). The water potential was measured in a WP4 potentiometer (Decagon Devices, Pullman, USA), which is based on the dew-point temperature of the air in equilibrium with the sample examined, following the methodology described by Delgado and Barbedo (2012), with results presented in MPa.

The germination test was performed in Gerbox plastic boxes with 200 mL of fine vermiculite saturated with distilled water. The Gerbox boxes were kept in a germination room with 90% relative humidity and constant temperature of 25 °C, under continuous light. The evaluation was performed 90 days after setting up the test, recording seeds with primary root protrusion of at least 5 mm in length (germination) and seeds that developed normal seedlings, according to Delgado and Barbedo (2007).

Seed respiratory rates were evaluated by analyzing the O_2 consumption and CO_2 release, incubating the seeds in respirometers and analyzing the gases in a Model 6600 gas analyzer (Illinois Instruments, Inc., Johnsburg, USA), with results expressed in $\mu mol.gDM^{-1}.h^{-1}$. The respiratory quotient (RQ) was also calculated by dividing the amount of CO_2 produced by the amount of O_2 consumed (Lamarca and Barbedo, 2012; Cécel and Barbedo, 2021).

The data were subjected to analysis of variance (5%), and the means were compared by Tukey test (5%) (Santana and Ranal, 2004).

RESULTS AND DISCUSSION

Eugenia brasiliensis seeds showed water content of 50.8%, water potential of -0.81 MPa, dry mass of 0.313 $g.seed^{-1}$ and germination of 100%, with 77% of seeds forming normal seedlings, values that are normally found for seeds of this species (Kohama et al., 2006; Delgado and Barbedo, 2007; Lamarca and Barbedo, 2014; Amador and Barbedo, 2015).

The decrease of seed incubation temperature reduced respiratory rates, as can be verified by the values of O_2 consumption and CO_2 release (Table 1). In addition, the reduction of temperature up to 8 °C increased the RQ to a value closer to 1.0, suggesting lower rates of oxidative processes (Lamarca and Barbedo, 2012). These values are in line with the better conservation of seeds stored at low temperatures (Bonjovani and Barbedo, 2020), because they point to lower metabolic rates. Surprisingly, however, the reduction of water availability, which supposedly would also cause reduction of seed metabolism (Farrant and Hilhorst, 2021), did not reduce respiratory rates, as verified by the values of CO_2 release (Table 2). Only when seeds were placed under extreme water deficit (-8 MPa) was there any reduction in O_2 consumption, but without equivalent reduction in CO_2 release, which promoted RQ closer to 1.0.

The storage of seeds for up to 120 days was efficient at the lowest temperature (8 °C), both using glass jars and plastic bags, making it possible to maintain their viability and their capacity to produce normal seedlings (Table 3). At the highest temperature (25 °C), the seeds kept in glass jars completely lost their viability, while those kept in plastic bags germinated inside the package itself (Table 3). When comparing these results with those presented in Table 1, it was observed that the more intense respiration of seeds incubated at 25 °C resulted in depletion of their reserves, or in the formation of an environment with concentration of potentially lethal gases to the seeds. As demonstrated by Cécel and Barbedo (2021), the increase in CO_2 to a concentration of 3.7%, with equivalent reduction of O_2 concentration, substantially reduces the respiratory rates of *E. brasiliensis* seeds. This may be due to either some induction of inhibition of respiration or death of the seeds.

Incubation in PEG solutions for up to 540 days resulted in different changes in water content (Table 4), water potential (Table 5) and conservation of viability of the seeds (Table 6), as well as in their capacity to produce normal seedlings (Table 7). This incubation was efficient for the conservation of *E. brasiliensis* seeds only when the water potential of the solution was -1 and -2 MPa (Tables 6 and 7). Interestingly, these were the potentials that caused minimal alteration in the water content of the seeds (around 52%; Table 4), which maintained their water potential around -2 MPa (Table 5).

Table 1. O_2 consumption, CO_2 release and respiratory quotient (RQ) of *Eugenia brasiliensis* seeds, incubated at four temperatures. Means followed by the same letter in the column do not differ from each other (Tukey, 5%).

Temperature (°C)	O_2 consumption ($\mu mol.gDM^{-1}.h^{-1}$)	CO_2 release ($\mu mol.gDM^{-1}.h^{-1}$)	RQ
35	7.07 a	3.84 a	0.55 b
25	3.60 b	2.12 b	0.59 b
15	0.71 c	0.40 c	0.61 b
8	0.29 d	0.23 d	0.83 a

Table 2. O_2 consumption, CO_2 release and respiratory quotient (RQ) of *Eugenia brasiliensis* seeds, incubated at 8 °C in airtight glass jars, loose or in paper rolls with water or PEG solution at -2 and -8 MPa. Means followed by the same letter in the column do not differ from each other (Tukey, 5%).

Incubation	O_2 consumption ($\mu mol.gDM^{-1}.h^{-1}$)	CO_2 release ($\mu mol.gDM^{-1}.h^{-1}$)	RQ
Loose	0.25 a	0.16 a	0.71 b
Water	0.23 a	0.14 a	0.63 b
PEG -2 MPa	0.29 a	0.17 a	0.64 b
PEG -8 MPa	0.16 b	0.15 a	0.98 a

Table 3. Germination and formation of normal seedlings (Seedlings) of *Eugenia brasiliensis* seeds stored for 60 and 120 days at two temperatures (25 and 8 °C) and in perforated plastic bags (Plastic) or airtight glass jars (Glass). Means followed by the same letter (lowercase comparing packages, uppercase comparing temperatures), within each storage period, do not differ from each other (Tukey, 5%).

Storage (days)	Temperature (°C)	Package	Germination (%)	Seedlings (%)
60	25	Plastic	100 a A	93 a A
		Glass	0 b B	0 b B
	8	Plastic	90 a A	87 a A
		Glass	87 a A	77 a A
120	25	Plastic	100 a A	70 a B
		Glass	0 b B	0 b B
	8	Plastic	93 a A	93 a A
		Glass	87 a A	77 b A

Table 4. Water content (%) of *Eugenia brasiliensis* seeds without storage (0 days) or stored at 8 °C for 60, 180, 360 and 540 days in plastic bags, loose (Control) or in paper rolls with PEG solution of different water potentials (-1 to -9 MPa). Means followed by the same letter in the columns do not differ from each other (Tukey, 5%).

Water potential of the solution	Storage periods (days)				
	Initial	60	180	360	540
Control	50.8	53.2 a	57.3 a	62.9 a	63.8 a
-1 MPa		50.4 ab	52.1 b	52.4 b	53.0 b
-2 MPa		49.6 b	50.1 b	51.1 b	51.1 b
-3 MPa		48.0 bc	45.7 c	47.4 c	47.1 c
-7 MPa		45.3 c	42.6 d	38.0 e	41.3 d
-8 MPa		45.0 c	40.9 d	42.0 d	42.4 d
-9 MPa		45.2 c	41.6 d	38.5 e	42.0 d

Seeds that remained without incubation in PEG solution (Control), only kept in perforated plastic bags, as traditionally stored, had no change in water potential (Table 5), but increased their water content (Table 4) and did not maintain viability for more than 180 days (Table 6). Seeds kept in solutions with -1 and -2 MPa, in turn, reached 540 days of storage with germination greater than 80% (Table 6). However, even these already showed signs of deterioration, as identified by the reduction in the capacity to produce normal seedlings (Table 7).

It is interesting to note that seeds incubated at -3 MPa had a slight reduction in water content, reaching a value of 47% (Table 4), which was sufficient to accelerate their deterioration (Table 6). Therefore, the water potential of -3 MPa may be very low for the seeds to be able to control the movement of water and keep the water content above a minimum value. This is also verified at the much lower water potentials, from -7 to -9 MPa, which caused a great

Table 5. Water potential (MPa) of *Eugenia brasiliensis* seeds without storage (0 days) or stored at 8 °C for 60, 180, 360 and 540 days in plastic bags, loose (Control) or in paper rolls with PEG solution of different water potentials (-1 to -9 MPa). Means followed by the same letter in the columns do not differ from each other (Tukey, 5%).

Water potential of the solution	Storage periods (days)				
	Initial	60	180	360	540
Control	-0.81	-0.90 c	-1.05 f	-1.01 e	-0.80 d
-1 MPa		-1.32 bc	-0.89 f	-1.74 d	-1.55 c
-2 MPa		-0.96 c	-2.19 e	-2.46 c	-2.16 bc
-3 MPa		-1.74 ab	-3.21 d	-2.97 c	-2.70 b
-7 MPa		-2.25 a	-4.84 c	-4.12 b	-4.07 a
-8 MPa		-1.76 ab	-5.10 b	-4.23 ab	-4.04 a
-9 MPa		-2.15 a	-6.30 a	-4.89 a	-4.36 a

Table 6. Germination (%) of *Eugenia brasiliensis* seeds without storage (0 days) or stored at 8 °C for 60, 180, 360 and 540 days in plastic bags, loose (Control) or in paper rolls with PEG solution of different water potentials (-1 to -9 MPa). Means followed by the same letter in the columns do not differ from each other (Tukey, 5%).

Water potential of the solution	Storage periods (days)				
	Initial	60	180	360	540
Control	100	100 a	30 c	0 c	0 c
-1 MPa		90 a	97 a	90 a	87 a
-2 MPa		97 a	100 a	90 a	80 a
-3 MPa		100 a	80 b	63 b	23 b
-7 MPa		40 b	0 d	0 c	0 c
-8 MPa		53 b	3 d	0 c	0 c
-9 MPa		13 c	0 d	0 c	0 c

Table 7. Production of normal seedlings (%) of *Eugenia brasiliensis* seeds without storage (0 days) or stored at 8 °C for 60, 180, 360 and 540 days in plastic bags, loose (Control) or in paper rolls with PEG solution of different water potentials (-1 to -9 MPa). Means followed by the same letter in the columns do not differ from each other (Tukey, 5%).

Water potential of the solution	Storage periods (days)				
	Initial	60	180	360	540
Control	77	77 a	23 b	0 b	0 a
-1 MPa		70 a	70 a	53 a	13 a
-2 MPa		70 a	73 a	50 a	10 a
-3 MPa		77 a	60 a	30 a	7 a
-7 MPa		17 b	0 b	0 b	0 a
-8 MPa		30 b	0 b	0 b	0 a
-9 MPa		7 b	0 b	0 b	0 a

reduction in the water content of the seeds at 60 days of storage (Table 4) and, consequently, in their viability (Table 6). At these potentials, curiously, precisely when the seeds completely lost their viability, at 180 days, their water potential showed a great variation (Table 5).

The data suggest an ideal range of hydration of these seeds, close to 52%, below or above which the rate of deterioration is more accelerated. Remaining within this range does not necessarily mean lower metabolic rates compared to the more hydrated ones, since the respiration of seeds within this range does not differ from that observed in more hydrated seeds (Table 2). Likewise, it does not also mean more active metabolism than that of seeds kept with lower water content, as observed by the CO_2 release values of these seeds and those maintained at -8 MPa (Table 2). It is possible, therefore, that the maintenance of an optimal hydration range of these seeds involves other factors besides the metabolic rate.

Incubation in PEG solutions seems to effectively prolong the storage of recalcitrant seeds (Andréo et al., 2006; Pereira et al., 2020; Pelissari et al., 2022) but, as demonstrated in this study, not necessarily for reducing the metabolic rates. Recalcitrant seeds remain metabolically active during storage (Barbedo, 2018) and, therefore, it is possible that they are germinating, and not exactly being stored. The reduction of storage temperature can thus reduce the germination speed, as demonstrated for *Inga vera* seeds (Bonjovani and Barbedo, 2019). Considering that incubation in PEG solution did not alter respiratory rates, the increase in storage time may also result from a reduction in germination speed, but without reduction in the level of metabolism. This could be, for example, the same observed in phase II of the germination of orthodox seeds, in which there is no substantial change in water content, there is still no cell division and growth, but there is intense restructuring metabolism of cellular components (Marcos-Filho, 2015). It is important to point out that, at water potential equal to or lower than -1 MPa, phase II of germination becomes relatively more extensive and phase III (primary root growth) may not occur (Castro et al., 2004). Andréo et al. (2006) obtained similar results, as embryos of recalcitrant seeds of *Inga vera* kept under more negative water potential (-1.6 MPa and -2.4 MPa) remained with water content close to 63%-69% after 288 hours, not initiating the growth of the primary root.

Finally, the results of the present study demonstrated that the maintenance of *E. brasiliensis* seeds under water control, with potential of -1 and -2 MPa, allowed maintaining their water content close to 52%, expanding the capacity for conserving the viability in storage of these seeds. They also demonstrated that the expansion of this conservation, unlike what occurs with the reduction of temperature, is not necessarily associated with the reduction of metabolic rates.

CONCLUSIONS

Incubation of *Eugenia brasiliensis* seeds in PEG solutions with water potential between -1 and -2 MPa and water content around 52% increases their storage capacity up to 540 days.

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