



Unveiling the reproduction of *Proteopsis argentea* (Asteraceae): how the complex pollination system might influence its conservation status

João Paulo Raimundo Borges¹ · Ilmara Aparecida Oliveira Ferreira² · Danubia Natalina Silva² · Cinthia Soares Novaes¹ · Ana Carolina Pereira Machado¹ · Gudryan Jackson Baronio³ · Leonardo Ré Jorge^{4,5} · André Rodrigo Rech^{1,6}

Received: 24 November 2023 / Revised: 30 May 2024 / Accepted: 20 June 2024
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Abstract

The reproductive success of organisms depends on the positive and negative interactions it establishes throughout its lifetime. Pollinators and mutualists increase adaptive value, while herbivory and antagonism impair plant performance. Asteraceae flowers, simultaneously interacting with pollinators, herbivores and parasitoids with the same structure (the flower head), serve as a model for studying these interactions. In this work we describe the multi-layered interactions between *Proteopsis argentea*, its pollinators, endophagous herbivores and parasitoids, aiming to understand how they influence the plant's reproductive success and whether these processes are associated with its conservation status as vulnerable. We found that *P. argentea* produces little, but highly energetic nectar, acting in interactions with bees, hummingbirds and lepidopterans in a generalist pollination system. Antagonistic interactions were much less diverse, dominated by *Xanthaciura* aff. *chrysur*. In addition, two morphospecies of parasitoid wasps used the flower heads of *P. argentea*, and may have a negative impact on antagonists. We also found that *P. argentea* presents a reproductive insurance strategy, via autogamy, producing seeds without pollinators. The flowers of *P. argentea* seem to act as efficient filters, promoting interactions with potential pollinators and parasitoids and limiting interaction with endophagous herbivores. The reproductive strategies of *P. argentea*, with an apparently efficient floral filter and reproductive insurance in the absence of pollinators, seems to indicate that reproduction is not a limiting factor for the species. Thus, for its conservation, measures ensuring the protection of its occurrence area should be prioritized.

Keywords Campos rupestres · Endophages · Parasitoids · Pollinators · Reproductive strategies

✉ João Paulo Raimundo Borges
jpaulorbio@gmail.com

¹ Programa de Pós-Graduação em Ciências Florestais – PPGCF, Universidade Federal dos Vales do Jequitinhonha e Mucuri – UFVJM, Rodovia MGT 367, número 5000, Alto da Jacuba, Diamantina, Minas Gerais CEP 39100-000, Brazil

² Departamento de Ciências Biológicas, Universidade Federal dos Vales do Jequitinhonha e Mucuri – UFVJM, Rodovia MGT 367, número 5000, Alto da Jacuba, Diamantina, Minas Gerais CEP 39100-000, Brazil

³ Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo (IB-USP), Rua do Matão 321, Travessa 14, São Paulo CEP 05508-090, Brazil

⁴ Department of Ecology, Institute of Entomology, Biology Centre, Czech Academy of Sciences, Branišovská 31, 370 05 Ceske Budejovice, Czech Republic

⁵ Department of Zoology, Faculty of Sciences, University of South Bohemia, Branišovská, 31, 370 05 Ceske Budejovice, Czech Republic

⁶ Programas de Pós-Graduação em Biologia Animal (PPGBA) e Estudos Rurais (PPGER), Universidade Federal dos Vales do Jequitinhonha e Mucuri – UFVJM, Rodovia MGT 367, número 5000, Alto da Jacuba, Diamantina, Minas Gerais CEP 39100-000, Brazil

1 Introduction

Interactions between organisms are ubiquitous in ecosystems and play a fundamental role in the stability of biological communities and the maintenance of biodiversity (Bronstein 2009). These interactions are diverse in nature, ranging on a *continuum* from mutualistic, when there is a positive balance for the species involved, to antagonistic, when those involved are impaired (Almeida-Neto et al. 2010; Rech and Brito 2012). Taken together every group of interactions established could be considered as a layer of connectivity within the interactive community (Kivela et al. 2014). In nature, plants are simultaneously exposed to these interaction layers, and they significantly influence the performance of individuals in populations (Morris et al. 2007). Understanding how traits vary between individuals and how they mediate the net effects of mutualistic and antagonistic interactions in a species throughout its lifetime is an important challenge for understanding the potential evolutionary forces operating on its genotype and phenotype (Siepielski and Benkman 2010).

In the context of mutualistic interactions, plants and their pollinators play a key role in maintaining biodiversity (Biesmeijer et al. 2006). Pollen transfer between flowers is essential for fertilization and the development of viable seeds in most flowering plants (Ollerton et al. 2011). Understanding the mutual dependence between plants and their pollinators is fundamental for the conservation of these interactions, and the loss or change in the behavior of pollinators can have negative effects on the plants relying on them (Biesmeijer et al. 2006). In this sense, we can find a myriad of structures and behaviors that different plant species may employ to attract, retain, and exploit pollinators as pollen dispersers (Pyke 2016; Gurevich and Hadany 2021).

By signaling the existence of resources, flowers also attract organisms that may not contribute to pollination or seed dispersal, but only feed on the resources offered, creating the so-called *defense-apparency dilemma* (Huang et al. 2022; Sasidharan et al. 2023). The presence of herbivorous organisms, such as florivores or seed parasites, represents a major cost to plant fitness (Almeida-Neto et al. 2010). Just as pollinator's absence impairs or prevents the reproductive success of a plant, the presence of a herbivore can have a similar effect. These herbivores feed directly on the reproductive structures of plants, resulting in a decrease in floral attractiveness, viable seeds and reproductive success (Boaventura et al. 2022). In this dilemma, natural selection must favor the functioning of flowers as filters, attracting mutualists and repelling or restraining the access of antagonists (Shuttleworth and Johnson 2009; Papiorek et al. 2015).

It is important to highlight that the traditional dichotomy between mutualism and antagonism may be too

simplistic to capture the complex dynamics of ecological relationships (Fontaine et al. 2011). A more realistic perspective, which recognizes the fluid and multifaceted nature of these interactions, is the objective addressing of interactive layers (Pilosof et al. 2017). This perspective recognizes that relationships between organisms are not static, but rather dynamic, varying in degrees of mutualism and antagonism over time and in different contexts (Bronstein et al. 2003; Chamberlain et al. 2014). Adopting this holistic view is fundamental to a deeper and more accurate understanding of the complex relationships that sustain the web of life in ecosystems.

Asteraceae is one of the largest and most diverse plant families, presenting a wide variety of species all over the world. In Brazil, the Asteraceae are represented by 335 genera, with 2228 species, of which 1369 are endemic (Flora do Brasil 2024). Furthermore, it is a highly suitable study model for investigating mutualistic and antagonistic interactions simultaneously (Almeida et al. 2006; Theis et al. 2007; Perre et al. 2011; Amorim et al. 2021; Lewinsohn et al. 2022). Theis et al. (2007) revealed that plants of this family, such as the *Cirsium* species investigated, can adjust the emission of fragrance to attract pollinators during periods of insect activity and minimize the attraction of floral herbivores. This is because the flower-head inflorescence (capitulum), where the flowers and fruits (achenes) are located, interact concomitantly with pollinators and herbivores (flower-head endophages) (Theis et al. 2007). This dual attraction of different groups of organisms with different net effects on fitness makes the Asteraceae an exemplary model for examining the complex relationships between plants and the various organisms they interact with (Romero and Vasconcellos-Neto 2005). It also allows us to understand the evolutionary mechanisms resulting from this balance of ecological effects on propagule production (Cosmo et al. 2023). Although the Asteraceae family has been considered in ecological studies focused on community assembly (Almeida et al. 2006; Nascimento et al. 2014; Bergamini et al. 2017), detailed analyses of the antagonistic forces operating with the organisms attracted to floral structures are needed. The intricate network of relationships between plants in the family, pollinators, endophages and their parasitoids, raises the need for a thorough investigation into the floral functioning of Asteraceae.

In this scenario, *Proteopsis argentea* Mart. & Zucc. ex Sch.Bip. (Asteraceae) stands out as an herb endemic to the *campos rupestres* classified as vulnerable to extinction (Ministério do Meio Ambiente 2022; Loeuille 2024), which is aggravated by its occurrence in geographically isolated populations (Jesus et al. 2001), many of them outside protected areas (Martinelli and Moraes 2013), which can lead to a loss of genetic variability. This spatial fragmentation, mediated by habitat loss and anthropogenic pressures, is one

of the main threats to the persistence of this species (Flora do Brasil 2024). Curiously, despite its critical position in the context of conservation, the natural history of this species remains largely unknown as it seems to be the case for endangered Asteraceae in general. It is in this context that our case study is inserted, aiming to fill this gap with a comprehensive investigation of the interactions occurring in the flower heads of *P. argentea*. We also sought to understand what are the potential consequences of these interactions to the conservation efforts of this species. Our primary objective was to evaluate seed production in *P. argentea* to determine the role of pollination and herbivory in its reproductive success. We particularly pursued how these factors influence the persistence of *P. argentea* and, based on this understanding, help develop more effective conservation strategies to protect vulnerable plant species.

2 Materials and methods

2.1 Study area

Our study was conducted between March and April 2023, in a natural area of *campos rupestres* belonging to the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM – Fig. 1). *Campos rupestres* are ancient ecosystems found at altitudes above 900 m, characterized by rocky outcrops and shallow, acidic, nutrient-poor soils (Alves et al. 2014).

Most *campos rupestres* are distributed in the Espinhaço mountain range, in the states of Bahia and Minas Gerais. These are megadiverse environments, concentrating almost 15% of the Brazilian flora in less than 1% of its national territory, with a large number of endemic species (Silveira et al. 2016). We sampled 22 *Proteopsis argentea* individuals, which produced a total of 54 glomeruli. Each glomerulus, in turn, produced on average 13 ± 2.2 inflorescences. *Proteopsis argentea* only occurs in *campos rupestres*, within occurrence sites smaller than 500 km². Despite their importance and the

little we know about how they function, *campos rupestres* have faced significant threats due to human activity.

2.2 Flower morphology

We measured the tube length of *P. argentea* flowers from nine different individuals. For each individual, we selected two flowers from different inflorescences. We measured floral tubes using photographs shot, with the Sony Handycam HDR-CX405 Full HD NTSC/PAL camera, against 10-mm-scaled paper (Schneider et al. 2012) and the ImageJ software.

2.3 Nectar soluble sugar production and concentration

We quantified nectar production and concentration in 10 individuals of *P. argentea*. We measured the sugar concentration in the nectar in all of them, but in only 8 it was possible to measure nectar volume. The nectar sugar concentration was measured using a portable refractometer (Hilitand) with a measuring range of 0–50% Brix and a resolution of 0.1%. To measure nectar volume, we bagged the inflorescences with organza bags at sunset the day before data collection. The following day, between 8 and 10 am, we measured the nectar volume using a microcapillary (1 µL) in a flower at anthesis per inflorescence. The nectar was absorbed by the microcapillary tubes due to capillarity. The amount of nectar withdrawn was then measured by the height of the nectar column inside the tube, and the filled proportion of the entire column (1 µL) was calculated. Subsequently, the nectar in the microcapillary tubes was washed with distilled water (Morrant et al. 2009). The number of inflorescences evaluated per plant varies between one and five (30 per volume and 36 per concentration) depending on the availability of inflorescences on the plant at the time of data collection.

Fig. 1 **A** Panoramic view of the study region. **B** *Proteopsis argentea* in its natural habitat in Diamantina, Minas Gerais, Brazil. The image board was created with Microsoft Office Professional Plus 2019 software (version 2203) for Windows 64 bits



2.4 Seed production

We selected 14 plants to quantify and qualify their seed production. The average number of inflorescences per individual was $5 (\pm 2.5)$. Following flower senescence, we bagged the inflorescences to prevent damage and protect the developing seeds. Once mature, seeds were collected, counted and separated into viable and non-viable based on morphological viability criteria. We considered full seeds (i.e., containing endosperm) as viable and empty seeds (without endosperm) as non-viable (Feitosa et al. 2009).

2.5 Dependence on biotic pollination for seed production

To assess pollinator dependence, we conducted an experiment with eight individuals, each subjected to two conditions: In the treatment, the flower buds ($n = 31$) were isolated from floral visitors with organza bags. In the control group, the same number of flower buds on each plant were marked with cotton strings, with free access to pollinators. Following the fruiting period, once the seeds were ripe, we counted and compared the total number of seeds produced in both groups.

2.6 Floral visitors

We observed 19 *Proteopsis argentea* individuals during the flowering period between March and April 2023. During this period, we collected a total of 31 h in data on interactions between plants and their floral visitors. We recorded the number of open flowers and the number observed per plant at each moment. In addition, we carried out focal observations at regular intervals of 30 min for each individual, taking notes and identifying the species of floral visitors that visited them.

2.7 Flower-head endophages

To assess the incidence and abundance of herbivory by flower-head endophages and abundance of parasitoids, we collected *P. argentea* inflorescences at the moment they entered senescence following standard methodology (Almeida et al. 2006). After collection, we placed the flower heads in plastic jars covered with organza to allow air to pass through and ensure the emergence of insects from inside the flower structures.

Every week, the emerged insects were euthanized with carbon dioxide and by freezing. This approach ensured

that their morphological features were preserved for later taxonomic identification at the lowest possible level.

2.8 Data analysis

Reproductive Success Analysis: To analyze reproductive success, we used a generalized linear mixed model (GLMM) with a binomial distribution and logit link function. The model was fitted using the `glmer` function from the `lme4` package in the R programming environment. The model formula was defined as the count of "Feasible" and "Infeasible" events relative to a constant predictor, with a random term for the effect of the individual. Akaike (AIC) and Bayes (BIC) information criteria, as well as deviation and log-likelihood estimates were recorded to assess the fit of the model to the data.

Furthermore, we calculated the average probability of success using the `emmeans` package and its function `emmeans`. We specified the response type as "response" to obtain the mean probability estimates and their 95% confidence intervals.

Pollinator Dependency Analysis: To assess pollinator dependence on seed production, we fit a linear mixed model (LMM) using the `lmer` function from the `lme4` package. The model formula included the number of seeds as the response variable, with treatment as a fixed predictor and a random term for the individual effect. We used the restricted maximum likelihood (REML) estimation method to fit the model. We recorded the deviations and parameters of the random and fixed effects.

Furthermore, we performed mean contrasts between the "With pollinator" and "Without pollinator" treatments using the `emmeans` package and its `emmeans()` function. We calculated the estimated means for each treatment along with their 95% confidence intervals. The estimated difference between treatments was recorded, along with the p-value associated with the contrast, to assess the statistical significance of the differences.

3 Results

3.1 Floral and phenological aspects and nectar soluble sugar production and concentration

Proteopsis argentea flowers present a tubular structure with an average size of 1.6 cm ($SD \pm 0.1$ cm) and a lilac color. They are arranged in flower-head inflorescences (capitula), organized in glomeruli. In these inflorescences, flower heads flower asynchronously. We observed an average production of accumulated nectar per flower of approximately 0.7 ± 0.2 μ L, composed of around $43 \pm 0.5\%$ SD of sugars.

3.2 Seed production and dependence on pollinators

Our results indicate a high probability of reproductive success among the individuals studied, with an overall estimate of approximately 95.1%. Furthermore, with a 95% confidence level, we can state that the proportion of viable seeds is between 89% and 97.9%. The adjusted model presented reasonable AIC and BIC values, indicating an adequate fit to the data. Finally, the random effects included in the model were considered, recognizing the possibility of variation not explained by the explanatory variables included.

Our results show similar estimated means for the number of seeds produced in both treatments in *P. argentea*. With pollinators the estimated average was 105.6 seeds, with a confidence interval of 92.3 to 119. Without pollinators, the estimated average was slightly lower, totaling 97.3 seeds, with a confidence interval of 84.0 to 111. When comparing these treatments, the estimated difference between them was 8.23 seeds and not significant ($p=0.2$, Fig. 2).

3.3 Floral visitors

We observed a rich diversity of pollinators visiting the inflorescences of *P. argentea*. Bees stood out as the most representative group of interactions (55.3%), with the species *Bombus morio*, *Agapostemon* sp. *Centris tarsata* and Megachilidae sp.1 (Fig. 3) as the most abundant in this group, with 232, 153, 121 and 100 interactions, respectively. In addition, the hummingbirds *Augastes scutatus* and *Chlorostilbon lucidus* also played an important role in visitation, with 96 and 44 interactions respectively, together accounting for 12% of the 1136 interactions observed involving all visitors (Fig. 3).

We also observed dipterans (9.2%) visiting the flowers of *Proteopsis argentea*, represented by 11 different morphotypes, totaling 105 interactions. In addition, we recorded

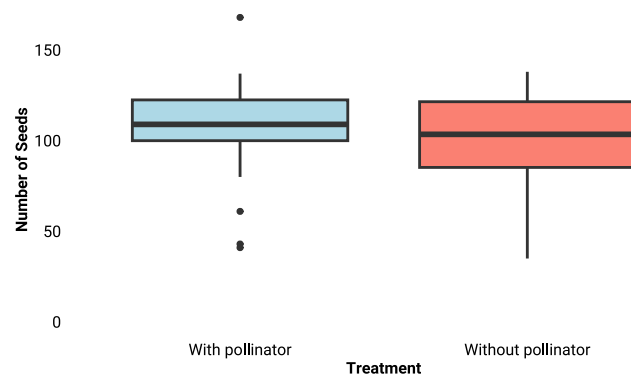


Fig. 2 Seed production with and without pollination in *Proteopsis argentea* in Diamantina, Minas Gerais, Brazil

other groups of visitors, such as beetles (9 morphotypes with 0.8% of interactions), butterflies including *Sarbia damippe* (25 visits) and *Synargis paulistina* (2 visits) which accounted for 2.4% of visits and a wasp, *Dielis trifasciata*, with a single interaction record (Fig. 3). The visit frequency distribution curve showed an inverted J shape with many rare species and few frequent ones (For a more detailed analysis, please refer to Supplementary Fig. 4).

3.4 Flower-head endophages

With 42 individuals collected, *Xanthaciura* aff. *chrysur*a was the main herbivore of *Proteopsis argentea*. In addition, a Tortricid was identified as a herbivore, with 14 occurrences. We also observed the morphospecies *Podagrion* sp. (Spinola, 1811) and another Torymid, both parasitoid wasps, with one occurrence each.

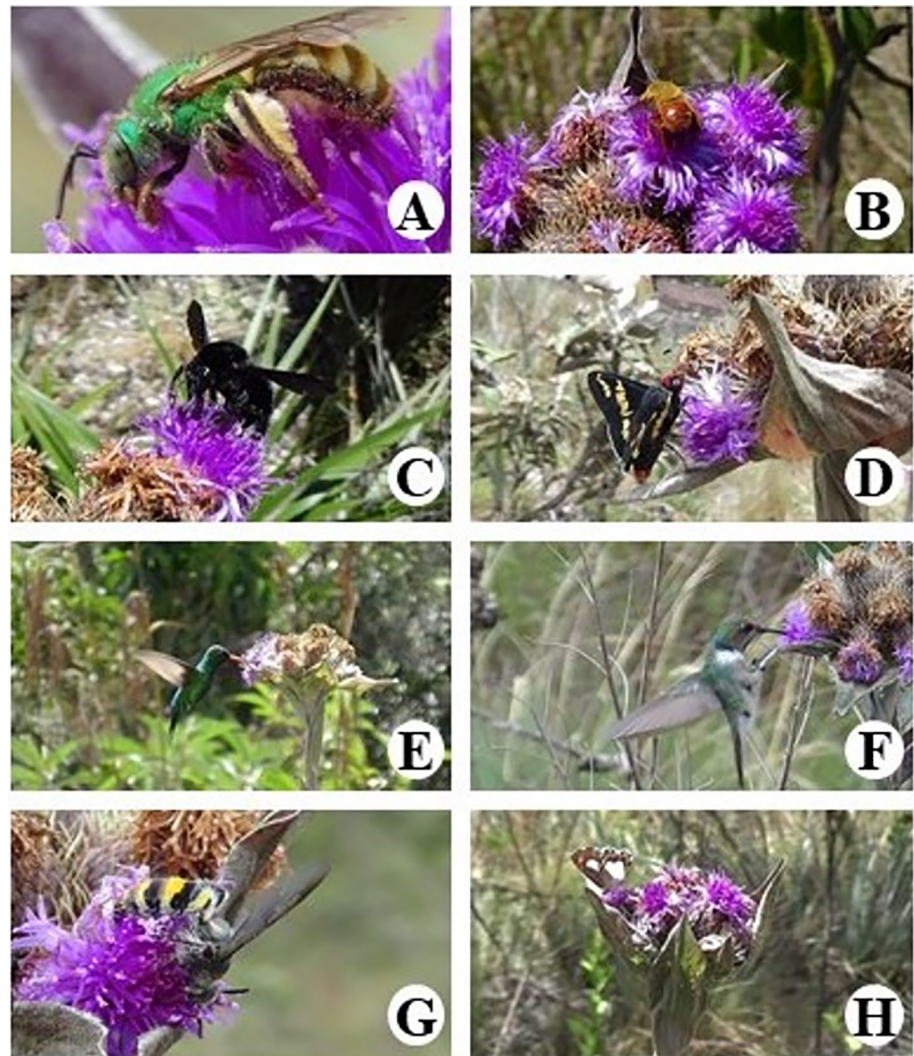
4 Discussion

Proteopsis argentea is an autogamous species that attracts a large number of floral visitors and has good reproductive success, as measured by the production of viable seeds in the wild. The generalization of interaction with pollinators contrasts with its restriction when it comes to endophagous herbivores. This dichotomy suggests an adaptive strategy involving a balance between dependence on pollinators and the need for self-defense against herbivores, reflecting an evolutionary response to environmental fluctuations (Zhang et al. 2022). While the floral traits of this species attract a wide range of pollinators, the concomitant repulsion mechanism directed at endophagous herbivores can be considered a form of specialized defense, resulting in the preservation of floral and progeny integrity and, consequently, the plant's reproductive success. In this sense, *P. argentea* seems to have effective floral filters to ensure that pollinators are attracted but not antagonistic herbivores.

While nectar production suggests a highly effective floral reward strategy to attract and maintain a variety of visitors, the fruiting rate did not differ between pollinated and bagged flowers, suggesting a reproductive security strategy in the absence of pollinators. Likewise, we can see a morphological specialization in the floral tubes, which allows legitimate access to nectar only for pollinators with long mouthparts. Moreover germination tests are required to define the quality of each group of seeds produced.

The high frequency of hummingbird visits to *Proteopsis argentea* indicates that these birds benefit from highly energetic nectar produced (Johnson & Nicolson 2008; Van der Kooi et al. 2021). Nectar production is a costly activity for plants (Pyke and Ren 2023). Therefore, it is likely that this activity has some benefit for plant reproduction, such as

Fig. 3 Floral visitors of *Proteopsis argentea*. **A** Halictidae sp. **B** *Centris tarsata*. **C** *Bombus morio*. **D** *Sarbia damippe*. **E** *Chlorostilbon lucidus*. **F** *Augastes scutatus*. **G** *Dielis trifasciata*. **H** *Synargis paulistinha*. The image board was created with Microsoft Office Professional Plus 2019 software (version 2203) for Windows 64 bits



genetic diversity. In fact, nectar attracts many visitors, which can increase the chances of pollination, which has also been reported in several other species, even in families predominantly composed of plants that offer pollen as a reward (Brito et al. 2017).

Mutualistic interactions predominated, with bees standing out as the main floral visitors. This observation corroborates the relevance of these insects as key pollinators in natural ecosystems (Pinheiro et al. 2014). In addition, previous studies, such as Monteiro et al. (2021), showed that bee pollination was predominant in a large sample of 636 plant species studied, totaling 56% of the interactions recorded in *campos rupestres*. Larger bees such as *Bombus morio* and *Centris tarsata* stood out as the species that interacted most with the flower heads of *P. argentea*. In addition, hummingbirds *Augastes scutatus* and *Chlorostilbon lucidus* also played an important role in visitation, demonstrating the importance of these birds as pollinators. The occurrence of *P. argentea* in small populations scattered throughout *campos rupestres*

highlights the importance of pollinators with large body sizes, such as bees and hummingbirds, capable of flying long distances and promoting the exchange of genetic material between distant populations. This contributes to genetic diversity and the conservation of the species (Monteiro et al. 2021).

Our results show that *Proteopsis argentea* is autogamous, resulting in a reproductive security mechanism (Rech et al. 2018). This reproductive flexibility can be interpreted as an advantageous adaptation to environments with a shortage or fluctuations in the availability of effective pollinators, as well as being beneficial for small, dispersed populations subject to recurrent local extinction events. However, although autogamy can represent a reproductive security mechanism, this strategy can also be associated with costs, such as the loss of genetic variability and increased inbreeding (Jesus et al. 2001; Herlihy & Eckert 2002). Therefore, the studied *P. argentea* population seems to be benefiting from a balance between cross-pollination by animals and autogamy to

optimize its reproductive efficiency under different ecological scenarios.

We observed a low diversity of antagonists, which, together with the presence of natural enemies, are integrated with the positive effects of pollinators and even autogamy. Such interactions can play a crucial role in population regulation and behavior of endophagous, exerting an indirect influence on plant reproduction. The relevance of these interactions is supported by studies such as Nunes et al. 2018, who investigated a scenario in which weevils pollinated orchids before their larvae feed on the growing fruit. The results revealed that this relationship has an adverse impact on the plant, since the endophagous larvae often consume the entire infested fruit. However, parasitoid wasps often *rescue* part of the infested fruit, eliminating the endophagous larvae before the entire fruit content is consumed. Fruits that are *rescued* exhibit seed viability and biomass comparable to that of uninfested fruits, thus highlighting the fundamental relevance of parasitoids in mitigating the effects of the interaction between the plant and its parasitic pollinator on the plant's fitness.

We conclude that the reproductive ecology of *Proteopsis argentea* is truly complex, showing a multilayered network of interactions between pollinators, endophages, parasitoids and the plant itself. The filter function performed by the flowers, the dual mechanism of reproductive security, as well as the presence of long-flight pollinators such as bees and hummingbirds, play vital roles in the efficient transfer of pollen between small and distant populations of *P. argentea* in the mosaic-like environment of *campos rupestres*. Our results seem to indicate that the vulnerable threatened status of *P. argentea* is not associated with its ability to produce seeds, but with other processes acting on germination, recruitment, establishment, and maintenance of viable populations in its potential area of occurrence. In addition, many of these populations are located outside protected areas, making them vulnerable to human activities and environmental degradation, which further accentuates the challenges for the preservation of *P. argentea* and its complex reproductive ecology in *campos rupestres*. Urban expansion, eucalyptus agriculture and silviculture, mining and other anthropogenic processes have contributed to the reduction of these natural areas, threatening the survival of various species, and negatively impacting biodiversity in the *campos rupestres* (Alves et al. 2014; Fernandes 2016; Fernandes et al. 2018).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s40415-024-01025-8>.

Acknowledgements Fundação de Amparo à Pesquisa do Estado de Minas Gerais—FAPEMIG (APQ-00932-21, APQ-03100-21, RED-00253-16, APQ-02806-22, APQ-03364-21, APQ-01151-22, APQ-01822-21), Conselho Nacional de Desenvolvimento Científico e

Tecnológico—CNPq (311665/2022-5, 400904/2019-5, 423939/2021-1), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—CAPES (Financial Code 001, 88887.837988/2023-00), Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP nº 2021/09247-5.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JPRB, IAOF, DNS and CSN. The first draft of the manuscript was written by JPRB. ACPM, GJB, LRJ and ARR reviewed and commented on previous versions of the manuscript. All authors read and approved the final manuscript. The authors have no conflict of interest to declare.

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