ASPECTS OF THE REPRODUCTIVE BIOLOGY OF SYMPATRIC MYRTACEAE: *MYRCEUGENIA EUOSMA* AND *SIPHONEUGENA REITZII* IN SOUTHERN BRAZIL

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Abstract

Background: The Myrtaceae family represents one of the most diverse groups in South America. Yet, there are few studies on their reproductive characteristics. Among the species with limited information on their reproductive behavior are *Myrceugenia euosma* (O. Berg) D. Legrand and *Siphoneugena reitzii* D. Legrand.

Questions: What are the floral attractions and resources of *M. euosma* and *S. reitzii* for pollinators? Who are the pollinators of these species? What is the predominant reproductive system in both species?

Studied species: *M. euosma* and *S. reitzii*, two sympatric Myrtaceae from southern Brazil.

Study site and dates: Urubici, Painel, and Rio Rufino, Santa Catarina state, southern Brazil. November 2021 - February 2022.

Methods: Information was obtained on morphological aspects, anthesis time, stigmatic viability, floral resources, structures attractive to visitors, olfactory testing, observation and identification of floral visitors, and reproductive system testing.

Results: The flowers of *M. euosma* opens between 11 am and 17 pm (- 20 pm), with full opening occurring mostly at night; *S. reitzii* flowers open between 4 a.m. and 6 a.m., with a highest peak by 8 a.m. Flowers of both species have receptive stigmas until senescence. Pollen is the primary floral reward and the scent coming from the anthers combined with the floral display attract native bees and *Apis mellifera*, legitimate pollinators of both species. The lack of fruit development in the self-pollination tests support cross reproduction in both taxa.

Conclusions: Both species exhibit traits consistent with melittophily syndrome and potential self-incompatibility. These results are essential for conservation efforts.

Keywords: anthesis, floral reward, melittophily, pollination, self-incompatibility.

Resumen

Antecedentes: La familia Myrtaceae es una de las más diversas en América del Sur, pero sus características reproductivas están poco estudiadas. *Myrceugenia euosma* (O. Berg) D. Legrand y *Siphoneugena reitzii* D. Legrand son dos especies con información limitada sobre su comportamiento reproductivo.

Preguntas: ¿Cuáles son las atracciones florales y los recursos de *M. euosma* y *S. reitzii* para los polinizadores? ¿Quiénes son los polinizadores de estas especies? ¿Cuál es el sistema reproductivo predominante en ambas especies?

Especies estudiadas: *M. euosma* y *S. reitzii*, dos Myrtaceae simpátricas del sur de Brasil.

Sitio y años de estudio: Urubici, Painel y Río Rufino, Santa Catarina, Sur de Brasil. Noviembre de 2021 - febrero de 2022.

Métodos: Se analizaron aspectos morfológicos, tiempo de antesis, viabilidad estigmática, recursos florales, estructuras atractivas para visitantes florales, pruebas olfativas, identificación de visitantes florales y ensayos del sistema reproductivo.

Resultados: Las flores de *M. euosma* se abren entre las 11 am y las 5 pm (completamente durante la noche), y las de *S. reitzii* entre las 4 am y las 8 am. Ambas especies tienen estigmas receptivos hasta la senescencia. El polen es la principal recompensa floral, y el aroma de las anteras, junto con la visualización floral, atrae a abejas nativas y *Apis mellifera*, sus polinizadores legítimos. La autopolinización no resultó en frutos, lo que sugiere la predominancia de la reproducción cruzada en ambas especies.

Conclusiones: *M. euosma* y *S. reitzii* muestran características de melitofilia y potencial autoincompatibilidad, lo que es crucial para su conservación.

Palabras clave: antesis, autoincompatibilidad, melitofilia, polinización, recompensa floral.

The family Myrtaceae has a cosmopolitan distribution, spreading to all continents except Antarctica, with wider representation in tropical and sub-tropical regions (Wilson 2011, Stadnik *et al.* 2016). However, loss and fragmentation of natural habitats negatively alters functional, ecological and reproductive interactions (Cascante *et al.* 2002, Mammides *et al.* 2002, Quesada & Stoner 2003). In fact, various species of Myrtaceae are threatened in the wild, partly because natural pollinators or seed dispersers are in decline. Additionally, relevant biological information regarding pollination and seed biology is lacking, or, in the case of South American taxa, only a few studies on pollination and other reproductive aspects are available (Gressler *et al.* 2006).

Myrceugenia euosma (O.Berg) D.Legrand (Myrtaceae) stands out as an early secondary, semi-deciduous and heliophytic species, which is distributed throughout southeastern and southern Brazil, Uruguay, northern Argentina and southeastern Paraguay (Silva *et al.* 2017, Wagner & Fiaschi 2020). It stands out for its ornamental, medicinal and restoration potential in degraded areas (Marchiori & Sobral 1997, Lorenzi 2008, Gomes *et al.* 2017). In addition, *M. euosma* has been gaining notoriety due to its essential oil being a source of bioactive compounds with high biotechnological potential (Cavalheiro *et al.* 2023). There is no information on its reproductive biology.

Siphoneugena reitzii D. Legrand is an arboreal, heliophytic and early secondary/late secondary (Sobral 2003). It only occurs in the Brazilian Atlantic Forest, in high-altitude locations in the south and southeast of the country (Wagner & Fiaschi 2020). It has volatile oils with anti-inflammatory and antimicrobial activity (Apel *et al.* 2001, Souza *et al.* 2011) that can be exploited in the pharmaceutical, food and cosmetic industries (Gomes *et al.* 2017). There is no information on its reproductive biology.

M. euosma and *S. reitzii* are two sympatric species of Myrtaceae in the tree community of the Caveiras River basin, located in the highland plateau region of Santa Catarina state, in southern Brazil (Higuchi *et al.* 2013). Both species share common characteristics, forming an integral part of the plant biodiversity of the Upper Montane Mixed Ombrophilous Forest (Martins-Ramos *et al.* 2011). Studying and understanding the coexistence between species can provide information on ecological interactions between familiar species in specific ecosystems, enriching the knowledge about the flora and its role in local ecology (Sigrist & Sazima 2015, Gomes 2017).

Considering that studies on the reproductive biology of the genus *Myrceugenia* are non-existent and of *Siphoneugena* are scarce, the research suggests that late self-incompatibility occurs in representatives of the latter (Proença 1990, Proença & Gibbs 1994, Lughadha & Proença 1996). Regarding the floral biology and pollinators of *M. euosma* and *S. reitzii*, the studies found propose that the genera are pollinated by bees (Proença 1990) while the pollen of *M. euosma* was represent in *Apis mellifera* honey in southern Brazil (Tonelli *et al.* 2022). No other aspects of pollination and reproduction were found for these species.

Thus, research into the reproduction of these Myrtaceae is crucial to understand biological and ecological aspects for the purposes of conservation and management of these species in the Atlantic Forest. The aim of this study was to investigate the floral attractants/resources, pollinators and reproductive system of *M. euosma* and *S. reitzii*. It is hypothesized that these species have a melittophily syndrome, are an important source of floral resources for the local entomofauna and are self-incompatible.

Materials and methods

Study area. The study was conducted at 'Fazenda das Nascentes', belonging to the 'Serra da Farofa' Complex Private Nature Reserve, owned by Klabin SA, located between the municipalities of Urupema, Painel and Rio Rufino, Santa Catarina state, southern Brazil (27° 54' 53.7" S 49° 52' 51.0" W) with an altitude of approximately 1,425 m asl (Costa *et al.* 2017). The region's climate is classified as Cfb - humid subtropical mesothermal - according to the Köppen classification, with mild summers, harsh winters with average annual temperatures of 10.9 °C and sporadic snow in the coldest months (Alvares *et al.* 2018). Rainfall is well-distributed, with 1,600 and 1,900 mm/year and no dry season.

The predominant forest formation in the area is the High-Montane Mixed Ombrophilous Forest, occurring in a mosaic with high-altitude fields (Costa *et al.* 2017). Soils are classified as Haplic Nitisols and Cambisols, which are almost always moist (Martins-Ramos *et al.* 2011, Costa *et al.* 2017).

The trees of *M. euosma* and *S. reitzii*, selected for the study, were randomly selected, with a distance of between 1 and 2 km between the trees. The trees were marked and collected along the pre-existing trail at the site. The flowering branches of the evaluated trees were collected, catalogued and incorporated into the LUSC Herbarium of the Santa Catarina State University (vouchers 1466, 11467 and 11468 for *M. euosma* and 11469, 11470 and 11471 for *S. reitzii*).

Flower biology. Initially, the morphology of the *Myrceugenia euosma* and *Siphoneugena reitzii* flowers were described and characterized in terms of the structural changes that occurred during anthesis until total senescence, using the terminology of Vidal & Vidal (2006).

The time of anthesis and the floral changes of *M. euosma* and *S. reitzii* were determined by marking floral buds in pre-anthesis (balloon stage) in three trees of each species (n = 50 and 155 flowers, respectively), at least 1.5 km apart. The longevity of the flowers was observed from pre-anthesis through the complete anthesis to the drying out of the structures and total senescence, counting upto 60 hours of observation over five days. At the same time, records were taken using a camera.

The receptivity of the stigma was checked by adding 3 % hydrogen peroxide for five minutes, which promotes bubbling, indicating the viability of the structure (Dafni 1992). This test was carried out on fifteen flower buds, flowers at the beginning of anthesis, anthesis and senescent flowers of *M. euosma* and *S. reitzii*. Stigma activity was observed under a stereomicroscope with a camera. The test was complemented by exposing the same number of flowers at different stages to a 1 % neutral red solution for 60 min, then washing them in distilled water and observing the coloration of the stigma under a stereomicroscope. The intensity and dark red coloration indicated positive receptive activity of the stigma (Versieux *et al.* 2014, Matias & Consolaro 2014).

The presence of nectar was assessed by placing glass microcapillaries under the base of the calyx and pistil in *M. euosma* and *S. reitzii* (Dafni *et al.* 2005). The test was complemented by observing the behaviour of visitors to the flowers, indicating the type of resource they were looking for.

To check for the presence of osmophores and/or nectaries and floral resource guides, the flowers of the two species were exposed to a 1 % solution of neutral red for 60 min, highlighting the areas of intense metabolic activity, then washed in distilled water and observed under a stereomicroscope (Dafni 1992, Willmer 2011). Floral resource guides were identified by making an adapted ultraviolet light chamber, where 50 flowers in anthesis were directly observed, highlighting the structures that reflected or absorbed the ultraviolet light (Buchmann *et al.* 1977).

In order to characterize and identify the time of greatest odour release, a bioassay was carried out with flowers placed in glass jars, sealed with plastic film, for 24 h, with subsequent sensory evaluation by six untrained volunteers, with flowers collected from anthesis until 18 h after opening, with evaluation between two-hour intervals (Versieux *et al.* 2014).

To identify the floral structure releasing odour from the flowers of *M. euosma* and *S. reitzii*, flowers at anthesis (n = 25) were collected and separated into pistils, petals, sepals and stamens, and each structure was sealed in falcon tubes for 24 h, with subsequent identification of the odour intensity per floral piece by eight untrained volunteers (Benezar & Pessoni 2006).

Floral visitors and pollinators. To characterize the floral visitors and pollinators, direct observations were made on five non-consecutive days of up to three *Myrceugenia euosma* and *Siphoneugena reitzii* mother plants at the ground level, using a ladder or climbing up to the top of the trees, where the collections, times of visits and number of flowers visited were obtained (Willmer 2011, Versieux *et al.* 2014). The observation period was at least 19 h a day for five days, with intervals of 15 min per hour, from 4 to 5 am, supplemented by night observations between 1 and 4 am. The total observation time was 73 h for *M. euosma* and 71 hours for *S. reitzii*. Temperature and humidity were measured every hour during all the days of observation using a thermo-hygrometer.

The individuals observed were counted and classified as pollinators (frequent and legitimate visits and contact with all reproductive structures), occasional pollinators (less frequent visits, even if they are legitimate visitors) and

plunderers (no contact with reproductive structures) (Matias & Consolaro 2014). The number of visits was counted based on the number of times the visitor landed on the flowers to obtain the reward. Every hour associated with a collection event, the visitor's behaviour was observed, noted and photographed. The observation of *M. euosma* was complemented with continuous filming of flowers at anthesis of two trees, where the behaviour of the visitors could be observed in detail.

The visitors were captured using an entomological net made of tulle and organza fabric and preserved in 70 % alcohol for later identification using an identification guide (Fujihara *et al.* 2016) with the aid of a specialist in the area.

Reproductive system. The initial assessment of the reproductive system was carried out by isolating at least 15 flower buds per treatment with organza or nonwoven fabric-type from five adult plants of *M. euosma* and three of *S. reitzii*. The pollen used in the crosses was previously checked for viability in a specific culture medium. Based on these results, the treatments carried out (Polatto & Alves-Junior 2009).

Spontaneous self-pollination.- The pre-anthesis flower buds were bagged and left unmanipulated until the floral structures were completely dry, at which point the protection was removed.

Geitonogamy.- At the beginning of anthesis, the flower was emasculated, followed by the manual transfer of pollen from one flower to the stigma of another flower on the same plant and then bagged.

Xenogamy (cross-pollination).- There was a manual transfer of pollen to the stigma of different plants (emasculated), followed by bagging.

Natural pollination or control.- The flowers at anthesis were marked, without any manual intervention.

Data analysis. The floral biology data was presented using percentages, descriptive statistics and graphs. The analysis of reproductive systems was presented according to the calculation indices for each type of test (Bullock 1985, Willmer 2011).

Results

Floral biology of Myrceugenia euosma. The greenish flower buds are axillary, pedicellar, pubescent and have a calyx with a valvar opening. The small flowers of *M. euosma* are bisexual and dichlamydeous, actinomorphic, gamosepalous and dialypetalous, solitary or arranged in pairs at the end of the stem axis. The hypanthium is densely hairy obdeltoid. Calyx: concave triangular, sparsely pubescent, open with lobes that are longer than the petals in the bud, valvar opening. Corolla: four creamy-white petals. Androecium: numerous stamens (between 30 and 60 filaments), creamy-white dialistemone*,* yellow anthers with longitudinal dehiscence. Gynoecium: punctiform stigma, erect style in a simple filament, inferior ovary with three locules containing 5 to 6 ovules per locule ([Figure 1](#)).

Observations of anthesis time took place from November 12th to 17th, 2021. The flowers of *M. euosma* started the anthesis process from around 11 am to 5 pm, with some flowers starting the process from 8 pm. The full flower anthesis occurred mostly during the night and early morning, between 10 pm to 3 am, where the release of scent could only be observed after 6 am in most flowers. It was noted that after dawn, the flowers that had already opened began to dehisce their anthers due to the exposure of the recesses of the locules in each anther theca.

The process of total opening took an average of 6 h. However, some flowers took up to 24 h to fully distend the anthers and stigmas. It should be noted that the flowers do not close during the night and there was no statistical difference between the trees.
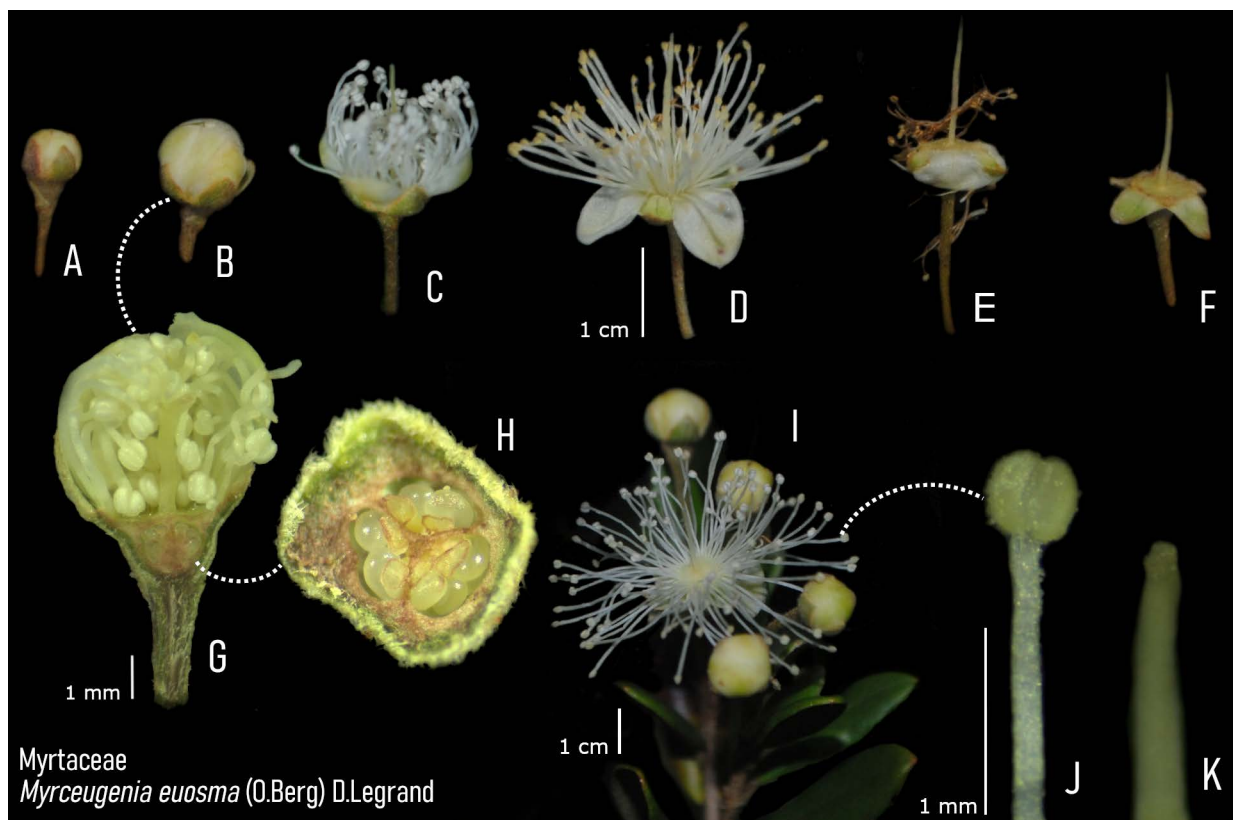
In the first 26 h after anthesis, there was a change in the colour of the anther from yellow to light-grayish yellowish brown, and the previously firm filaments began to soften. After approximately 40 h, the floral structures began

to dry out and darken, starting with the style and moving on to the anthers and petals/sepals, indicating the onset of senescence. On the third day after anthesis, with the fall of the floral structures (anthers, stigma and style, petals and sepals), the flower was considered to have completed senescence. If fertilized, the immature fruit remained attached to the peduncle and if aborted, the whole flower was detached.
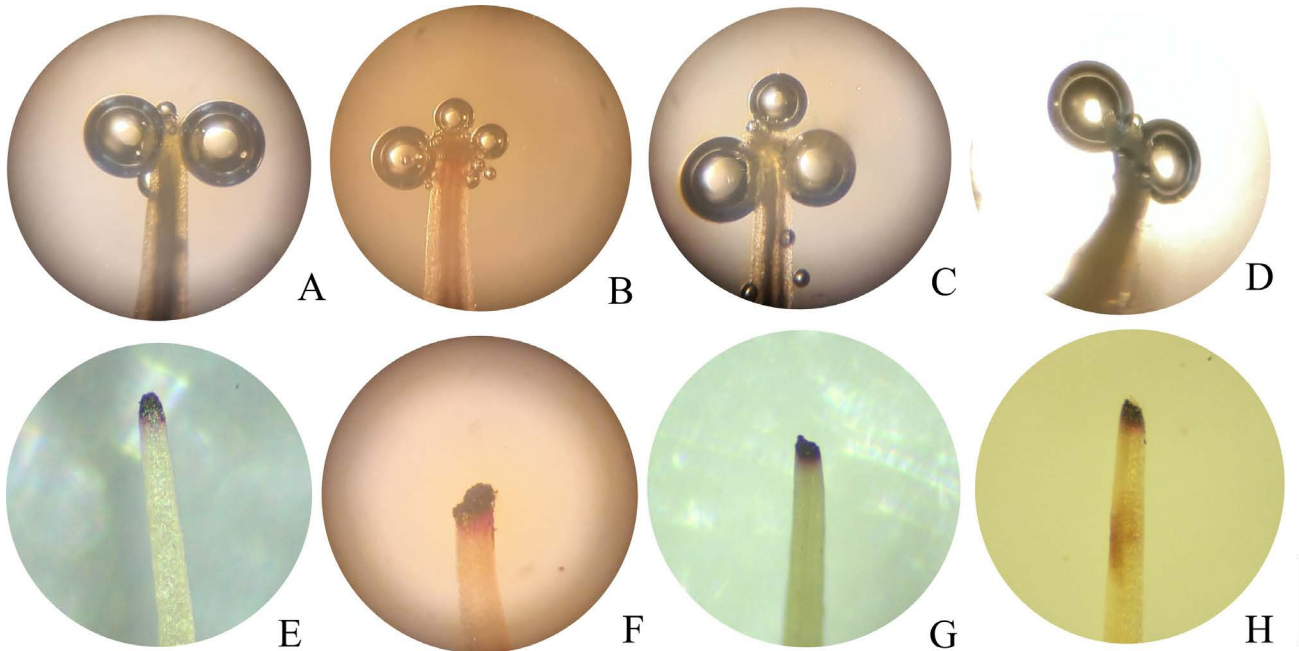
The hydrogen peroxide stigma receptivity test showed that 89 % of the flowers had a receptive stigma in pre-anthesis (flower bud stage), and during flower opening this percentage reached 100 %. However, the intensity of the bubbling was considerably lower than the flowers in full anthesis, which showed peroxidase enzyme activity in all the flowers. Similarly, all the flowers in senescence also showed stigma activity, albeit at a lower bubbling intensity (Figure 2). The neutral red test converged with these results, showing 78 % of receptive stigmas in pre-anthesis and 100 % both at the start of anthesis and in full anthesis and senescence.

The microcapillary tube test did not indicate the presence of nectar/nectaries in the flowers of *M. euosma*, which was confirmed by the neutral red test. In addition, no floral resource guides were visualized by observing the flowers under ultraviolet light. This was also confirmed by the neutral red test, which indicated the anthers as a region of activity for *M. euosma* (Figure 3).

The olfactory bioassay indicated that the flowers of *M. euosma* have a sweet and slightly herbal floral aroma. According to the volunteers, the flowers picked almost every hour after anthesis had a light smell, only 4 h after anthesis had a moderate smell and after 8 h an intense smell. The floral part that released the odour indicated by all the volunteers was the anther/pollen. In fact, as mentioned above, this structure remains in intense activity in *M. euosma* flowers, according to the neutral red test, indicating that it is the source of the odour of these flowers (Figure 3).

Figure 1. Floral morphology of *Myrceugenia euosma*. A) Flower bud. B) Flower bud in balloon stage. C) Flower initiating anthesis. D) Full anthesis. E) Flower starting senescence. F) Completely senescent flower. G) Detail of flower bud in longitudinal section with stamens curved inwards. H) Transverse section, showing the ovary. I) Branch with leaves, flower buds and flowers. J) Stamen. K) Stigma and style.

Figure 2. *Myrceugenia euosma*. Stigma receptivity according to hydrogen peroxide in flower buds: A) Pre-anthesis. B) Beginning of anthesis. C) Full anthesis. D) Senescence. Stigmas stained with neutral red 1 %: E) Pre-anthesis F) Beginning of anthesis) G) Total anthesis. H) Senescence. Bar: 0.5 mm.

Figure 3. *Myrceugenia euosma* flower anthers after immersion in 1 % neutral red for 60 minutes. A) general view, B) detail of anthers.

Floral visitors and pollinators of Myrceugenia euosma. Pollinator observations took place between November 12th and 17th, 2021, on sunny days and nights with dense fog and rain. The minimum temperatures were 9 ºC, the average 18 ºC and the maximum 24 ºC.

A total of 495 insect visitors (Table 1, Figure 4) to *M. euosma* were observed, of which 78.9 % belonged to the order Hymenoptera, with a predominance of the Apidae family, specifically one species, *Melipona* sp 1. (Table 1, Figure 4). The order Diptera was represented in 7.8 % of the samples, all belonging to the Syrphidae family. The order Coleoptera had 12.9 % of visits, while the other orders were recorded in low numbers, such as Lepidoptera (0.2 %) and Hemiptera (0.2 %) (Figure 4).

Table 1. Flower visitors to *Myrceugenia euosma* from November 12th to 17th, 2021 at Fazenda das Nascentes (RPPN Serra da Farofa), on the border between the municipalities of Painel and Urupema, Santa Catarina state, Brazil.

Species	Percentage of occurrence (%)	Species	Percentage of occurrence (%)
Hymenoptera		Coleoptera	
Melipona sp. 1 (Apidae)	61.5	Galerucinae	10.6
Apis mellifera (Apidae)	0.6	Elateridae	2.3
Augochlora sp. (Halictidae)	2.6	Hemiptera	
Polistes sp. (Vespidae)	6.4	sp1	0.2
Tetramesa sp. (Vespidae)	4	Lepidoptera	
Polybia sp. (Vespidae)	3.8	sp2	0.2
Diptera			
Baccha elongata (Syrphidae)	3.8		
Platycheirus sp (Syrphidae)	2.4		
Parasyrphus sp. (Syrphidae)	0.8		
Toxomerus sp. (Syrphidae)	0.8		

All the insects from the Apidae and Halictidae families were observed foraging, visiting various flowers and touching the reproductive structures, so they are considered legitimate pollinators. *Melipona* sp. (Figure 5) showed extensive foraging dynamics, as it visited an average of six flowers, staying an average of eight seconds per flower and 58 seconds per tree.

Although less common, Diptera, Lepidoptera and Vespidae insects remained for prolonged periods on one or two flowers, occasionally touching the anthers and stigmas, thus characterizing them as occasional pollinators.

The insects from the Coleoptera (Table 1, Figure 5) and Hemiptera families did not contact the stigma, remaining for a long time on the flower and peduncle without moving between the flowers. The representatives of Coleoptera, although they were not observed feeding on the flowers, remained inert, occasionally feeding on pollen dropped on the perianth. Both groups can therefore be considered pillagers of the species.
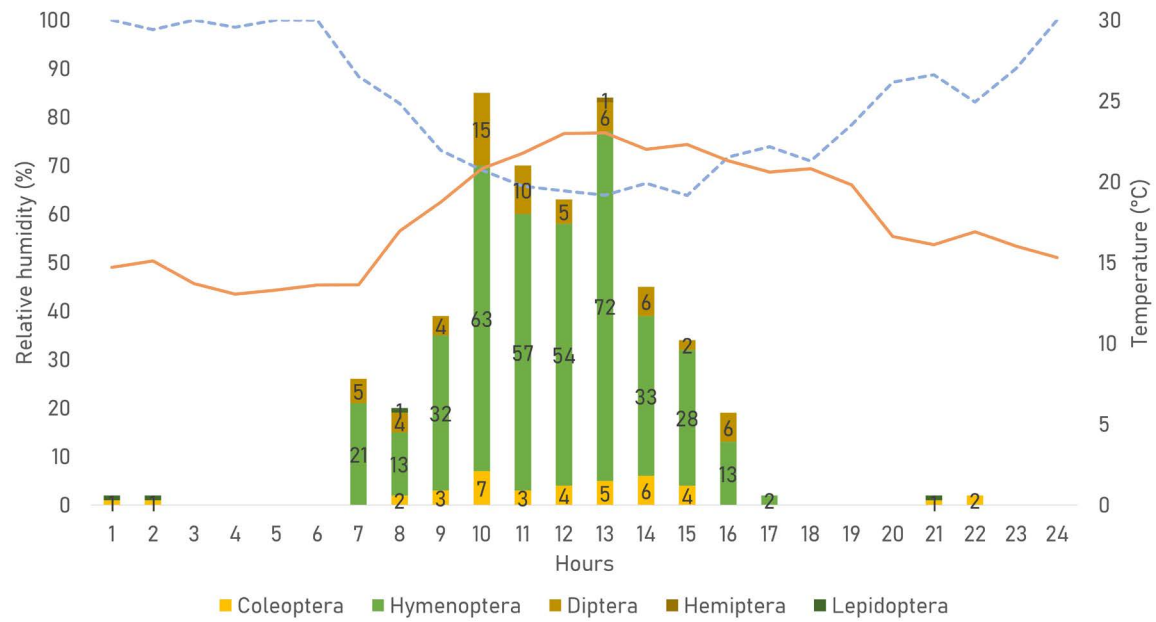
Reproductive system of Myrceugenia euosma. The pollen from the matrix used for the tests showed 86.3 % viability according to the *in vitro* germination test using 30 % sucrose in the culture medium, confirming the fertility of the pollen grain.

The reproductive system tests were evaluated according to the production of mature fruit per test, which took around 30 to 45 days after bagging. The treatments to verify the reproductive system showed that fruit formation occurred only for xenogamy and in the control group, with open pollination and, of the 100 flowers marked, 24 % fruited through natural pollination (Table 2).
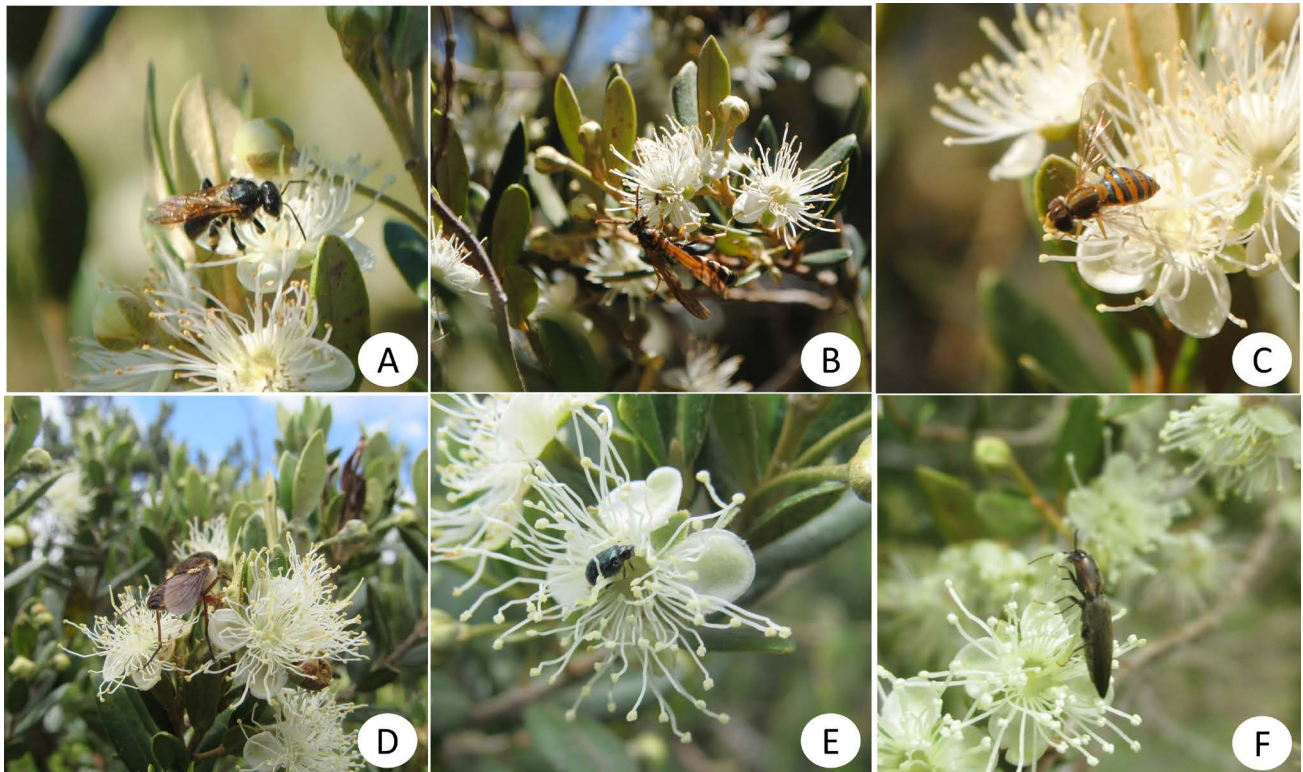
For the xenogamy treatment (manual pollination between different plants), there was a total of 7 % fruit formation, while the self-pollination and geitonogamy treatments did not form any fruit.

Floral biology of Siphoneugena reitzii. The flowers of *S. reiztii* are bisexual, dichlamideous, actinomorphic, dialypetalous and gamosepalous with 1 to 7 flowers per raceme. They have persistent deltate bracteoles and reddish flower buds with conspicuous calyx lobes. Hypanthium: glabrous, oblong and deciduous after anthesis of the lobes. Calyx: closed in the bud and arranged in lobes that form a continuous tubular structure from the hypanthium, with four creamy-white sepals. Corolla: four obovate, pubescent white petals. Androecium: dialistemone stamens (between 70 and 85 filaments), white stamens, yellowish anther with longitudinal dehiscence. Gynoecium: punctiform stigma, erect style in a single simple filament, bilocular inferior ovary with 3 to 6 ovules per loculus (Figure 6).

Figure 4. Mean temperature per hour, relative humidity, main visiting orders and visiting times in three *Myrceugenia euosma* trees from November 12th to 17th, 2021. Location: Fazenda das Nascentes (RPPN Serra da Farofa), on the border of the municipalities of Painel and Urupema, Santa Catarina state, Brazil.

Figure 5. Flower visitors of *Myrceugenia euosma* from February 17th to 22th, 2022, at Fazenda das Nascentes (RPPN Serra da Farofa), on the border between the municipalities of Painel and Urupema, Santa Catarina state, Brazil. Hymenoptera. A) *Melipona* sp. 1 (Apidae). B) Vespidae. Diptera. C) D) Syrphidae. Coleoptera. E) Galerucinae. F) Elateridae.

Table 2. Pollination tests obtained on *Myrceugenia euosma* plants at Fazenda das Nascentes, on the border between the municipalities of Painel and Urupema, Santa Catarina state, Brazil.

Reproductive tests	Used flowers	Ripe fruits
Spontaneous self-pollination	288	0
Natural Pollination	291	71
Xenogamy (cross-pollination)	15	1
Geitonogamy (manual self-pollination)	21	0
Self-Incompatibility Index (SII)		n/a
Spontaneous self-pollination index (SPI)		n/a
Reproductive efficacy (RE)		3.64

The anthesis of the flower occurs when the sepals and petals distend, showing the curved stamens, with the stylus arranged over them. Complete anthesis occurs when all the stamens are aligned. As soon as the anthers dry out, there is a change in the colour of the flower to brown, characterizing the onset of senescence (Figure 6E).

The flowers of *S. reitzii* began to open from 4 am, with greater intensity around 6 am. From 8 am onwards, some flowers were already fully open, with the largest number of open flowers observed around 9 am. Out of the 155 flowers observed, 86 % became fully open during the observation period. The anthesis of this species took place mainly in the early hours of the morning, with complete opening after 2 to 4 h, and no flowers closed during the night.
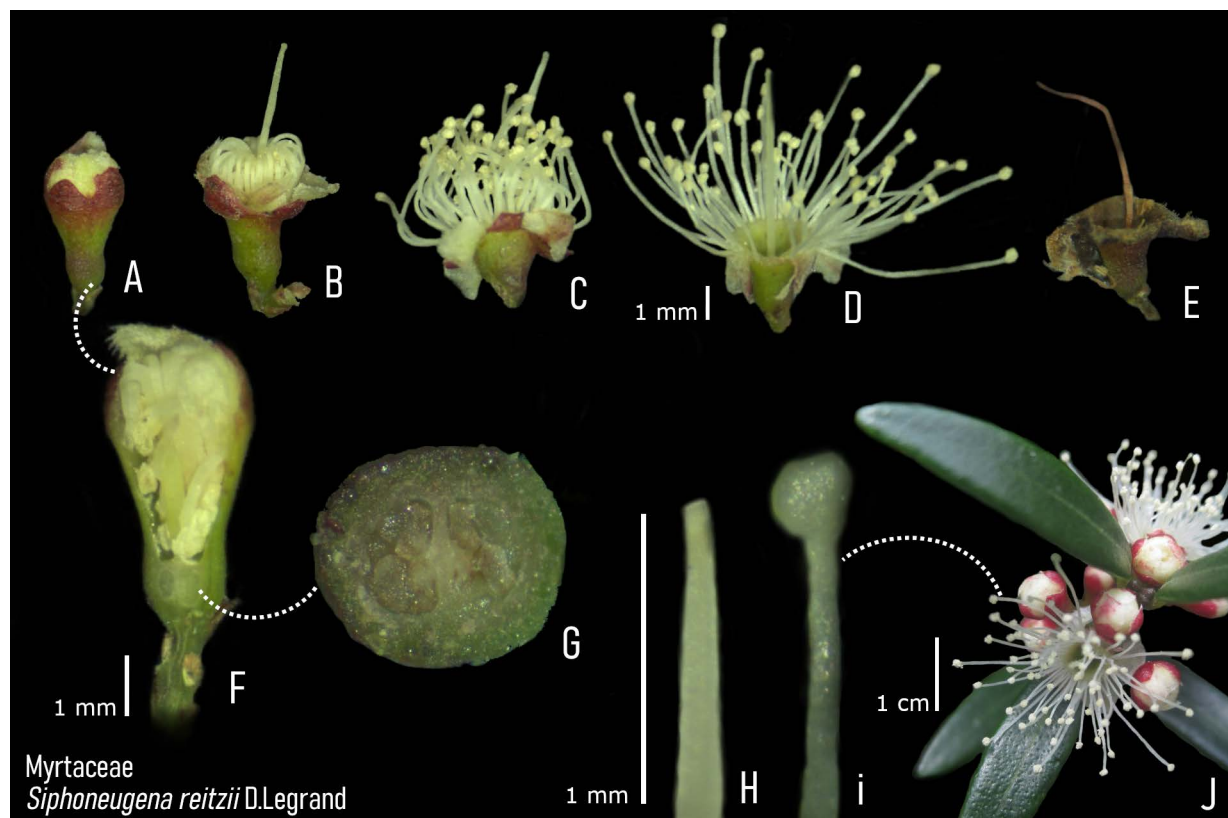
Flowers lasts an average of 46 hours, when the flowers go into a process of dehydration/senescence with the reproductive structures drying out and falling off, leaving only the fascicle and the stigma/stylus for 3 to 4 months, at which point the morphology of the ovary changes due to fruiting.

The receptivity of the stigma in pre-anthesis indicated 33 % activity with the hydrogen peroxide test, which was corroborated by the 1% neutral red test, indicating the same percentage of stigmas receptive to pollen. Flowers at the beginning of opening already showed a 90 % response to hydrogen peroxide and a 100 % response to neutral red. Some stigmas of flowers in pre-anthesis and at the beginning of anthesis showed bubbling, but with less intensity than the activity seen in all flowers that were complete floral open (Figure 7), which reached 100 % enzymatic activity of hydrogen peroxide and 93.3 % through neutral red. In senescent flowers, it was observed that 100 % reacted with hydrogen peroxide, but 86 % showed a characteristic neutral red colour.

The microcapillary and neutral red tests did not indicate the presence of nectar/nectaries or secretory structures in the flowers of *S. reitzii*. Likewise, the flowers exposed to ultraviolet light did not indicate reflectance in any floral structure.

The olfactory bioassay reported that the flowers have a strong sweet and slightly herbal smell. According to the volunteers, during the early hours of the morning, after the start of anthesis between 6 and 8 am, the odour released is intense (2 h after anthesis), decreasing to moderate towards absent after the first 10 h after anthesis. All the volunteers indicated the anthers/pollen as the structure that concentrates the smell in *S. reitzii* flowers. This can be seen from the coloration indicating activity using the neutral red test.

Floral visitors and pollinators of Siphoneugena reitzii. A total of 201 insect visitors were observed on the flowers of *S. reitzii* between February 17th and 22th, 2022, on sunny days and nights with high fog, with average temperatures of 17 ºC, minimum of 10 ºC and maximum of 25 ºC. Among the total number of flower visitors, 90.2 % were from the Hymenoptera order (Figure 9), specifically the Apidae family, with a predominance of visits from the same species of stingless bee, *Melipona* sp. This was followed by *Apis melifera* bees and solitary bees from the genera *Xylocopa* and *Bombus*. The Vespidae family accounted for 12 % of visits from the Hymenoptera order. Other insect orders that visited *S. reitzii* were Diptera (6 %) and Coleoptera (4.8 %) (Table 3, Figure 8).

Figure 6. *Siphoneugena reitzii*. A) Closed flower bud. B) Pre-anthetic flower bud with stamens curved inwards. C) Flower initiating anthesis. D) Full anthesis. E) Completely senescent flower, F) Detail of flower bud in longitudinal section. G) Transverse section, showing the ovary with the two locules. H) Stigma and style. I) Stamen. J) Flower branch.

Melipona sp 2. Bees ([Figures 8](#), [9](#)) predominated in terms of the number of visits and visitors to the flowers of *S. reitzii*, with foraging activity in four flowers on average, staying around 5 seconds in each flower, *Apis mellifera* (± 8 visits, ± 7 seconds/flower); *Xylocopa* sp. and *Bombus* sp. were also seen visiting the reproductive structures of the flowers. In this way, all these genera can be considered legitimate pollinators of the species.

The representatives of Diptera made brief visits with sporadic contact with all the structures, which classifies them as occasional pollinators. In contrast, the insects of the Coleoptera order remained for long periods in the flower, without moving and without contact with the anthers and stigmas ([Figure 9](#)). In the case of the Vespidae family, sporadic visits were observed, in which, as well as not having contact with all the reproductive structures, there was evidence of active searching for other insects during their interaction with the flowers, thus revealing a resource plundering behaviour for these groups.
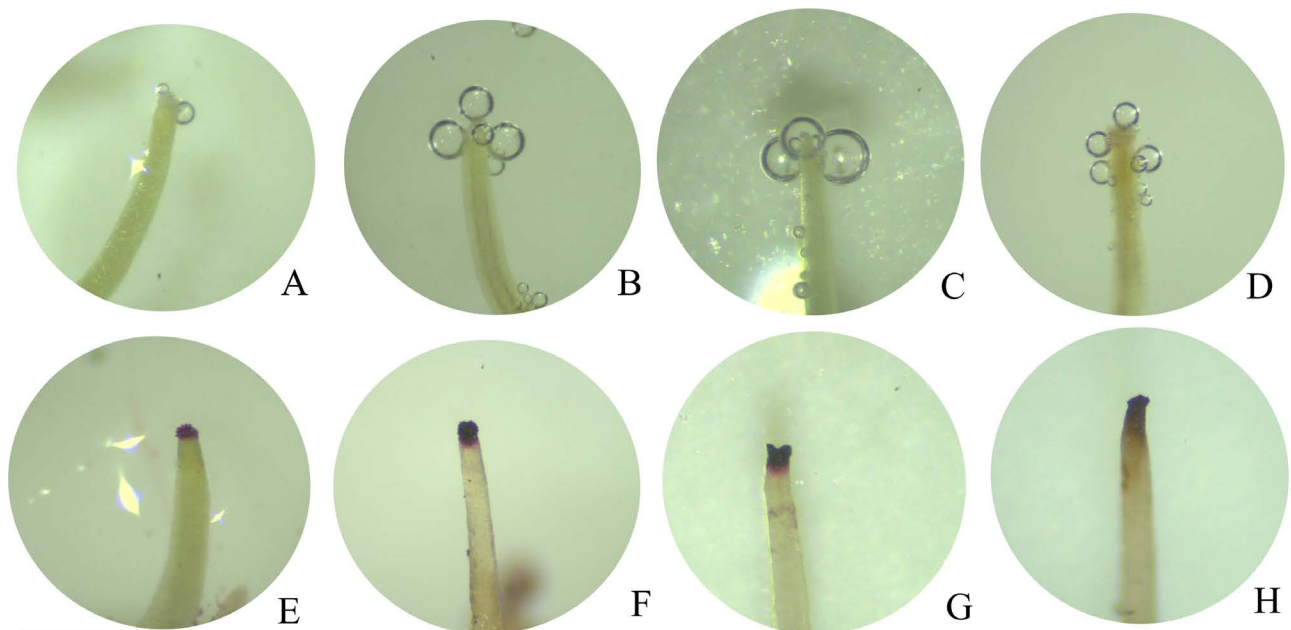
Reproductive system of Siphoneugena reitzii. In the cross-pollination tests, the pollen from the mother tree previously tested obtained 40 % germination potential in vitro with a culture medium containing 20 % sucrose. From the crosses made for *S. reitzii*, fruit formation was observed both through natural pollination (19 %) and manual cross-pollination (13 %). The self-pollination treatments showed aborted flowers in the first few days after senescence, both without fruit formation ([Table 4](#)).

Due to the lack of self-fertilization of *S. reitzii* flowers, there were no values for the self-incompatibility index (SII) and the spontaneous self-pollination index (ISA), while the species' reproductive efficiency (RE) was 1.50.

Discussion

Floral biology. One of the aspects common to several representatives of the Myrtaceae group and also found in *M. euosma* is the number of stamens, which through the anthers offer a good amount of pollen, the main floral reward for visitors and pollinators (Gressler *et al.* 2006), since the species is devoid of nectars and nectar. In fact, as we have seen, the anther is constantly active in the flowers of *M. euosma*, as it is responsible for the attractive smell, as well as providing the pollen that will be transported to other flowers or to the bee hive. The scent concentration in this structure can be related to anther volatiles (Jürgens & Dötterl 2004) a similar characteristic to that observed in *Campomanesia guazumifolia* (Guollo *et al.* 2021) and *Eugenia myrcianthes* (Guollo *et al.* 2023). The genus *Siphoneugena*, in turn, is notable for its botanical uniqueness, especially in relation to the morphology of its flowers, which have a "siphon" shape due to the elongated hypanthium and taper towards the apex (Proença 1990, Dos Santos & Marchiori 2010). However, it is important to note the scarcity of research dedicated to this genus as a whole (Proença 1990). There are only 9 species representing the genus in Brazil (Caldas *et al.* 2020), which limits efforts to expand knowledge about *Siphoneugena*. The characteristics of the flowers of *S. reitzii*, such as size, colour and shape, are similar to those observed in other Myrtaceae (Vasconcelos *et al.* 2019, Oliveira *et al.* 2021), especially in the subtribe Pliniinae (Varela & Sanchez-Vindas 2021, Lucas *et al.* 2019), and in other representatives of the genus (Proença 1990, Caldas *et al.* 2020). Notably, the presence of small white flowers stands out, which, in contrast to the dark green of the tree crown, constitutes a visual attraction at close range for floral visitors, as previously described for *S. densiflora* (Proença 1990).

Despite the apparent lack of specific secretions to attract pollinators, it is believed that the flowers of *M. euosma* and *S. reitzii* adopt a combined (olfactory + visual) strategy, using the scent of the anthers/pollen to attract potential pollinators from a long distance, and their floral display, marked by the contrasting white colour of the numerous flowers in the canopy, to attract pollinators from a short distance (Faegri & Van Der Pijl 1979, Nucci & Alves-Junior 2019).
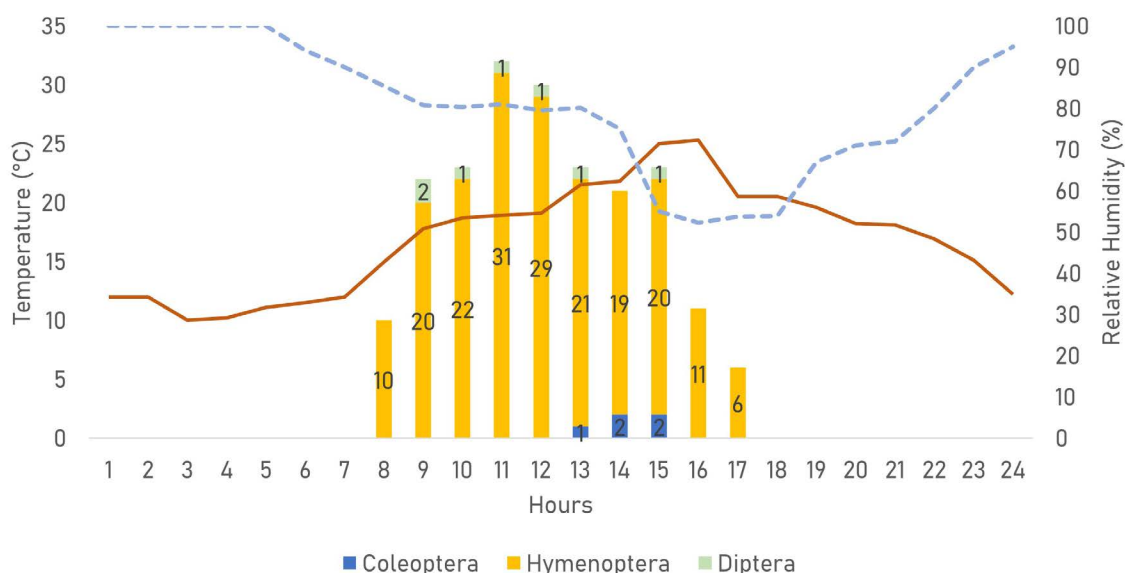

Figure 7. *Siphoneugena reitzii*. Stigma receptivity using the hydrogen peroxide test on flower buds: A) Pre-anthesis. B) Beginning of anthesis. C) Full anthesis. D) Senescence. Stigma receptivity as revealed by staining with neutral red 1 %: E) Pre-anthesis. F) Beginning of anthesis. G) Complete floral opening. H) Senescence. Bar: 0.5 mm.

Table 3. Floral visitors of *Siphoneugena reitzii* from February 17th to 22th, 2022 at Fazenda das Nascentes (RPPN Serra da Farofa), on the border between the municipalities of Painel and Urupema, Santa Catarina state, Brazil.

Species	Percentage of occurrence (%)	Species	Percentage of occurrence (%)
Hymenoptera		Diptera	
Melipona sp. 2 (Apidae)	60.2	*Toxomerus* sp. (Syrphidae)	4.2
Apis mellifera (Apidae)	8.4	*Baccha* sp. (Syrphidae)	0.8
Xylocopa sp. (Apidae)	7.2	Coleoptera	
Bombus sp. (Apidae)	2.4	Galerucinae	4.8
Polistes sp. (Vespidae)	12		

The odour of the flowers of *M. euosma* and *S. reitzii* is attributed to volatile oils, common in other Myrtaceae, and can be exhaled both by the anther itself and by the pollen grains (Pernal & Currie 2002, Gressler *et al.* 2006). The sweet smell noted in *M. euosma* is commonly reported for the most diverse Myrtaceae, such as *Psidum cattleyanum* (Costa *et al.* 2015) and *Campomanesia adamantium* (Nucci & Alves-Junior 2017). For *S. reitzii*, the olfactory bioassay presents a different perspective, highlighting that the flowers of *S. reitzii* have a strong sweet and slightly herbal odour, similar to that reported for *Psidium cinereum* (Gressler *et al.* 2006), *Myrcia guianensis* and *Myrcia laruotteana* (Pires & Souza 2011).

Floral volatile compounds can vary in their composition and characteristics, creating the distinctive odour associated with different types of flowers, so pollinators often develop an association between the specific odour of a flower and the food reward (such as pollen) that the flower offers, establishing a mutualistic relationship that is beneficial for both the plant and the pollinator (Lughadha & Proença 1996, Rech *et al.* 2014). The specific odour emitted by Myrtaceae can be an important primary signal for specific pollinators (Cordeiro *et al.* 2019), a factor that may be related to the particularities of the aroma emitted by both species studied.

Figure 8. Mean temperature per hour, relative humidity and, main visiting orders and visiting times in three *Siphoneugena reiztii* trees from February 17th to 22th, 2022 at Fazenda das Nascentes (RPPN Serra da Farofa), on the border between the municipalities of Painel and Urupema, Santa Catarina state, Brazil.

Figure 9. Floral visitors of *Siphoneugena reiztii* from February 17th to 22th, 2022 at Fazenda das Nascentes (RPPN Serra da Farofa), on the border of the municipalities of Painel and Urupema, Santa Catarina state, Brazil. Hymenoptera. A, B) *Melipona* sp. 2, C) Coleoptera.

The flowers opening at night usually indicates the possibility of nocturnal pollination (Cordeiro *et al.* 2017), a characteristic not observed in *M. euosma*. However, the olfactory bioassay showed that it was only after dawn that its flowers emitted a perceptible smell, a behaviour that converged with the senescence of the anther. This behaviour demonstrated that during this period, the flower was already able to receive floral visitors in the early hours of the morning, characterizing an efficient strategy to optimize the window of availability of the flower for pollinators (Silva & Pinheiro 2007). A similar behaviour has already been observed in *M. pungens* (Guollo *et al.* 2019), where nocturnal anthesis also did not necessarily indicate the presence of floral visitors.

The total receptivity of the stigma during the early morning is a common characteristic for Myrtaceae (Fidalgo & Kleinert 2009) and observed also in *M. euosma*. The already receptive stigma in the balloon stage is a temporal separation strategy, dichogamy, due to the dehiscence of the anther occurring only after the floral opening is complete, as seen in *M. pungens* (Guollo *et al.* 2019) and *C. xanthocarpa* (Homczinski 2021). Among the greatest advantages of this behaviour is the increased likelihood of cross-pollination. The flowers of *M. euosma* senesce after two days, this characteristic allows cross-pollination to take place within the first 24 hours, ensuring its reproductive success, since from this period onwards, both the smell and the visual display of the flower decline (darkening of the flower) (Nucci & Alves-Junior 2017, Pires & Souza 2011, Cavalcante *et al.* 2023).

The flower opening began mostly in the morning in *M. euosma*, as soon as the first rays of sunlight appeared, and within a matter of hours the flowers were fully open. The anther dehiscence began within the first few hours of the flower fully opening, accompanied by the peak of odour release indicated by the olfactory bioassay. These factors coincided with the arrival of the first floral visitors (Apidae = Meliponini) around 9 am, which may be related to the big bang flowering strategy, a common behaviour for several Myrtaceae (Proença & Gibbs 1994, Pires & Souza 2011, Guollo *et al.* 2019). This big bang flowering strategy exposes resources/attractants from the full anthesis of a high quantity of flowers to ensure reproduction.

Divergent behaviour was observed in *S. reitzii*, where it was found that the stigmas of *S. reitzii* were not completely receptive during pre-anthesis, but became susceptible throughout the opening of the flower, reaching full receptivity when the flower was fully open, despite this, the flowers did not show protandry, since the maturation of the stigma preceded the senescence of the anther, a characteristic similar to that observed for *Citrus* (Ramos *et al.* 2008).

The tests with microcapillaries and neutral red did not indicate the presence of nectar/nectaries or secretory structures in the flowers of *S. reitzii*, favouring the pollen as the main floral reward and established an initial basis that suggests the absence of characteristics traditionally associated with attractiveness to pollinators, but usual in Myrtaceae (Lughadha & Proença 1996, Silva & Pinheiro 2007, Guollo *et al.* 2019, Guollo *et al.* 2021). At the same time, exposing the flowers to ultraviolet light did not reveal reflectance in any floral structure, corroborating that only the contrast caused by the flower in the canopy may be its visual signal to attract its pollinators, a characteristic similar to that observed in *C. adamantium* (Nucci & Alves Junior 2017).

Table 4. Pollination tests carried out on *Siphoneugena reitzii* plants at the Fazenda das Nascentes, on the border between the municipalities of Painel and Urupema, Santa Catarina state, Brazil.

Reproductive tests	Used flowers	Ripe fruits
Spontaneous self-pollination	94	0
Natural Pollination	107	20
Xenogamy (cross-pollination)	32	4
Geitonogamy (manual self-pollination)	27	0
Self-Incompatibility Index (SII)		n/a
Spontaneous self-pollination index (SPI)		n/a
Reproductive efficacy (RE)		1.50

Floral visitors and pollinators. The behaviour observed in *M. euosma* and *S. reitzii* showed that the main floral reward is pollen, since the pollinators rarely stayed still on the flowers. Instead, they walked around the stamens collecting pollen, a behaviour mostly observed in bees. In addition, only one insect of the order Lepidoptera was observed on *M. euosma*, which usually visit flowers with a nectar supply (Rech *et al.* 2014).

The analysis of floral visitors to *Myrceugenia euosma* indicated that the main syndrome associated with the species is melittophily. Among the visitors evaluated, the Apidae family stood out as the main pollinating group of *M. euosma*, reinforcing the claim that the group plays an essential role in the reproductive process in Myrtaceae (Lughadha & Proenca 1996, Gressler *et al.* 2006, Silva & Pinheiro 2007). Bees of the genus *Melipona* (Apidae = Meliponinae), the main pollinators of *M. euosma*, are recognized as one of the main pollinating groups for Myrtaceae in southern and southeastern Brazil (Gressler *et al.* 2006, Fidalgo & Kleinert 2009).

A species of Halictidae (Apidae) was observed foraging on *M. euosma*, proving to be even more expressive than *Apis mellifera*. It is not uncommon for bees from the Halictidae family to be considered efficient pollinators of Myrtaceae (Gressler *et al.* 2006, Fidalgo & Kleinert 2009). The representatives of Diptera, in turn, followed a pattern previously observed in Syrphidae, a group recognized for containing flies that feed on pollen and occasionally contribute to pollination (Gressler *et al.* 2006, Silva & Pinheiro 2007, Guollo *et al.* 2021). Similarly, insects from the Vespidae order and the Lepidoptera family, despite not having suitable behaviour and morphology, can be characterized as occasional pollinators (Rech *et al.* 2014).

Visitors from the Coleoptera and Hemiptera families were observed exploring the floral resources of *M. euosma* flowers, showing particular interest in pollen. Both groups did not provide reproductive benefits for the plants, reflecting behaviour similar to that observed in *Eugenia myrcianthes* (Guollo *et al.* 2023).

S. reitzii also showed melittophily syndrome, since the main floral reward for visitors is pollen, which was observed in good quantity in the anthers. These structures were the most sought after by representatives of the order Apidae, characterizing a trait similar to that already observed in several Myrtaceae (Proença & Gibbs 1994, Lughadha & Proença 1996, Gressler *et al*. 2006, Silva & Pinheiro 2007).

The main pollinator of *S. reitzii* is a small bee of the genus *Melipona* (Apidae = Meliponini) different from the species that predominated on *M. euosma*. In fact, the flowers of *M. euosma* are not only larger in all respects (sepal, petal, stamens) and have more stamens and ovules per flower when compared to those of *S. reitzii*, but they are also visited by larger *Melipona* bees (1 cm vs. 0.5 cm). The larger flower of *M. euosma* and the greater number of stamens and ovules may be adapted to attract and be pollinated by larger bees, while the smaller flowers of *S. reitzii* are visited predominantly by smaller bees, possibly due to the correspondence between the size of the flower and the size of the pollinator (Dafni & Kevan 1997, Cavalcante *et al*. 2023). In any case, *Melipona* is recognized as one of the main pollinating genera of Myrtaceae, being closely linked to the reproductive biology of Myrtaceae and other botanical families (Lughadha & Proença 1996, Gressler *et al*. 2006, Fidalgo & Kleinert 2009, Rech *et al*. 2014).

Previous research by Proença & Gibbs (1994) revealed that *S. densiflora*, similar to *S. reitzii* did not show a high diversity of pollinating insects. In this case, a crepuscular bee of the genus *Ptiloglossa* (Colletidae) was identified as

the main pollinator of *S. densiflora*. In this context, the statement by Proença & Gibbs (1994) that the flowers of *S. densiflora* can be visited by insects of different sizes is confirmed, as *S. reitzii* was approached by small (*Melipona* sp 2.), medium-sized (*Apis mellifera*) and robust bees (*Bombus* sp. and *Xylocopa* sp.), all of which carried out legitimate pollination on the small flowers of the species.

The two species of syrphids (Diptera) observed were classified as occasional pollinators, similar to those found in *Myrciaria dubia* (Maués & Couturier 2002) and *Eugenia myrcianthes* (Guollo *et al.* 2023). This group is rarely classified as a legitimate pollinator (Silva & Pinheiro 2007), despite the fact that they have exerted intrafloral and inter-plant movement. Unlike the representatives of the orders Coleoptera and Vespidae, which were classified as resource plunderers, in line with the observations reported in *Campomanesia guazumifolia* and *E. myrcianthes* (Guollo *et al.* 2021, 2023).

Melipona sp. bees, the main pollinators of *M. euosma* and *S. reitzii*, seek pollen from Myrtaceae flowers to feed the offspring of their colonies (Pimentel *et al.* 2023). In turn, the flowers of various Myrtaceae depend on those of this group to ensure their reproduction through effective pollination, due to the adherence of pollen to their legs (Guollo *et al.* 2021).

Given the results, it can be deduced that *M. euosma* and *S. reitzii* play complementary roles as sources of resources for the local bee entomofauna, since they dominate the region's tree community (Martins-Ramos *et al.* 2011). Likewise, the presence of Myrtaceae in the landscape can provide a source of food for different groups of insects (Gressler *et al.* 2006, Nucci & Alves-Junior 2017), thus promoting the health and resilience of insect populations and, consequently, the ecosystems in which they are found.

Reproductive system. Tests on the reproductive system of *M. euosma* indicated a low fruiting rate with the open pollination treatment, possibly due to the quantity and quality of pollen transported (Polatto & Alves-Junior 2009). High flower abortion may also be a common behaviour of flowers with high sensitivity (Link 2000), similar to that observed in *Plinia cauliflora*, which has massive flowering with the formation of few fruits (Semensato *et al.* 2020).

Despite the low reproductive efficiency for *M. euosma*, it can be seen that the efficiency in fruit production by natural pollination was higher than the test with manual pollination (geitonogamy and xenogamy), indicating the need for a specific pollen transfer vector in the reproductive process. In this case, the pollinator deposits specific amounts of pollen under the flower's stigma, increasing the chances of successful fertilization and, consequently, in the fruit development (Polatto & Alves Junior 2009). The results presented here are similar to those reported for *Psidium cattleyanum* Sabine (Neto 2018) for testing natural and controlled pollination.

The null rate of fruit formation for the self-pollination and geitonogamy tests suggests the presence of self-incompatibility for *M. euosma* (SI < 0.25). This phenomenon can occur in Myrtaceae (Fidalgo & Kleinert 2009, Guollo *et al.* 2023) and implies that an individual cannot fertilize its own ovules, preventing self-pollination. In fact, there are no comprehensive investigations into the dynamics of the reproductive biology of the *Myrceugenia*. The closest study, conducted by Lughadha & Proença (1996) for the subfamily Myrtoideae, indicates the probable presence of late (post-zygotic) self-incompatibility in representatives of *Myrceugenia*, due to the scarce production of seeds in relation to the quantity of ovules present in the ovaries. This may also be a characteristic of *M. euosma*, since a maximum of one seed is produced per locule, totalling three seeds per fruit (Wagner & Fiaschi 2020).

In the case of *Myrceugenia euosma*, self-incompatibility favours cross-fertilization, ensuring greater genetic diversity in the offspring, which is fundamental for resistance to diseases, environmental variations and other selective pressures (Goldberg *et al.* 2010). Therefore, the probable presence of this mechanism in *Myrceugenia euosma* highlights the importance of understanding the reproductive mechanisms for the conservation and the sustainable management of this species, and further tests are suggested to define whether its probable self-incompatibility is pre-zygotic (gametophytic or sporophytic) or post-zygotic (Lughadha & Proença 1996, Schifino-Wittmann & Dall'Agnol 2002).

Similarly, the tests on the reproductive system of *S. reitzii* indicated a probable self-incompatibility due to the zero fruiting of the self-pollination treatments (SII < 0.25). It is known that the reproductive system of Myrtaceae can vary

from complete self-sterility to the occurrence of apomixis (Silva & Pinheiro 2007, Fidalgo & Kleinert 2009). For the genus *Siphoneugena*, Proença & Gibbs (1994) suggest a predominance of self-incompatibility. According to their studies, *Siphoneugena densiflora* showed self-incompatibility, since no fruit was formed in the treatment with its own pollen. Furthermore, it was observed that the abscission of the self-pollinated flowers of *S. densiflora* occurred four weeks after the pollination, indicating the presence of late (post-zygotic) self-incompatibility. It is also worth noting the slow development of the fruit in this species, a characteristic similar to *S. reitzii* (Proença & Gibbs 1994). Despite this hypothesis, future studies are suggested to determine whether the probable self-incompatibility in *S. reitzii* is pre-zygotic (gametophytic or sporophytic) or post-zygotic.

Given the limited number of studies on the genus *Siphoneugena*, the results presented here provide resources on reproductive biology and ecology. This scarcity of information highlights the importance of encouraging more detailed investigations to expand knowledge of the genus and, by extension, contribute to a broader understanding of the reproductive mechanisms of Myrtaceae.

Acknowledgements

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