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ARTICLE

Regeneration of roots and shoots as a propagation strategy in *Eugenia candolleana* DC. (Myrtaceae) seeds

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ABSTRACT: Seeds of the genus *Eugenia* have high regenerative capacity, producing roots and shoots from seeds with reduced cotyledon matter. In addition, seeds of this genus regenerate new roots and shoots when the first roots and shoots are eliminated, and this characteristic is maintained even when the seed reserves are reduced by up to half. The aim of this study was to analyze the limits of new root and shoot regenerative capacity in whole and fractionated *Eugenia candolleana* seeds at different maturity stages. The regenerative capacity of seeds stored for six months was also evaluated. The seeds were sown; and when the first roots and shoots were produced, they were eliminated, simulating herbivory conditions. The results showed that the seed not only has high regenerative capacity at different maturity stages, but also that it maintains root regeneration when the seed is reduced by half, even after the storage period. Such information may indicate that seeds of the *Eugenia* genus can await the next crop season by regenerating several times as a way to withstand or tolerate predation. This ability can be understood as a propagation strategy of the species.

Index terms: fractionation, maturation, recalcitrant seed.

RESUMO: Sementes do gênero *Eugenia* apresentam alta capacidade regenerativa, produzindo raízes e partes aéreas a partir de sementes que tiveram redução da massa cotiledonar. Também, foi observado, para sementes deste gênero, a regeneração de novas raízes e partes aéreas, quando as primeiras raízes e partes aéreas são eliminadas, ainda, tal característica se mantém quando a semente teve suas reservas reduzidas pela metade. Este estudo teve como objetivo analisar os limites das regenerações de novas raízes e partes aéreas em sementes inteiras e fracionadas de *Eugenia candolleana,* em diferentes estádios de maturação. Avaliou-se também, a capacidade regenerativa em sementes armazenadas por seis meses. As sementes foram semeadas e quando emitiram as primeiras raízes e partes aéreas, estas foram eliminadas, simulando uma condição de herbivoria. Os resultados mostraram que, além de apresentar alta capacidade regenerativa em diferentes estádios de maturação, a semente reduzida pela metade, a regeneração de raízes se manteve, inclusive após o período de armazenamento, tais informações podem indicar que sementes do gênero *Eugenia* são capazes de aguardar a próxima safra regenerando diversas vezes, como maneira de suportar ou tolerar a predação. Essa habilidade pode ser entendida como estratégia de propagação da espécie.

Termos para indexação: fracionamento, maturação, semente recalcitrante.

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INTRODUCTION

The genus *Eugenia L.* (Myrtaceae Juss.) includes forest and fruit-bearing species with potentially high economic value, as they produce fleshy fruit, some sweet, suitable for consumption in their natural form or for processing (Amador and Barbedo, 2015; Lamarca et al., 2020; Amorim et al., 2020). The fruit of this species is dispersed by animals (zoochory), mostly by birds and mammals (especially monkeys), but also by reptiles and fish (Castro and Galetti, 2004; Christianini and Martins, 2015).

Field observations show that the fruit of *Eugenia* spp. is picked up by birds that feed on it or its seeds; in this operation, the seeds may be naturally split or fractionated. In addition to the action of birds, insect larvae were also observed as feeding on seeds.

One of the *Eugenia* species, *E. candolleana* D.C. (rainforest plum), has not yet been widely studied. Its seeds do not tolerate desiccation and, consequently, just as occurs in seeds of other species of *Eugenia*, they cannot be stored for long periods of time (Barbedo, 2018; Amorim et al., 2020). In addition, as for other species of the genus, the seeds of *E. candolleana* are able to regenerate new roots and shoots after the first germinations and the first developments have been eliminated (Alonso et al., 2019; Alonso and Barbedo, 2020). This capacity for regenerating roots, or even whole seedlings, was also observed when the seeds passed through water restriction; they were able to regenerate new roots from preserved moist plant tissue within the seed in *Eugenia brasiliensis* Lam., *E. involucrata* DC.*, E. pyriformis* Cambess.*,* and *E. uniflora* L. (Inocente and Barbedo, 2019).

Eugenia seeds have potential for generating new roots and even whole plants after removal of a large part of their reserves (Prataviera et al., 2015). There is clear interest in understanding the factors involved in this regenerative capacity that allow the formation of new seedlings, even when the seed is fractionated (Teixeira and Barbedo 2012; Prataviera et al. 2015; Calvi et al. 2017; Alonso et al. 2019). After seed fractionation, regenerative capacity was also observed in *Garcinia L.* of the Clusiaceae Lindl. family (Joshi et al., 2006; Asomaning et al., 2011).

In *Eugenia* species, seed fractionation can result in values higher than 100% germination, considering the number of roots obtained in the fractions in relation to the number of seeds placed to germinate, because two or more fractions of the same seed can produce roots. In other words, a single seed can produce more than one set of roots. However, it is noteworthy that germination will not likely exceed 100% when these seeds are not fractionated, suggesting some internal control mechanism in germination, impeding the reserves from being used up in various simultaneous germinations (Amador and Barbedo, 2015). The capacity for regeneration of structures and of internal control of simultaneous germinations and the quantity of reserves superior to that which is necessary for formation of a seedling may be related to some natural selection pressure that ensured establishment of the seedling even when the environment was not favorable to establishment in the first germinations, such as in cases of predation or in a period of water deficit. Studies indicate that for species of the *Eugenia* genus, even when a seed is nearly totally consumed by an insect, it still maintains reserves sufficient for producing a seedling, preserving its capacity for propagating the species by means of successive germinations (Teixeira and Barbedo, 2012; Alonso et al., 2019).

Furthermore, it has been shown that for some species of *Eugenia*, the regenerative capacity of *Eugenia* seeds is present over a long period, from still immature seeds up to those that have already begun germination (Teixeira and Barbedo, 2012; Delgado and Barbedo, 2020). However, the limits of regeneration of new structures and the time of each regeneration, which could increase the continuity and conservation of these seeds in the environment, are still little known. Therefore, the aim of this study was to analyze the limits of regeneration of new roots and shoots in whole and fractionated seeds of *Eugenia candolleana* at different maturity stages and after storage.

MATERIAL AND METHODS

Obtaining plant material – In the period from January to May 2019, *E. candolleana* fruit was collected, coming from mother plants in the São Paulo Botanical Gardens (Jardim Botânico de São Paulo - JB, Figure 1A) in the municipal region of São Paulo (23°38'27.38'' S and 46°37'34.57'' W) and on a private property in the city of Rio Claro (RC, Figure 1B) (22°25'09,5" S and 47°33'37,1" W), both in the state of São Paulo. After the collections, the fruit was taken to the seed laboratory for classification, and then the seeds were extracted.

The JB fruit was separated into five maturity stages, according to epicarp color. Totally unripe fruit, which constituted stage 1 (E I), was discarded for being in very small number. Fruit that had 75% of the epicarp of green color were considered as belonging to stage 2 (E II); those that had 75% of the epicarp of red color, stage 3 (E III); totally red fruit, stage 4 (E IV); and fruit with 75% to 100% purple color, stage 5 (E V) (Figure 1A).

The RC fruit was collected when fully ripe (Figure 1B); and the seeds, after extraction, were stored in perforated polyethylene bags at low temperature (10 °C ± 2 °C) for a period of six months.

Seeds were extracted manually, removing the pulp residues with the aid of a sieve and running water and removing excess water with a paper towel. After that, the seeds were stored in perforated polyethylene bags under low temperature (10 °C ± 2 °C) up to the time of setting up the experiments, not exceeding the period of seven days for JB.

Physical and physiological evaluations – moisture content (in %, wet basis) and dry matter (mg. seed^{-1}) of the seeds were determined by the laboratory oven method at 105 °C for 24 hours (Brasil, 2009), with four replications of ten seeds. The water potential of the seeds was obtained in a WP4 potentiometer (Decagon Devices, Inc., Pullman) based on dew point temperature.

The germination tests, with daily evaluations, were performed in a germination room with temperature controlled at 25 °C under continuous white light. The seeds were placed in 11 × 11 × 3.5 cm plastic boxes (gerbox) containing a 2-cm layer of vermiculite as substrate, previously moistened with water up to saturation limit. A seed was considered as germinated when it showed emergence of 0.5 cm of a primary root. A normal seedling was considered one with normal development and without defects (Inocente and Barbedo, 2021).

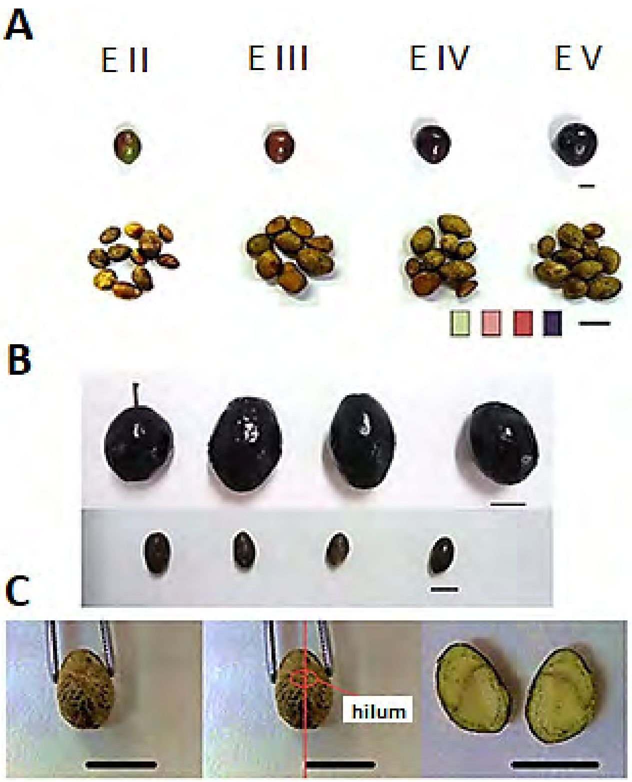

Figure 1. *E. candolleana* fruit and seeds collected in JB, separated into four maturity stages (A); *E. candolleana* fruit and seeds collected in RC when fully mature and stored for six months (B); and the whole seed, indicating the position of the hilum and of fractionation in the longitudinal direction, and the fractionated seed in two parts (C). Scale – 1 cm

Limit of regenerability of roots and shoots in whole seeds and fractionated seeds at different stages of maturity – whole seeds and fractionated seeds, from E II to E V, were placed to germinate in moistened vermiculite, as previously described. For fractionation, a surgical scalpel was used to cut them in half longitudinally, with the cut passing through the hilum, evaluating only half of the seed, as a way of reducing cotyledon matter (Figure 1C).

When the primary roots coming from the whole seeds or fractionated seeds reached 1.5 cm length, they were eliminated with the aid of a surgical scalpel. After that, the seeds were placed in the plastic germination boxes to check their capacity for regenerating new roots. New roots that were generated from those seeds were also removed upon reaching 1.5 cm length, and the seeds were once more placed in the germination box. This process was repeated until the seeds produced the seventh root (that is, until the sixth regenerated root). Thus, the following treatments were performed: SE = without elimination of roots, ER1 = elimination of the root of the first germination, ER2 = elimination of the first regenerated root, ER3 = elimination of the second regenerated root, ER4 = elimination of the third regenerated root, ER5 = elimination of the fourth regenerated root, ER6 = elimination of the fifth regenerated root, and ER7 = elimination of the sixth regenerated root (Figure 2).

The same procedure was carried out to evaluate regeneration of whole seedlings, eliminating the shoots and roots when the seedlings reached 2.5 cm total length. These treatments were represented by SE, EP1, EP2, and EP3 (Figure 2).

Limit of regenerability of roots and shoots in stored seeds – whole seeds of *E. candolleana* coming from fully ripe fruit exhibiting 92% germination and 77% production of normal seedlings were kept in storage in semi-perforated polyethylene bags under low temperatures (10 °C ± 2 °C) for six months, until setting up the regenerability experiments. After that period, they were placed to germinate as in the previous experiment and also underwent the same treatments from SE to ER10 and from SE to EP3 (Figure 2).

In all experiments, a completely randomized experimental design was used, with four replications of twenty-five seeds for all the tests. Analysis of variance was used on the results obtained at the level of 5%, and the mean values were compared by Tukey's test ($P < 0.05$).

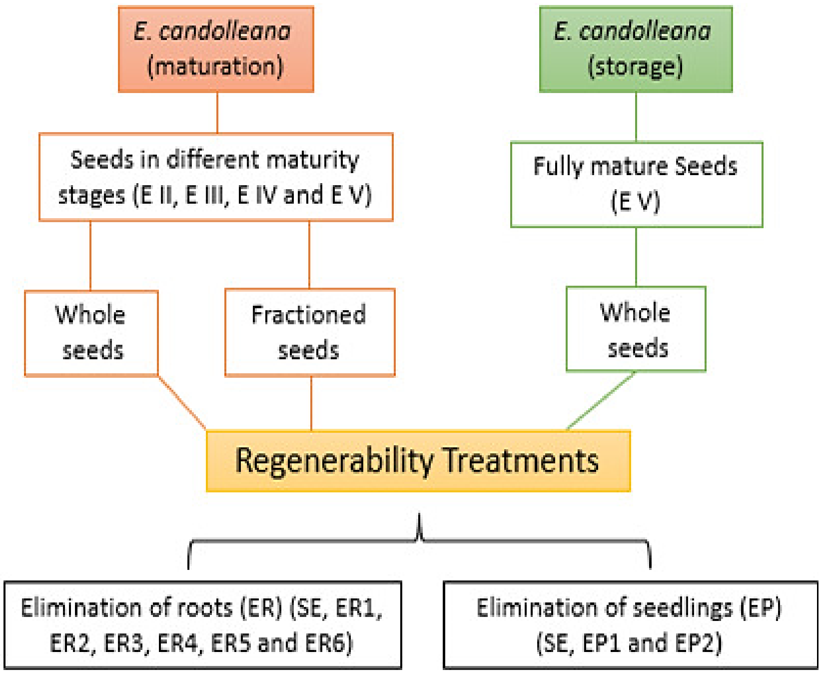

Figure 2. Flowchart of the sequence of regenerability treatments applied to *E. candolleana* seeds from two different origins.

RESULTS AND DISCUSSION

Fruit and seed diameter, as well as seed moisture content and dry matter content, did not show differences among the maturity stages studied (Figure 3). Seed fresh matter was slightly greater in E III. These data suggest that there is little difference in the degree of maturity of the seeds between those coming from more immature fruit (E II) and those from the more mature fruit (E V), as already observed in another species of the genus (Lamarca et al., 2013). The data of germination and of normal seedling formation (Figure 4, SE) reinforce this small difference between the maturity stages. Only water potential showed a substantial difference, especially among the seeds of more immature fruit in relation to the others (Figure 3D). The water potential of the immature fruit was quite less, perhaps for being at the beginning of formation, receiving a larger amount of assimilates from the mother plant, making the water present in the seed contain a greater concentration of solutes and, therefore, showing reduction in the fluidity of the aqueous medium (Marcos-Filho, 2015; Alonso et al., 2019).

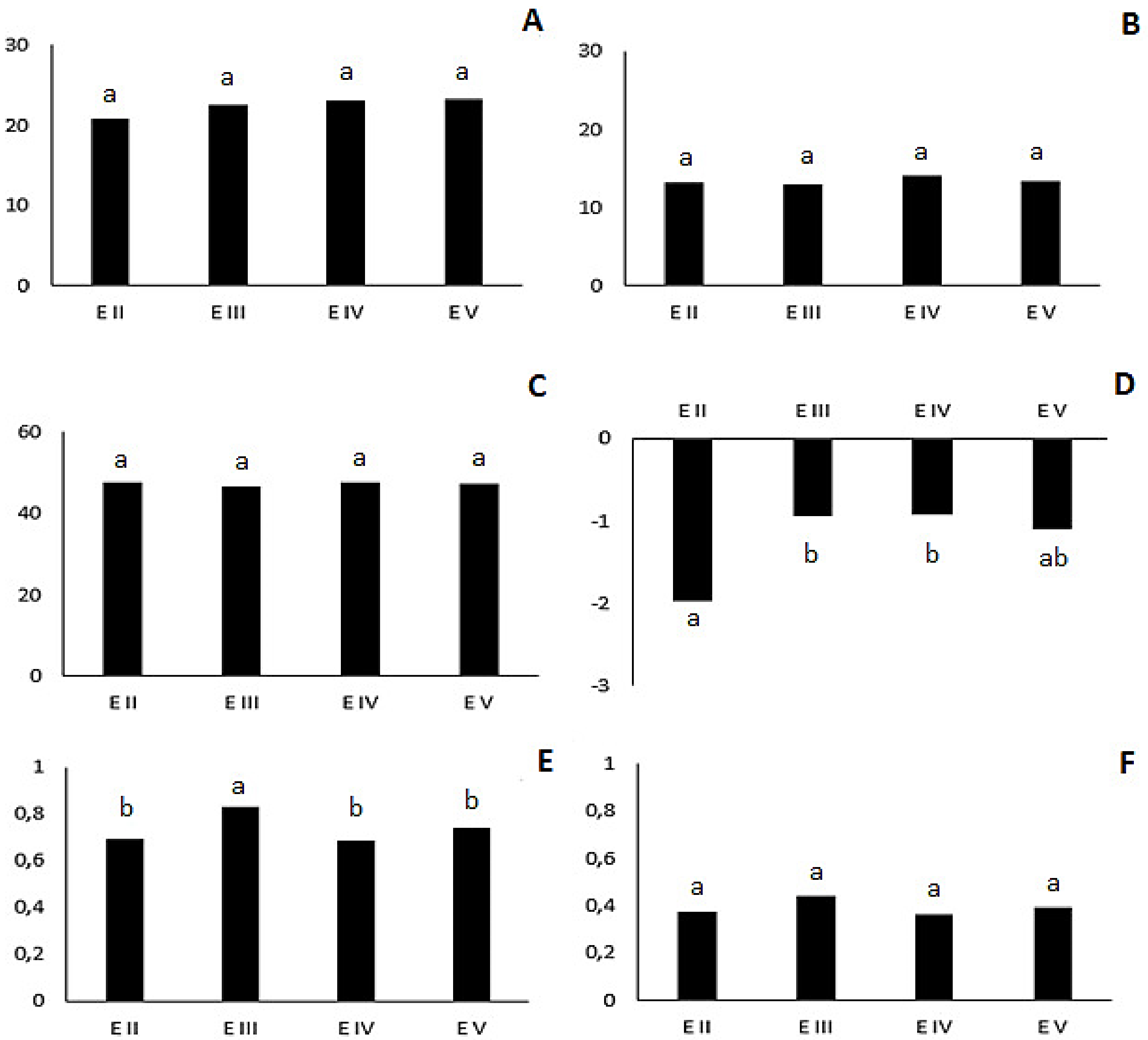

Figure 3. Diameter (in mm) of the fruit (A) and of the seeds (B), moisture content of the seeds (C, in %), water potential of the seeds (D, in MPa), and fresh matter (E) and dry matter (F) of the seeds (in g. seed^{-1}) of *E. candolleana* DC., according to the different physiological maturity stages (E II to E V). Columns with the same letter do not differ from each other (Tukey, 5%).

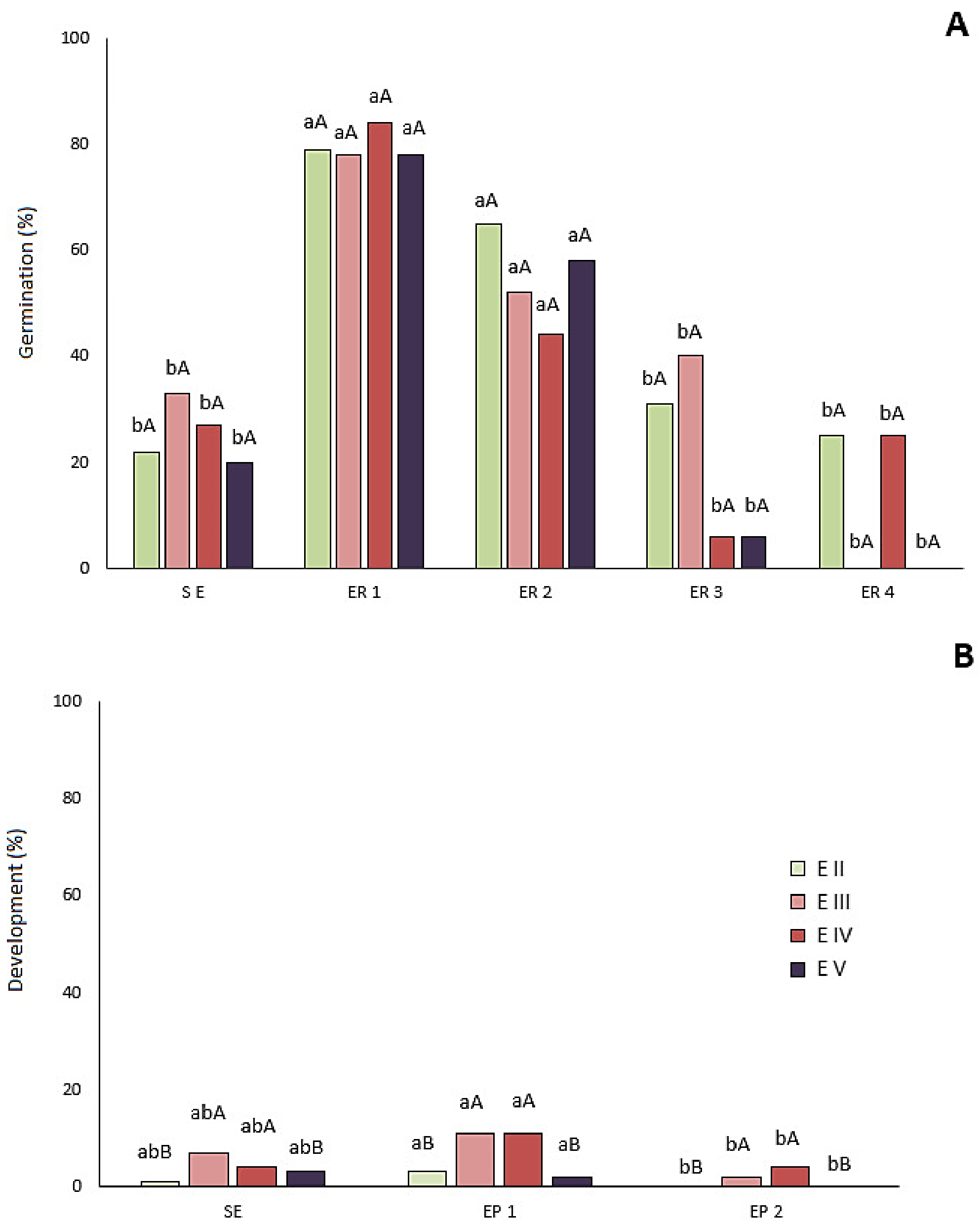

Figure 4. Regeneration of roots in whole seeds of *E. candolleana* at different maturity stages described as E II, E III, E IV, and E V; and SE, ER1, ER2, ER3, and ER4 being the root regenerability treatments (SE = without eliminations, ER1 = elimination of the first root, ER2 = elimination of the second root, ER3 = elimination of the third root, and ER4 = elimination of the fourth root) (A); regeneration of normal seedlings in whole seeds of *E. candolleana* at different maturity stages (E II, E III, E IV, and E V) and seedling regenerability treatments (SE, EP1, and EP2). SE = without eliminations, EP1 = elimination of the first seedling, and EP2 = elimination of the second seedling (B). Lowercase letters compare the eliminations and uppercase letters compare the maturity stages.

In whole seeds, the regeneration of roots occurred up to removal of the third root in all maturity stages and up to the fourth root in stages II and IV (Figure 4A), but the regeneration of seedlings in whole seeds was less (Figure 4B). The regenerative capacity of new roots after elimination of the first roots in seeds of *E. candolleana* may be related to the large quantity of reserves that these seeds have, similar to other seeds of the same genus for which the reserves were quantified (Teixeira and Barbedo, 2012), as well as the capacity of producing new roots from the cells of the cotyledon (Delgado et al., 2022). It may also be associated with evolutionary selection, allowing these seeds to be greatly preyed upon and, nevertheless, continuing to be able to germinate even with low energy reserves.

This characteristic can also be understood as an evolutionary adaptation to the environment in which these species were selected, modifying the characteristics during maturation (Guardia et al., 2018; Barbedo, 2018; Barbedo, 2021). In *Eugenia* species, the large accumulation of reserves and the acquisition of regenerative capacity in the seeds while still quite immature (Teixeira and Barbedo, 2012) may have allowed the species to perpetuate itself in the environment through successive germinations from the same seed. The big advantage of this strategy is continuity of the species in the environment, even an adverse environment, for long periods. In this respect, it is noteworthy that at each new formation of roots and/or shoots, when some root/shoot is lost, there is an increase in the time necessary for this new formation (Figure 5). Therefore, when there is loss of a germinated root or formed seedling, there is not only the possibility of a new root/shoot being produced, but also the time for this new production will be greater than the time for the previous production. That prolongs even more the period in which the seed can remain in the environment

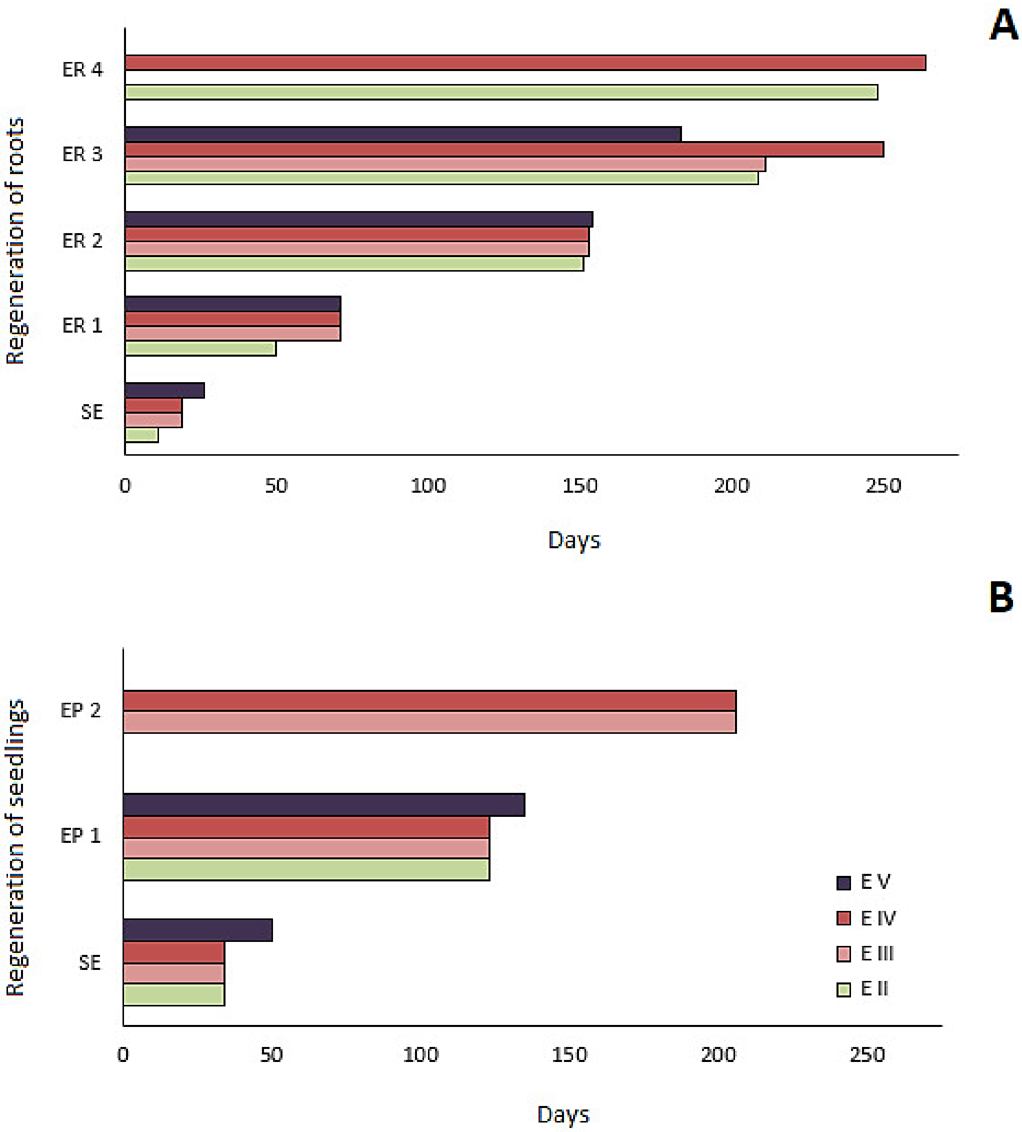

Figure 5. Time (days) of regeneration of new roots (A) and of new seedlings (B) in whole seeds of *E. candolleana* at different maturity stages (E II, E III, E IV, and E V). In A: SE = without elimination of roots, ER1 = elimination of the first root, ER2 = elimination of the second root, ER3 = elimination of the third root, and ER4 = elimination of the fourth root; and in B: SE = without seedling elimination, EP1 = elimination of the first seedling, and EP2 = elimination of the second seedling.

in conditions of propagating the species. Since these seeds are sensitive to desiccation (recalcitrant), they could not create seed banks in the soil, as occurs in many species with orthodox seeds, because they would have to germinate immediately or would deteriorate rapidly (Barbedo, 2018). However, the capacity of producing successive germinations, associated with the mechanisms of tolerance to water deficits in the environment (Inocente and Barbedo, 2019) and of regenerating roots and shoots under water deficit (Inocente and Barbedo, 2021), produces an effect similar to the seed bank in the soil, perhaps until the new crop of seeds is dispersed.

When a fraction of the seed is eliminated, that is, when its nutritional reserves are reduced by half, simulating an herbivorous attack, as if an insect or bird had consumed half the seed reserves, even then the seeds were able to regenerate new roots (Figure 6) and new seedlings (Figure 7). The regeneration occurred in fractionated seeds;

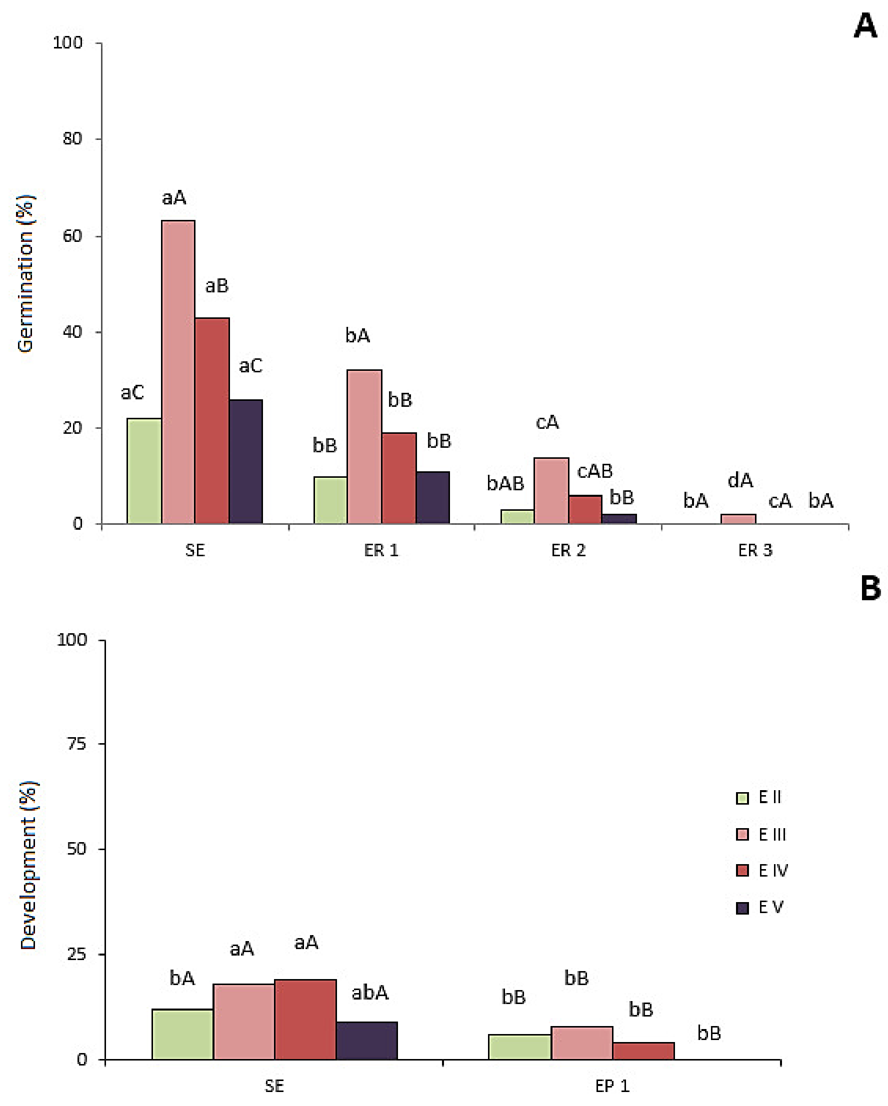

Figure 6. Regeneration of roots in fractionated seeds of *E. candolleana* at different maturity stages described as E II, E III, E IV, and E V. In A: percentages of regenerability of the roots in the treatments SE, ER1, ER2, and ER3, where SE = without eliminations, ER1 = elimination of the first root, ER2 = elimination of the second root, and ER3 = elimination of the third root; and in B: percentages of seedling development in fractionated seeds of *E. candolleana* in the treatments SE and EP1, where SE = without eliminations, and EP1 = elimination of the first seedling. Mean values followed by the same letter do not differ from each other (Tukey 5%). Lowercase letters compare the eliminations and uppercase letters compare the maturity stages.

production of a third root was observed in all the maturity stages, and even a fourth root for E III, indicating that these seeds have much more cotyledon matter than necessary for only one germination (Barbedo, 2018; Delgado and Barbedo, 2020). Teixeira and Barbedo (2012) highlighted the importance and the effect that the maturity stage has on the regenerative capacity of *Eugenia* seeds; however, the intensity of the effect depends on the species and the degree of maturity, with mature seeds having greater regenerative capacity than immature ones.

The regeneration time in the half seeds was also lengthened. After the first root elimination (ER1), the second root regenerated in 64, 61, and 60 days for stages II, III, and IV, respectively (Figure 7). Meanwhile, in seedling production, when the first seedling was eliminated, the second regenerated in 180 days for stages II and III and in 144 days for stage IV; and in stage V, a second seedling was not regenerated (Figure 6). These data indicate that *E. candolleana* seeds have high regenerative capacity, producing roots and shoots from seeds with reduction in their cotyledon matter, as found in other species of the genus (Teixeira and Barbedo, 2012; Prataviera et al., 2015; Amorim et al., 2020; Delgado and Barbedo, 2020). They are able to remain for a longer time in the environment (Alonso et al., 2019; Alonso and Barbedo, 2020), allowing survival under unfavorable environmental conditions.

The *E. candolleana* seeds of JB had initial values of germination and normal seedling formation below that normally found for the species (Alonso et al., 2019). That may be the result of unfavorable conditions in the environment during

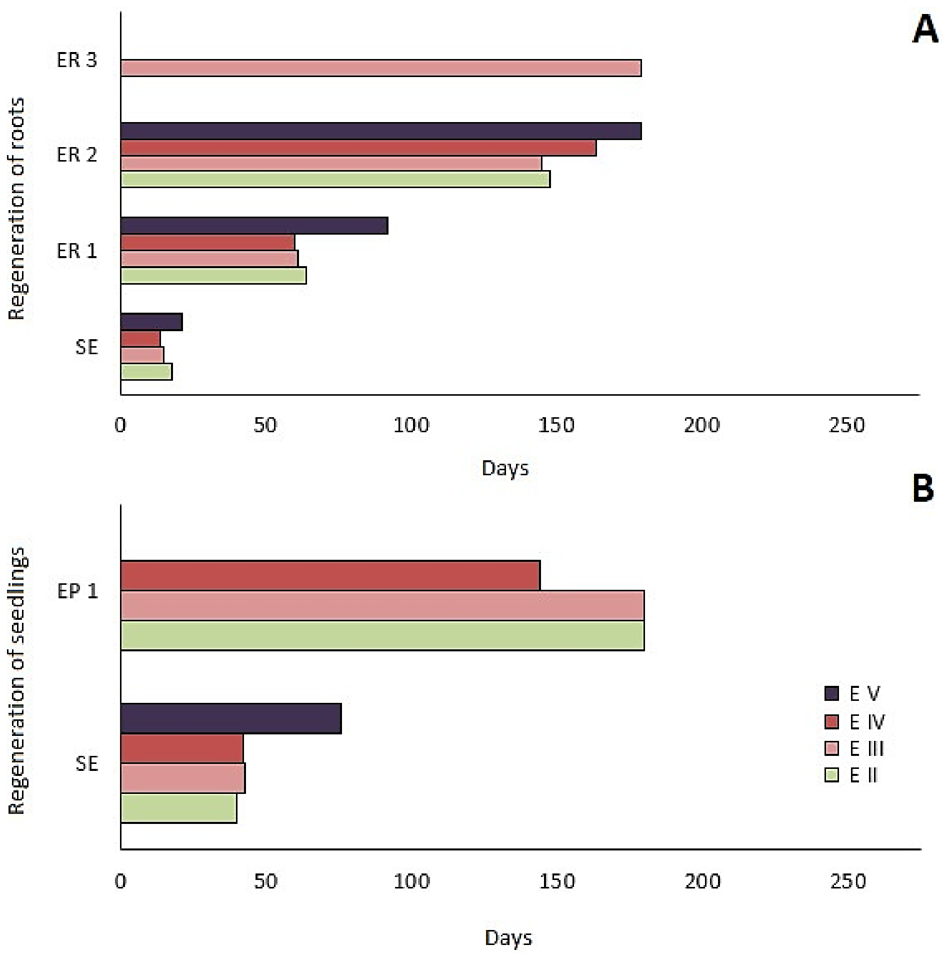

Figure 7. Time (days) of regeneration of roots (A) and seedlings (B) in fractionated seeds of *E. candolleana* at different maturity stages described as E II, E III, E IV, and E V, in which SE = without eliminations, ER1 = elimination of the first root, ER2 = elimination of the second root, and ER3 = elimination of the third root; and SE = without eliminations and EP1 = elimination of the first seedling.

seed formation and maturation (Marcos-Filho, 2015; Guardia et al., 2018). Seeds with higher initial quality were able to bring about even better regeneration of roots and shoots. Seeds from RC, for example, had high values of germination and normal seedling production even after storage (Figure 8). After the eliminations of roots and shoots, these seeds also had high regenerative capacity, producing up to the seventh root (Figure 8A).

Considering the successive germinations, the RC seeds would be able to remain for a longer time in the field, constituting a propagation strategy for this species (Barbedo, 2018; Alonso et al., 2019), as seen in the results of the time necessary for each regeneration (Figure 9). In evaluation of seedling production, regeneration can be observed

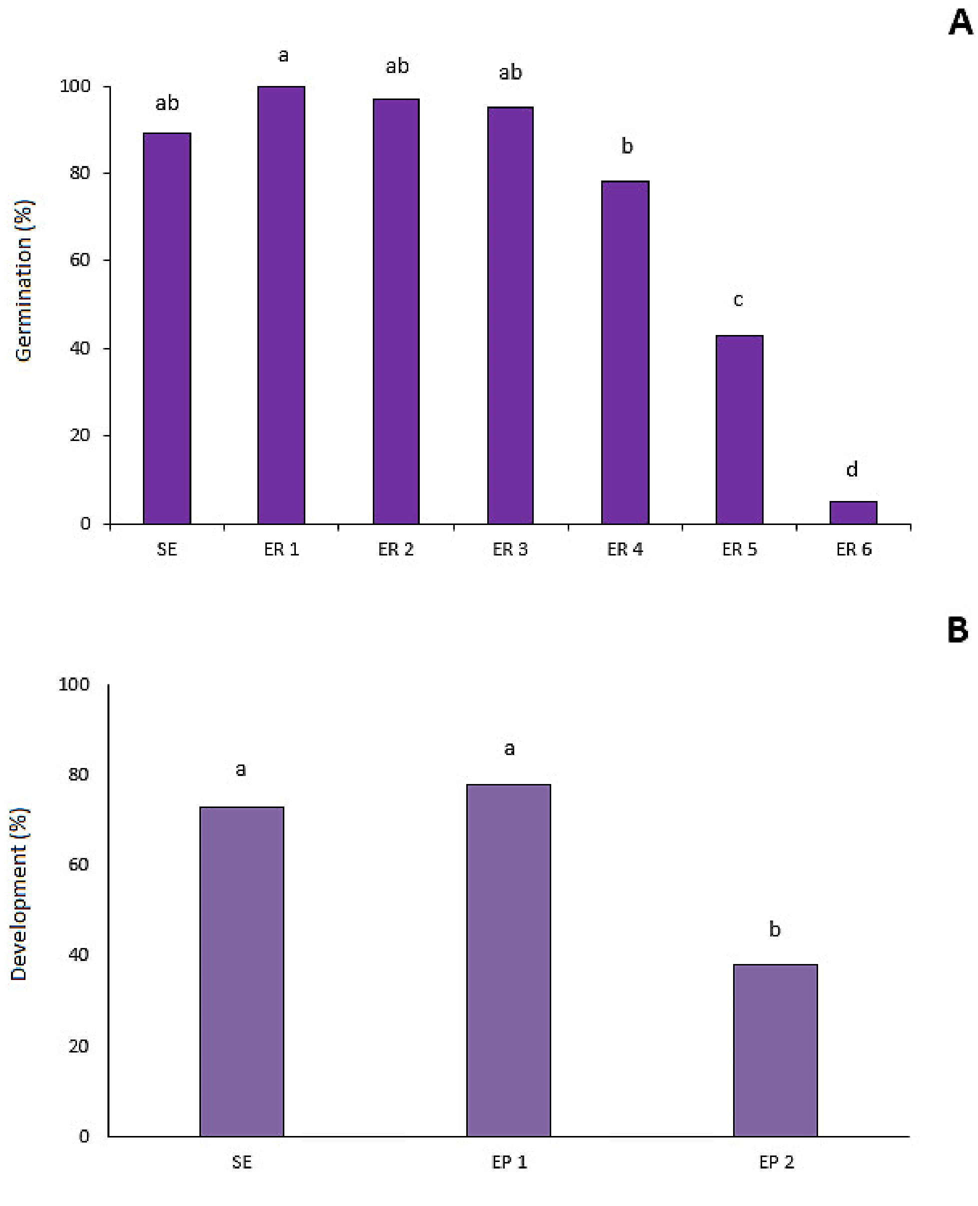

Figure 8. Regeneration of roots in whole and mature seeds of *E. candolleana* of RC, stored under low temperature for six months. SE, ER1, ER2, ER3, ER4, ER5, and ER6 are root regenerability treatments (SE = without eliminations, ER1 = elimination of the first root, ER2 = elimination of the second root, ER3 = elimination of the third root, ER4 = elimination of the fourth root, ER5 = elimination of the fifth root, and ER6 = elimination of the sixth root) (A); and SE, EP1, and EP2 are seedling regenerability treatments (SE = without eliminations, EP1 = elimination of the first seedling, and EP2 = elimination of the second seedling) (B). Mean values followed by the same letter do not differ from each other (Tukey, 5%)

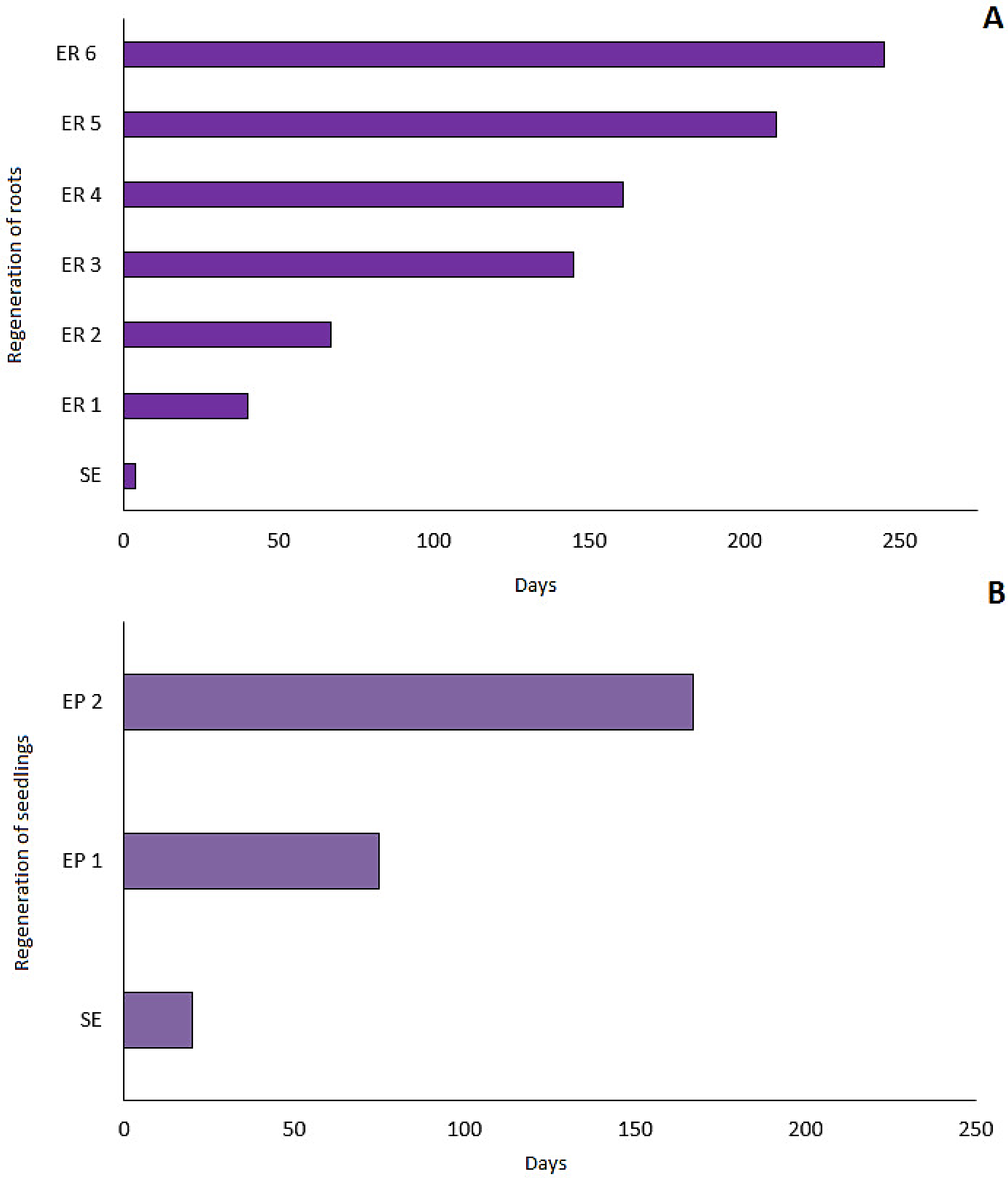

Figure 9. Time (days) of root regeneration (A) and seedling development (B) in whole and mature seeds of *E. candolleana*. SE, ER1, ER2, ER3, ER4, ER5, and ER6 are root regenerability treatments (SE = without eliminations, ER1 = elimination of the first root, ER2 = elimination of the second root, ER3 = elimination of the third root, ER4 = elimination of the fourth root, ER5 = elimination of the fifth root, and ER6 = elimination of the sixth root) (A); and SE, EP1, and EP2 are seedling regenerability treatments (SE = without eliminations, EP1 = elimination of the first seedling, and EP2 = elimination of the second seedling) (B).

only up to the third seedling. And observation of the period of regeneration of new roots showed that the time for each root regeneration increased as each root elimination occurred. Thus, the first root required 4 days to be produced; however, the seventh root was produced in 245 days; that is, 241 days separated the first root from the last regenerated root in this experiment. These seeds passed through around eight months trying to produce a new root (Figure 9); that time, added to the six months of storage, meant a total of 14 months that the seed survived, and it exceeded the period up to the next (annual) crop season (Barbedo, 2018; Alonso and Barbedo, 2020).

In relation to new seedling production, the stored *E. candolleana* seeds also lengthened the time of the regenerations. After the first seedling elimination, the seed needed 75 days to produce the second seedling and 167 days to produce the third, equivalent to nearly 5 and a half months of the seed trying to produce a new seedling (Figure 9). These data also show that even after having been stored for six months, they still maintained the capacity of regenerating new roots successive times.

This study showed that regenerative capacity may have lengthened the time the seeds remained in the soil, regenerating successive times. Thus, they may be able to produce new roots or shoots as roots and shoots are lost through predation (Barbedo 2018; Alonso and Barbedo, 2020).

CONCLUSIONS

The *Eugenia candolleana* seeds showed that they regenerated roots and shoots even with reduction in their cotyledon matter by half, maintaining the capacity to germinate successive times when the first roots produced are lost. They also showed that this capacity occurs even in stored seeds.

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