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Viability and storage of *Myrceugenia euosma* (O. Berg) D. Legrand and *Siphoneugena reitzii* D. Legrand (Myrtaceae) pollen

Viabilidade e armazenamento do pólen de Myrceugenia euosma (O. Berg) D. Legrand e Siphoneugena reitzii D. Legrand (Myrtaceae)

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ABSTRACT

This study evaluated aspects of the pollen viability of the species *Myrceugenia euosma* (O. Berg) D. Legrand and *Siphoneugena reitzii* D. Legrand (Myrtaceae), both native trees with ornamental, medicinal and industrial potential. The study used different concentrations of sucrose to determine pollen viability *in vitro* and through colorimetric tests with specific dyes (Lugol, carmine acetic, and tetrazolium chloride – TTC – 0.1 and 0.05%). The two species also had their pollen stored for 30 and 90 days in freezers, refrigerators, and natural environments to check the best method for preserving their viability. The results showed that the highest average germination of *M. euosma* pollen was in a culture medium containing 30% sucrose. After 30 and 90 days of storage, pollen viability was significantly reduced, having preserved part of its potential only in the freezer. The pollen of *S. reitzii* had a higher average germination rate in a medium with 20% sucrose, but also showed a reduction in viability after 30 and 90 days of storage. The colorimetric tests showed that the pollen of both species responded to the dyes. However, the viability of Lugol and carmine acetic was considered to be overestimated in relation to the *in vitro* germination tests. Tetrazolium at 0.1% proved to be an alternative for checking the viability of *S. reitzii* pollen. It can be concluded that different concentrations of sucrose affect germination, and that storing pollen at a low temperature can preserve some of its viability in the short term.

KEYWORDS: *in vitro* germination; tetrazolium; colorimetric analysis; freezer.

RESUMO

Neste trabalho foram avaliados aspectos da viabilidade polínica das espécies *Myrceugenia euosma* (O. Berg) D. Legrand e *Siphoneugena reitzii* D. Legrand (Myrtaceae), ambas arbóreas nativas com potencial ornamental, medicinal e industrial. No estudo foram utilizadas diferentes concentrações de sacarose para determinar a viabilidade do pólen *in vitro* e por meio de testes colorimétricos com corantes específicos (Lugol, carmim acético e tetrazólio – TTC – 0,1 e 0,05%). As duas espécies tiveram ainda seu pólen armazenado por 30 e 90 dias em freezer, geladeira e ambiente natural para verificar o melhor método para conservar sua viabilidade. Os resultados indicaram que a maior média de germinação do pólen de *M. euosma* foi em meio de cultura com 30% de sacarose. Após 30 e 90 dias de armazenamento, a viabilidade do pólen foi significativamente reduzida, preservando parte de seu potencial somente em freezer. Já o pólen de S. *reitzii* teve maior média de germinação em meio com 20% de sacarose, mas também apresentou redução de viabilidade após 30 e 90 dias de armazenamento. Os testes colorimétricos mostraram que houve resposta do pólen, de ambas as espécies, aos corantes, mas a viabilidade acessada com Lugol e carmim acético foi considerada superestimada em relação aos testes de germinação *in vitro*. O tetrazólio a 0,1% se apresentou como uma alternativa para verificar a viabilidade do pólen de *S. reitzii*. Conclui-se que diferentes concentrações de sacarose afetam a germinação e que o armazenamento do pólen em baixa temperatura pode conservar parte da viabilidade a curto prazo.

PALAVRAS-CHAVE: germinação *in vitro;* tetrazólio; testes colorimétricos; freezer.

INTRODUCTION

The species *Myrceugenia euosma* (O. Berg) D. Legrand and *Siphoneugena reitzii* D. Legrand are Myrtaceae, with scarce information on their reproductive behavior. *Myrceugenia euosma* (Subtribe Myrciinae) is an early secondary, semi-deciduous and heliophytic tree that occurs in different Brazilian forest formations (VIEIRA & MEIRELES 2023). It is known for its ornamental and medicinal potential, and for restoring degraded areas (GOMES et al. 2016). *Siphoneugena reitzii* (Subtribe Pliniinae) is also heliophytic, early secondary/late secondary, and it occurs in high-altitude locations in southern and southeastern Brazil, where it is endemic (WAGNER & FIASCHI 2020, PROENÇA 2023). It has volatile oils with anti-inflammatory activity and antimicrobial action, which makes this a species with potential in the pharmaceutical, food, and cosmetics industries (APEL et al. 2002). Both native species occur in areas of upper montane mixed ombrophilous forest in southern Brazil (GOMES et al. 2016). These species have also been gaining notoriety due to their promising source of bioactive compounds with potential for biotechnological applications (COSTA et al. 2021, CAVALHEIRO et al. 2023).

Pollen viability refers to the ability of a pollen grain to germinate and produce a pollen tube, which is essential for the fertilization of ovules. Research into the pollen viability of plants is important for understanding their reproductive capacity and for developing plant genetic conservation and improvement strategies (FRANZON et al. 2007). Knowing the pollen viability of a species can also help determine the fertilization rate in seed formation and identify factors that may affect reproduction. In addition, this research is fundamental for determining pollen storage parameters, which can be used in hybridization and breeding programs (DENISOW et al. 2014, SOUSA et al. 2021).

In vitro pollen germination is a technique for determining whether pollen grains are capable of germinating and producing a pollen tube when exposed to a culture medium containing agar and specific nutrients, and this is the most common method for testing viability (FRANZON & RASEIRA 2006, LUO et al. 2020). In this method, it is necessary to adjust the culture medium, as viability can vary due to its composition, the storage period of the pollen grain, and the physiological aspects of the species (FRANZON et al. 2007). In fact, the standardization of the medium is conducted by initially checking the concentration of sucrose to promote germination. From this parameter, the inclusion of other components is tested, such as boric acid (H3BO3) or calcium nitrate ($Ca(NO_3)_2$), among other substances (PATEL & MANKAD 2014, NOGUEIRA et al. 2015).

The colorimetric method is based on histological parameters, using specific chemical dyes applied to the pollen grains. The dyes are absorbed by the pollen grains, and they become easily identifiable under the microscope because of the filled protoplasm. This allows viable and non-viable pollen grains to be counted and, consequently, the pollen viability of the plant studied to be determined. This method provides results more quickly and at a lower cost, being useful for both basic research and practical applications, such as selecting plants for genetic improvement and assessing pollination success in agricultural and forestry crops (FRANZON & RASEIRA 2006, LUO et al. 2020, SOUSA et al. 2021).

The objective of this study was to evaluate the germination of pollen grains *in vitro* at different concentrations of sucrose, as well as their viability, using colorimetric tests. Another aim was to check the germination capacity of *M. euosma* and *S. reitzii* pollen after low temperature storage conditions.

MATERIAL AND METHODS

In vitro germination and pollen storage

The botanical material was obtained by removing branches with flower buds in pre-anthesis (balloon stage) from three marked accessions (genotypes) of *Myrceugenia euosma* and *Siphoneugena reitzii,* collected in the same locality. This was done in the upper montane mixed ombrophilous forest in the municipality of Urupema, Santa Catarina, on December 20, 2022, and March 8, 2023.

The branches were immediately placed in moistened floral foam and taken to the Forest Seeds Laboratory at UDESC-Lages, where they remained until the flower buds opened. First, the anthers of *M. euosma* and *S. reitzii* flowers were detached and placed on paper trays at room temperature (±20 °C) for four to six hours for natural dehydration, anther dehiscence, and pollen release.

The growth medium was produced following the methodology of DAFNI (1992) and GUOLLO (2021) for Myrtaceae, with 1% agar in the culture medium and the different concentrations of sucrose (0%, 10%, 20%, 30%, and 40%) dissolved in distilled water with the aid of heating in a microwave oven. While still hot,

the medium was poured into Petri® dishes. After cooling and solidifying, cuts were made using a spatula to form rectangular blocks. These were placed on microscope slides, on which the pollen was sprinkled using a brush or pipette.

The slides were placed in Gerbox-type boxes with lids, containing moistened blotting paper (simulating a humid chamber), and incubated in a Biological Demand Oxygen (B.O.D.) oven at a temperature of 25 °C for approximately 12 to 15 hours (FRANZON et al. 2007). Three replicates were used for ocular evaluation, with each slide being considered an experimental unit.

The germination of the pollen grains was assessed under a binocular microscope. Only those with a pollen tube length equal to or greater than the diameter of the pollen grain itself were considered germinated (FRANZON & RASEIRA 2006). To calculate the percentage, 100 pollen grains were considered.

To check the best storage conditions, pollen grains from the two species were stored in a freezer (-20 ºC), a refrigerator (4 ºC), and under natural laboratory conditions (± 20 ºC). Pollen germination was checked after 0 (pre-storage), 30, and 90 days of storage. Pollen grains stored in freezers and refrigerators were subjected to slow thawing at room temperature for 40 minutes. For *M. euosma*, the pollen was exposed to a culture medium containing 1% agar and 30% sucrose, while *S. reitzii* pollen was germinated in a culture medium containing 1% agar and 20% sucrose (FRANZON & RASEIRA 2006). To calculate this percentage, 100 pollen grains were considered.

Pollen viability by colorimetric tests

The botanical material for the study was obtained from the same accessions (genotypes) of *Myrceugenia euosma* and *Siphoneugena reitzii* that were tested for *in vitro* germination (DAFNI, 1992). For the pollen grains tested with the tetrazolium dye, 20 floral buds were collected at the pre-anthesis stage and taken to the laboratory in floral foam. The same quantities of pre-anthesis buds that were exposed to Lugol and carmine acetic at 2% were immediately placed in falcon tubes containing Carnoy's fixative. They were kept in the solution for 24 hours, then washed and stored in 70% alcohol.

The visual examination was carried out using the dyes described as follows.

-2,3,5 triphenyltetrazolium (TTC): the fresh anthers, after anthesis of the flower bud, were detached and fragmented using a glass rod on a slide containing two solutions of tetrazolium (concentrations of 0.1% and 0.05%). Then, a coverslip was added. The material was placed in Gerboxes containing moistened germ paper and incubated in a B.O.D. at 25 ºC, for six hours, in the dark. The pollen with protoplasm entirely stained light red, indicating oxidative activity, was considered viable.

Lugol at 1%: first, the flower buds fixed in Carnoy/70% alcohol were opened. This was followed by the removal and fragmentation of the anthers with a glass rod, releasing the pollen grains onto the microscope slide containing drops of Lugol's dye, with the subsequent addition of the coverslip (SOUZA et al. 2002). The extracted content remained exposed to a temperature of 25 ºC in the dark. The fertility of the pollen was determined by the pigmentation of the grains. The pollen that had protoplasm with a characteristic dark brown color were considered viable and those that did not have a defined color or transparent color were considered non-viable.

Carmine acetic at 2%: the dye was applied to the microscope slide containing the pollen, with the subsequent addition of the coverslip. After five minutes in natural laboratory conditions, the pollen was evaluated under a microscope. Full pollen showed red coloration of the protoplasm, while non-viable pollen did not stain due to non-existent protoplasm.

The scanning method was used to analyze the slides. 300 pollen grains were counted per slide, with three repetitions for each treatment (dye), totaling 900 pollen grains per test and 2,700 per access (genotype). The microscope slides were observed under a binocular optical microscope with 40x objective lens.

The pollen viability data (storage and *in vitro* germination of pollen, and viability using dyes) were subjected to the Shapiro-Wilk test of normality and homogeneity of variance (Bartllet). Given the assumptions of the model, the data was submitted to analysis of variance (ANOVA) to verify the significance of the factors and their interactions. The means were compared using the Tukey test at a 5% probability level. The data was analyzed using RStudio software (R CORE TEAM 2022) with the "ExpDes" statistical package.

RESULTS

In vitro germination and pollen storage

In the *M. euosma* pollen germination tests, it was found that the culture medium with 30% sucrose allowed more than half of the pollen to germinate and that the pollen responded with statistical differences between the sucrose concentrations (Table 1, Figure 1A).

Table 1. Viability of *Myrceugenia euosma* and *Siphoneugena reitzii* pollen expressed as the number of germinated grains in different concentrations of sucrose. Data expressed as percentages.

Sucrose concentrations	Germination (%)	
	Myrceugenia euosma	*Siphoneugena reitzii*
0%	0 c	22.6 b
10%	13.3 bc	26.3 b
20%	33 ab	45 a
30%	53.3 a	16 b
40%	21 bc	15.3 b
C.V (%)	35.57%	19.44

*Means followed by the same letter do not differ statistically by the Tukey test at 5%.

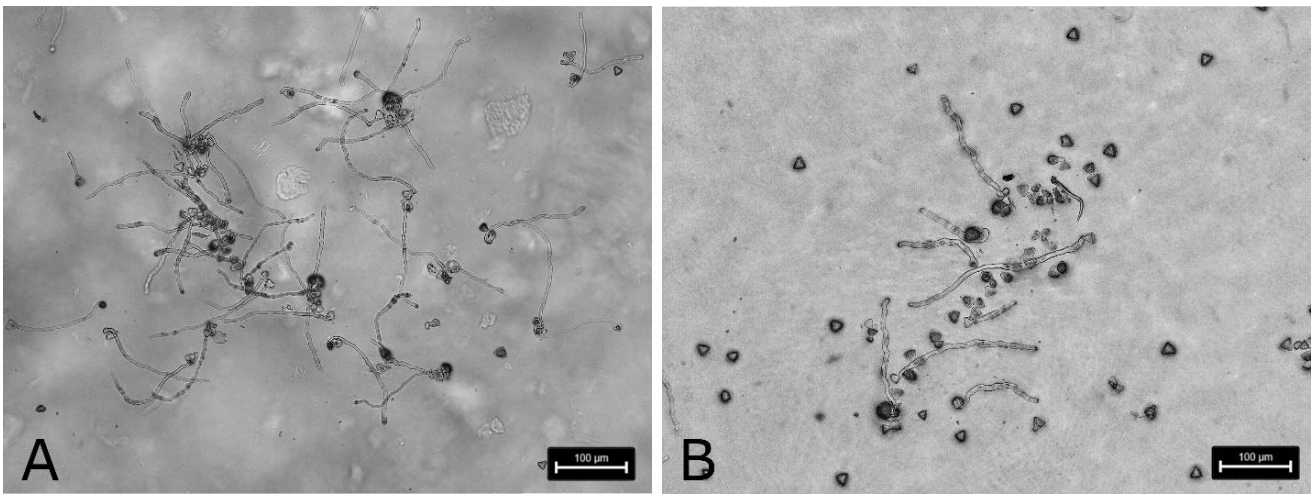

Figure 1. General view of *in vitro* germinated pollen grains. A - *Myrceugenia euosma* with 30% sucrose in growth medium. B - *Siphoneugena reitzii* with 20% sucrose in growth medium. 10x magnification.

The data obtained from the methodological adjustment of sucrose (30%) indicated that the germination of the pollen grains of this genotype of *M. euosma* reached up to 53%, which was the value used to estimate their viability prior to storage (time zero). The pollen grain storage tests showed that, after 30 days, only pollen stored in the freezer retained some of its viability, with 10% germination, followed by storage in the fridge, which showed only 1% germination. After 90 days, the pollen had not germinated in any of the environments tested, indicating a total loss of viability.

The highest germination percentage was observed with the 20% sucrose treatment for *S. reitzii*, where up to 45% germination was obtained (Table 1; Figure 1B), and this value was statistically different from the other concentrations. According to this result, this was the viability value of the pre-stored pollen.

The results of storing pollen grains in different environments showed no significant differences in the preservation of their germination capacity. After 30 days, only 2% of the pollen grains stored in both the freezer and refrigerator had germinated, while in the natural environment of the laboratory there was no germination. After 90 days, none of the environments were able to preserve pollen viability, resulting in zero germination.

Pollen viability by colorimetric tests

The evaluation of *M. euosma* pollen using colorimetric tests showed a predominance of full pollen grains. Their protoplasm was stained using both Lugol and carmine acetic at 2%, theoretically showing high pollen viability (Table 2; Figure 2A, 2B). Tests with the 0.1% tetrazolium dye showed up to 27% pollen viability when the reactive pollens were visualized (Figure 2C). This indicates disparate results in relation to the viability by the same genotype in the *in vitro* germination tests.

Table 2. Pollen viability of *Myrceugenia euosma* and *Siphoneugena reitzii* in different colorimetric methods (Lugol, carmine acetic at 2% and TTC at concentrations of 0.1 and 0.05%); data in percentages (%). C.V.= Coefficient of Variation.

		Lugol	Carmine acetic 2%	Tetrazolium (TTC)	
				0.1%	0.05%
Myrceugenia euosma	Mean (%)	86	91	27	11
	C.V.	5.54	1.23	2.65	3.51
Siphoneugena reitzii	Mean (%)	98	99	47	34
	C.V.	1.46	0.87	11.85	8.50

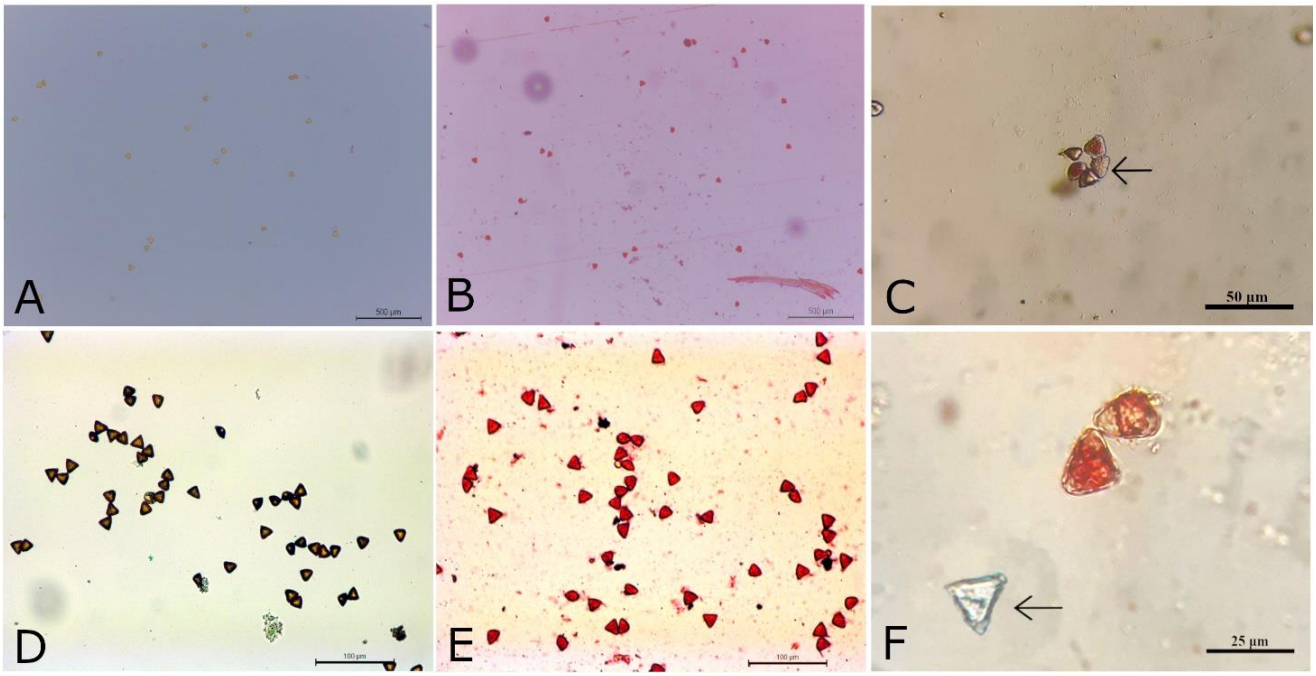

Figure 2. *Myrceugenia euosma*: A - Pollen stained with Lugol. B - Pollen stained withcarmine acetic at 2%; C - Pollen stained with 0.1% tetrazolium (TTC); viable and non-viable (arrow indication). *Siphoneugena reitzi*i. D - Pollen stained with Lugol. E - Pollen stained with Lugol. E - Pollen stained with carmine acetic at 2%; F – Pollen stained with 0.1% tetrazolium (TTC); viable and non-viable (arrow).

According to the test with Lugol dye and carmine acetic at 2%, the colorimetric tests on the pollen of *S. reitzii* showed a response in which numerous full pollens with filled protoplasm were seen (Figure 2D, 2E). However, the tests using tetrazolium obtained contrasting results. Even though many pollen grains had intracellular content, they did not interact with the tetrazolium solution (Table 2, Figure 2F).

DISCUSSION

The different concentrations of sucrose provided significant changes in the germination responses of the pollen of the two species. This may be linked to the osmotic nature of the sugar solution and the supply of energy to promote pollen tube growth (LUO et al. 2020). *Myrceugenia euosma* had more than half of its pollen grains being reactive in 30% sucrose, a recommended percentage for accessing the germination of different *Eucalyptus* species (POTTS & MARSDEN-SMEDLEY 1989). It can be seen that, in the absence of sucrose, *M. euosma* pollen did not germinate.

This may indicate that this component is indispensable in germination for this species, as observed in banana pollen genotypes, which showed virtually zero germination in the absence of this carbohydrate (REIS et al. 2011). The maximum germination of *M. euosma* was close to that of another Myrtaceae native to southern Brazil, *Eugenia involucrata* DC., which had an average germination of 58.3% (FRANZON & RASEIRA 2006). This is a considerable rate, given that good pollen should have 50 to 80% germinated grains with well-developed tubes (SCORZA & SHERMAN 1995).

The pollen of *S. reitzii* obtained almost half of its germination in 20% sucrose. This concentration was efficient in promoting the germination of another Brazilian tree*, Spondias mombin* L. (ZORTÉA et al. 2019). It is also recommended for the pollen of several bromeliad species (PARTON et al. 2002). It is suggested that the ideal percentage of sucrose for *S. reitzii* may be at a concentration of around 20%, so future studies with the addition of other components are recommended.

Siphoneugena reiztii showed pollen with germination behavior similar to different genotypes of another genus of the Plininae subtribe, *Plinia cauliflora*, which showed a maximum germination potential of 41% (DANNER et al. 2011). The germination of *S. reitzii* pollen is below what can be considered good viability, a characteristic already observed in other Myrtaceae, such as *Campomanesia aurea* O. Berg (43.7%) (TEDESCO et al. 2020) and *Eugenia uniflora* L. (up to 30.7%) (FRANZON & RASEIRA 2006).

The colorimetric tests with Lugol and carmine acetic at 2% in both species showed a reaction of the pollen to the dyes. This indicates only the existence of intracellular material, demonstrating that many pollen grains, although full, did not react with the dye. When assessing pollen viability using dyes, the results can be overestimated and it is recommended to compare them with the results obtained *in vitro* (FRANZON et al. 2007, ROCHA et al. 2023). In fact, the results found for both *S. reitzii* and *M. euosma* with Lugol and carmine acetic at 2% were similar to those obtained with pollen analysis of *Psidium cattleianum* Sabine (Mirtaceae) with acetic orcein at 2% (HISTER & TEDESCO 2016), in which the viability of the pollen grains was overestimated.

The use of tetrazolium on pollen is based on the dye's response to the hydrogen produced by the pollen grain's cellular respiration. Research on the consistency of TTC with *in vitro* germination tests has already been verified in *Carica papaya* L. (MUNHOZ et al. 2008) and *Corylus avellana* L. (NOVARA et al. 2017), which reported the reliability of the test for these species. For *S. reitzii*, the concentration of 0.1% tetrazolium was close to the results observed with *in vitro* germination with 20% sucrose, and it is possible that this test can be carried out for this species, provided that the different concentrations, time for analysis, and temperature are adjusted.

For *M. euosma*, pollen viability was underestimated regarding the two concentrations of tetrazolium tested. This result converges with what was stipulated by WANG et al. (2022) with *Taxodium distichum* (L.) Rich. Therefore, it confirms the controversy over the accuracy of the test in some species; thus, it is recommended that other concentrations be tested, in addition to the fact that the use of this dye should always be accompanied by *in vitro* results (FRANZON et al. 2007).

The comparison of results obtained through different viability assessment methods, such as *in vitro* germination tests and colorimetric tests with specific dyes, offers insights into the strengths and limitations of each method. Researchers studying other plant species of the genus or family can use this information to choose appropriate viability assessment methods. They must base their choices according to the specific characteristics and requirements of the species under investigation (LUO et al. 2020).

The storage of *M. euosma* pollen at low temperatures (freezer), despite the remarkable decrease even after 30 days, proved to be a promising method for preserving pollen in the short term. It is recommended to explore this potential with future tests, especially with storage in liquid nitrogen, which has proved ideal for preserving the germination potential of *Campomanesia guazumifolia* (Cambess.) O. Berg (Myrtaceae) pollen grains for up to 30 days (GUOLLO et al. 2021).

The stored pollen of *M. euosma* and *S. reitzii* did not show significant viability after 90 days. This may be due to their high sensitivity to short-term storage, which is common in other Myrtaceae, such as *Campomanesia xanthocarpa* (Mart.) O. Berg and *Eugenia uniflora* L. (FRANZON et al. 2006, FRANZON et al. 2007). This highlights the importance of studying the different storage conditions for storage-sensitive pollen.

The presented approach seeks to support future studies on conservation programs, hybridization, and cross-breeding for other representatives of the *Myrceugenia* and *Siphoneugena* genera, as well as the Myrtaceae family in general, since few species have information on their pollen biology.

CONCLUSION

Sucrose concentrations have a significant impact on the germination of *Myrceugenia euosma* and *Siphoneugena reitzii* pollen, with different optimum concentrations for each species (30 and 20% respectively). In addition, the colorimetric tests with Lugol and carmine acetic at 2% may show overestimated results, which suggest the need to complement these analyses with *in vitro* germination tests. The use of

tetrazolium as a pollen viability method showed variable results, highlighting the importance of adjusting concentrations and following up with *in vitro* tests.

Storing *Myrceugenia euosma* pollen grains at low temperatures showed promising potential, while both species showed sensitivity to long-term storage. These findings highlight the need to investigate different pollen storage conditions in the short term.

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