UNIVERSIDADE FEDERAL DO PARANÁ

LUAN SALLES PASSOS

ESTRATÉGIAS REPRODUTIVAS EM MELASTOMATACEAE DA FLORESTA ATLÂNTICA

CURITIBA
2021

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ESTRATÉGIAS REPRODUTIVAS EM MELASTOMATACEAE DA FLORESTA ATLÂNTICA

Dissertação apresentada como requisito parcial à obtenção do título de Mestre pelo Programa de Pós-Graduação em Botânica, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientador: Prof. Dr. Fabiano Rodrigo da Maia
Coorientador: Prof. Dr. Renato Goldenberg
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CURITIBA
2021

Universidade Federal do Paraná
Sistema de Bibliotecas
(Giana Mara Seniski Silva – CRB/9 1406)

Passos, Luan Salles
Estratégias reprodutivas em Melastomataceae da Floresta Atlântica. / Luan Salles Passos. – Curitiba, 2021.
128 p.: il.

Orientador: Fabiano Rodrigo da Maia.
Coorientador: Renato Goldenberg.
Coorientadora: Francismeire Jane Telles da Silva.

Dissertação (mestrado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Botânica.

1. Melastomataceae. 2. Antera. 3. Polinização. I. Título. II. Maia, Fabiano Rodrigo da. III. Goldenberg, Renato, 1968-. IV. Silva, Francismeire Jane Telles da. V. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Botânica.

CDD (22. ed.) 583.76

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIÊNCIAS BIOLÓGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO BOTÂNICA -
40001016004P9

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em BOTÂNICA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **LUAN SALLES PASSOS** intitulada: **Estratégias Reprodutivas em Melastomataceae da Floresta Atlântica**, sob orientação do Prof. Dr. FABIANO RODRIGO DA MAIA, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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Dedico esta dissertação à Paulina, Antônio e Marcos, que, de forma tão sutil e particular, mostraram-me a complexidade e beleza dos padrões e processos da Natureza! À Fran, pelo zelo por mim e minha irmã, além dos presentes (“de grego”) que nos deixou. Eu os amo muito!

Gostaria que estivessem aqui para curtir esse fim de ciclo comigo.

A saudade tem tamanho diretamente proporcional a esta nossa conquista!
Obrigado!

AGRADECIMENTOS

Entre subidas e descidas ao morro da UHE Parigot de Souza, muitas vezes realizadas apenas sobre meus pés e viajando em meus solitários e constantes pensamentos, cercado pela deslumbrante e imponente vegetação da Mata Atlântica, percebi que este projeto e sua finalização só foram possíveis graças aos esforços de muita, mas muita gente mesmo. Muitas pessoas investiram tempo e dinheiro para dar seguimento a este trabalho, muitas pessoas dobraram seus esforços para dar conta de um lar onde não me fiz presente, muitas pessoas me apoiaram e me deram suporte durante minhas próprias dificuldades. Portanto, esta seção é obrigatória para que eu possa considerar "nosso trabalho" concluído.

Primeiramente, agradeço às pessoas que me possibilitaram não só concluir esta dissertação, mas também minha graduação, além de terem sido meu 'porto-seguro' durante um longo e difícil período. São elas, Joana D'Arc (sim, a guerreira, Minha Mãe) e Tainã. Sem elas e suas "insistências" nada disso seria possível, nada mesmo. Muito obrigado pelo apoio e entusiasmo. Sem a força dessas Mulheres não seria possível tocar em frente. Obrigado!

Outras duas Mulheres foram e são essenciais para a concretização desta conquista: Naiana e Aiyra. Agradeço a compreensão por todas as ausências durante as expedições de campo, pelo apoio diário durante o período de escrita (principalmente na pandemia), e pelas flores que a Aiyra coleta diariamente e deixa em minha mesa! Obrigado meus amores, é impressionante e deliciosa a forma como vocês modificaram minha vida!

Agradeço aos meus irmãos Ian e Cauã pela compreensão, apoio e suporte durante todos os perrengues. Agradeço também a toda minha família que sempre teve paciência para ouvir sobre meu trabalho com as "abelhinhas". Em especial a minha avó Rosa, nosso xodozinho, Cris, às minhas tias e meus tios sempre presentes, além dos meus primos e primas! Obrigado, Família!

Eu tive, sem sombra de dúvidas, uma das melhores orientações que a vida acadêmica poderia ter me dado. Meus orientadores, de forma idiossincrática, se complementavam e me forneceram ferramentas essenciais para a conclusão desta dissertação e para meu desenvolvimento pessoal! Trabalhamos muito, não é?

Fabiano Rodrigo da Maia, meu orientador e amigo. Graças a ele tive o prazer de conhecer o magnífico universo da interação entre plantas e polinizadores. Através dele ingressei na área acadêmica e científica. Sou imensamente grato pela oportunidade que me ofereceu em um momento que muito precisava! Obrigado, parceiro!!!

Ao meu coorientador Renato Goldenberg, pela paciência e palavras amigas nos momentos de necessidade. Grande exemplo de pesquisador e de pessoa! Aprendi muito não somente no âmbito acadêmico, mas também sobre dedicar amor e excelência nas coisas que se constrói, principalmente na Família. Como dizem pelos corredores: Meu “pai-científico”... (hahahaha) brincadeira!

Agradeço à Meire Telles, coorientadora e amiga. Mulher, guerreira e inspiradora. A pessoa que injetou uma enorme dose de ânimo e esperança na minha busca por uma vivência acadêmica e científica livre do patriarcado, do abuso e do conservadorismo tóxico. Obrigado por tudo, Meire! Aprendi muito (de verdade) contigo!

Agradeço às professoras e professores do PPG-Botânica da Universidade Federal do Paraná por compartilharem o conhecimento e espaços necessários para o desenvolvimento desta dissertação. Agradeço especialmente a Professora Isabela Galarda Varassin por “me adotar” em seu laboratório e por toda atenção em momentos de dúvidas, sempre adicionando ideias e contribuindo com meu crescimento.

Agradeço aos meus “irmãos-científicos”: Thuane Bochorny, Lucas Bacci e Gessica Bisewski. Muito aturaram sobre as mesmas perguntas ao longo da construção deste trabalho: “Você tem foto?”, “Você tem flor fixada?”, “Sabe isso?”, “Sabe aquilo?”... Obrigado pessoal pelas trocas de ideias acerca desses grupos distintos, mas tão maravilhosos que trabalhamos. Agradeço também a parceria de sempre, pelas conversas, pelos cafés e pelo aprendizado sobre tudo!

Agradeço imensamente a toda a equipe da UHE Parigot de Souza pela presteza, permissão e companheirismo em todos os campos realizados. Agradeço principalmente ao Marcos Cruz por ser meu contato direto e pela confiança! Sem vocês e seus cuidados com a área, este trabalho não existiria. Espero uma oportunidade para poder apresentar nossos achados a todos vocês que me ajudaram nessa caminhada, é o mínimo que posso fazer para agradecer a atenção e dedicação prestadas!

Quero agradecer ao pessoal que vestiu sua roupa mais “mateira” e foi comigo a campo: Jéssica (“Jessicrazy”), minha grande amiga e irmã, maior parceira de campo; Miguelito, curando ressaca de Natal (hahahah), força indispensável em campo; Thiaguera e Leonardo (“Little Jesus”), esse com certeza foi um dos melhores campos que fiz! Valeu mesmo!

Agradeço imensamente às botânicas e botânicos mais amados da região austral do planeta: Dani, Ana, Carla, Andreyzera, Laura, Nicolás, Brunão, Jean, Camila, Mabi, Anna, Luciele, Gabi, Andressa, Joana, Ângela, Ethi, Mateus, Murilão, Maurício, Robin... Pessoas com as quais dividimos cafés, filas do RU, angústias, festinhas, mesas de bar, ou seja, todo o ócio criativo para a construção de uma vivência acadêmica crítica e vanguardista. Com alguns, o laço é tão forte que constituímos uma grande família ‘polifilética’! Né não, Dani?

Agradeço imensamente ao Lucas Katsumi por me emprestar/doar uma muda de *Bertolonia violacea*! Graças a ti, um artigo está publicado (da Gé) e outro está por vir (espero), desculpa o sumiço!

Um obrigado especial às minhas manas e meus manos do laboratório mais inclusivo que há: o LASBINTER! Agradeço a Lô, Ana Lula (Livre!!!), Fefê, Thais, Rafa, Isra, Alejandro, Elena, Artur, Lary, Rafaela, Tiagão, seja pelas trocas de ideia, incentivos em momentos difíceis, cervejinhas, níver surpresa (foi fantástico), ou simplesmente pela presença nessa caminhada! É muito bom saber que sempre terei um lugar aconchegante para cafezinhos e discussões.

A conclusão desta dissertação só foi possível com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) - Código de Financiamento 88882.382585/2019-01.

Por fim, agradeço a Universidade Federal do Paraná, instituição que forneceu, de forma gratuita, infindáveis oportunidades para meu crescimento profissional, crítico e pessoal. Mesmo diante de tantos ataques e do obscurantismo, destaca-se como referência de ensino, extensão, pesquisa de qualidade e resistência. Agradeço sinceramente a cada trabalhadora e trabalhador que possibilitaram com que eu fizesse parte desta admirável instituição, seja diretamente: professores, técnicos ou terceirizados que zelavam pelo transporte, segurança, alimentação e limpeza das áreas da instituição; ou indiretamente, contribuindo com impostos que garantiram a mim o privilégio de me graduar e finalizar um mestrado acadêmico. Lutarei todos os dias para que eu possa pagar esse investimento que fizeram, visando a construção de uma sociedade mais justa, igualitária e progressista!

Enfim, a todas e todos (a ELE NÃO) que contribuíram, de maneira direta ou indireta, na construção desta dissertação o meu mais sincero

MUITO OBRIGADO!!!

"O amor à complexidade sem reducionismo gera Arte.
O amor à complexidade com reducionismo gera Ciência."
Edward Wilson

"Representei com um do verdinho na mente, ok
Não desandei, eu me empenhei,
me dediquei também"
Mauro Mateus dos Santos (Sabotage)

RESUMO

Estratégias reprodutivas refletem processos adaptativos que direcionam a aptidão dos indivíduos no ambiente. Nas angiospermas, muitos desses processos estão associados a atributos florais, com consequências no sucesso reprodutivo das espécies. A família Melastomataceae apresenta vários casos interessantes para o estudo de estratégias voltadas para a polinização e reprodução em suas espécies. O principal objetivo desta dissertação foi estudar as estratégias reprodutivas, através de investigações baseadas na biologia reprodutiva e da polinização, que evoluíram em dois gêneros (i.e., *Huberia* e *Bertolonia*) de Melastomatáceas na Mata Atlântica. Para tanto, dividimos esta dissertação em três capítulos: no Capítulo 1, investigamos possíveis atribuições funcionais dos apêndices estaminais de *H. insignis* através de experimentos de polinização, anatomia floral e observação de polinizadores, a fim de averiguar seu efeito no sistema de polinização da espécie. *Huberia insignis* é autocompatível e dependente de polinizadores para a formação de frutos e sementes, principalmente abelhas do gênero *Bombus*. Não constatamos nenhuma função dos apêndices no sistema de polinização, seja na atratividade dos polinizadores ou na taxa de remoção de pólen durante a vibração das anteras pelas abelhas, portanto, concluímos que esta estrutura seja vestigial em *H. insignis*. No Capítulo 2, investigamos, por meio de experimentos de polinização e técnicas de microscopia, uma potencial capacidade de autopolinização espontânea em espécies de *Bertolonia*; ainda, verificamos se a orientação do poro das anteras poderia estar associada com tal evento. Tanto *B. paranaensis* quanto *B. mosenii* são capazes de se autopolinizar, algo raro em Melastomataceae, independente da orientação dos poros das anteras. Além disso, descrevemos o curioso mecanismo pelo qual se dá a autopolinização e o denominamos "banho de tubo polínico" (em inglês, *pollen tube shower*), que ocorre em flores em pós-antese, garantindo a segurança reprodutiva destas espécies. Por fim, no Capítulo 3, expandimos a mesma pergunta do capítulo anterior para outras duas espécies, *B. violacea* e *B. acuminata*. Desta forma, utilizamos experimentos de exclusão de polinizadores, além de buscar dados em exsicatas e trabalhos taxonômicos das espécies do gênero, que nos permitissem inferir se *Bertolonia* é um gênero puramente autogâmico. Ambas as espécies realizam o *pollen tube shower*. Também encontramos informações taxonômicas que sugerem a possibilidade de autogamia nos três clados de *Bertolonia*, o que nos faz acreditar que, de fato, a autogamia deva estar presente no grupo todo. Em conjunto, os três capítulos mostram que, embora muitos caracteres florais sejam conservados em Melastomataceae, múltiplas estratégias reprodutivas podem ser selecionadas por diferentes grupos e espécies dentro da família ao longo do tempo.

Palavras-chave: Anteras poricidas; *Bertolonia*; biologia floral; *Buzz-pollination*; flores de pólen; *Huberia*; polinização.

ABSTRACT

Reproductive strategies can be driven by different adaptive mechanisms that direct the fitness of individuals in the environment. In angiosperms, many of these processes are associated with floral traits, with consequences on the reproductive success of the species. Melastomataceae presents several interesting cases for the study of strategies for pollination and reproduction in their species. The main objective of this dissertation was to study reproductive strategies, based on reproductive biology and pollination, which evolved in two genera (i.e., *Huberia* and *Bertolonia*) of melastomes from Atlantic Forest. We have divided this dissertation into three chapters: in Chapter 1, we investigate a potential function of the staminal appendages of *H. insignis* through pollination experiments, floral anatomy and observation of pollinators, to verify their effect on the pollination system of the species. *Huberia insignis* is self-compatible and dependent on pollinators for fruit and seed set, especially bees of the genus *Bombus*. We have not found any function of the appendages in the pollination system, neither in the attractiveness of pollinators nor in the pollen removal rate during the vibration of the anthers by buzzing bees, therefore, we conclude that this structure is vestigial in *H. insignis*. In Chapter 2, we investigated a potential capacity for spontaneous self-pollination in *Bertolonia* species through pollination experiments and microscopy analysis; we also verified if the pore orientation of the anthers could be associated with selfing. Both *B. paranaensis* and *B. mosenii* are capable of selfing, something rare in Melastomataceae, independent of the orientation of the anthers pores. Also, we described the curious mechanism by which self-pollination occurs and we call it "pollen tube shower", which occurs in post-anthesis flowers, ensuring the reproductive assurance of these species. Finally, in Chapter 3, we expanded the same question of the previous chapter to two other species, *B. violacea* and *B. acuminata.* Thus, we use pollinator exclusion experiments, in parallel to searching for data on vouchers and taxonomic works for the genus that would allow us to infer whether *Bertolonia* is an autogamous genus. Both species perform the pollen tube shower. We also found taxonomic information that suggests the possibility of autogamy in the three clades of *Bertolonia*, which makes us believe that autogamy should be present in the whole group. The three chapters of this dissertation show that although many floral traits are preserved in Melastomataceae, multiple reproductive strategies can be selected by different groups and species within the family.

Keywords: *Bertolonia*; Buzz-pollination; floral biology; *Huberia*; pollen flowers; pollination; poricidal anthers.

LISTA DE TABELAS

CAPÍTULO 1

TABLE 1. Number and frequency of visits (visits.flower-1.h-1) in flowers (n=265 flowers; during 35 h) of *Huberia insignis* from Antonina, Paraná, Brazil 58

TABLE S1. Results of controlled pollination treatments: Fruit set, production and germination of seeds of *Huberia insignis* (Melastomataceae) from Antonina, Paraná, Brazil.................... 58

TABLE S2. Floral visitors observed in *Huberia insignis* (Melastomataceae) from Antonina, Paraná, Brazil 59

CAPÍTULO 2

Table 1. Abundance, visitation rates (abundance/total number of flowers observed) and behaviour of visitors of *Bertolonia paranaensis* (n = 29 flowers) and *B. mosenii* (n = 26 flowers) 93

CAPÍTULO 3

Table 1. Orientation of pore dehiscence in anthers and length (in mm) of stamens and style in *Bertolonia* Raddi species..108

LISTA DE FIGURAS

CAPÍTULO 1

Figure 1. (A) Flower of *Huberia insignis*. (B) Detail of anthers with the arrows indicating the filiform appendages. (C) Flower with appendages removed for preference and pollen removal tests 59

Figure 2. Results of controlled pollination treatments in flowers of *Huberia insignis*. (A) Fruit set from each pollination treatment; (B) Distribution of the quantity of seeds set in each pollination treatment; (C) Germination rate of seeds from each pollination treatments. Treatments: Manual self-pollination (SP), Control (C) and Cross-pollination (CP). Letters indicate differences or similarities among treatments. Bars represent 95% of confidence intervals 59

Figure 3. Results of staminal appendages experiments of *Huberia insignis*. (A) Proportion of visits in flowers with (Control) and without staminal appendages (Treatment). (B) Quantity of pollen removed in flowers with (Control) and without staminal appendages (Treatment) 60

Figure 4. Scanning electron microscopy images of *Huberia insignis* anthers. (A) Detail of the rough cuticle on anther epidermal cells. (B) Apex of staminal appendages. (C) Elongated cells that in the appendages. (D) Detail of the anther cells, shorter than the cells of the appendages.60

Figure 5. (A) Details of antesepalous (left) and antepetalous stamens (right) of *Huberia insignis*. (B) Anatomical structure of the stamen in a longitudinal section; connective with several layers of parenchyma cells. (C) Transverse section of the proximal portion of the appendage, with the epidermis intensely stained. The arrow indicates the vascular bundles in a central position. (D) Longitudinal section of the proximal portion of the appendages indicating a positive reaction with ferric chloride in the epidermal cells. (E) Transverse section of the distal portion of the appendage subepidermal cells with dense cytoplasmic content. (F) Longitudinal section of the distal portion of the appendages; arrows indicate subepidermal cells that reacted positively to ferric chloride. (G) Longitudinal section; the highlighted area indicates vascular tissue. (H) Positive reaction with PAS in the proximal region of the appendages; starch grains densely stained. Appendages (ap), connective (cn), epidermis (ep) and vascular bundles (vb) 60

Figure S1. (A) *Bombus brasiliensis* and (B) *B. morio* buzz pollinating *H. insignis*. (C) Anthers chewed by *Trigona spinipes* to steal pollen; arrows indicate intact appendages 60

Figure S2. Frequency of visits (visits.flower^{-1}.h^{-1}) in *Huberia insignis* flowers. Letters indicate differences or similarities among treatments........ 60

Figure S3. Wavelength (in nm) reflected by the anthers (dashed line), appendages (dotted line) and petals (whole line) of *Huberia insignis* 60

CAPÍTULO 2

Figure 1. Flowers and stamens of (A) *Bertolonia paranaensis* and (B) *B. mosenii*. (C) Illustration of lateral view and introrse pore detail of a stamen of *B. paranaensis*. (D) Stamen of *B. mosenii*, dorsal view and detail of extrorse double pore 93

Figure 2. Stigma diameter (mm) of *Bertolonia paranaensis* (white circles) and *B. mosenii* (black circles), in pre- and post-anthesis. * represents statistical differences between stages. 93

Figure 3. Fruit-set resulting from the manual pollination experiments in (A) *Bertolonia paranaensis* and (B) *B. mosenii*. Letters indicate differences or similarities among treatments. Ns = non-significant differences among treatments. Control (C), manual cross-pollination (MCP) and autonomous self- pollination (ASP) 93

Figure 4. Seed-set resulting from the manual pollination experiments in (A) *Bertolonia paranaensis* and (B) *B. mosenii*. Letters indicate differences or similarities among treatments. Control (C), manual cross-pollination (MCP) and autonomous self-pollination (ASP) 94

Figure 5. (A) Exudate on the stigmatic surface in a flower of *B. paranaensis.* (B) Flowers of *Bertolonia paranaensis,* details of a flower in anthesis and another one in post-anthesis (in the background) with the corolla removed, showing the stamens positioned around the style. (C) Stigmatic exudate in a post-anthesis flower with the corolla removed of *B. mosenii.* (D) Anthers in contact with the stigma of B. mosenii. (E) Mass of pollen tubes leaving the anther and penetrating the stigma in *B. mosenii*. (F) Selfing pollen tubes growing in the transmitting tissue of *B. paranaensis* and (G) penetrating the ovules. (H) Selfing pollen tubes reaching the ovary and (I) penetrating ovules of *B. mosenii*. (J) *Trichocerapis sp*. pollinating *B. paranaensis*. (K) *Ariphanarthra palpalis* and (L) *Trigonopedia sp.* pollinating *B. mosenii*. Anthers (a), style (s), pollen tubes (pt), transmitting tissue (tt) and ovules (o)....................... 94

Figure 6. Scanning electron microscopy images of reproductive whorls of (A-C) *Bertolonia paranaensis* and (D-F) *B. mosenii*. (A) Pollen grains germinating inside anthers and pollen tubes penetrating the stigma in *B. paranaensis*; (B) detail of pollen tube exiting the anther pore and (C) penetrating the stigma. (D) Pollen grains germinating inside anthers and pollen tube penetrating the stigma in *B. mosenii*; (E) pollen tubes contacting the dilated stigma. (F) Detail of stigma with unicellular papillae receiving pollen tubes in *B. mosenii*. Anthers (a), style (s) and pollen tubes (pt) 94

Figure S1. Dynamics of stamens in flowers of (A-E) *Bertolonia paranaensis* and (F-J) *B. mosenii* during anthesis. (A) Flower of *B. paranaensis* at the beginning of anthesis; (B) at 0800 h, (C) 1000 h, (D) 1500 h, and (E) at 1700 h. (F) Flower of *B. mosenii* at the beginning of anthesis, (G) at 0830 h, (H) 1200 h, (I) 1300 h, and (J) at 1500 h 99

CAPÍTULO 3

Fig. 1 (A) Flowers of *Bertolonia violacea*. (B) *Bertolonia acuminata*. (C) Detail of *B. acuminata* flower. Mass of pollen tubes leaving the anther and penetrating the stigma in (D) *B. violacea* and (E) *B. acuminata*. Flower of *B. violacea* at the end of the anthesis: (F) stamens gathering in the center of the flower, around the style; (G) petals closing with stamens and style included, and (H) flower in post-anthesis with reproductive structures included. (I) Pollen on stigma of *B. maculata*, anther and stigma were connected through pollen tubes but separate during handling. Subtitle: (a) anthers, (s) style and (pt) pollen tubes 109

SUMÁRIO

INTRODUÇÃO GERAL **20**

ESTRATÉGIAS REPRODUTIVAS EM MELASTOMATACEAE 20
MATA ATLÂNTICA: UM EXUBERANTE E AMEAÇADO CENÁRIO 22
ESTRUTURA DA DISSERTAÇÃO 23
REFERÊNCIAS 23

CAPÍTULO 1: TESTING THE FUNCTION OF THE STAMINAL APPENDAGES ON THE POLLINATION SYSTEM OF *HUBERIA INSIGNIS* (MELASTOMATACEAE), A BUZZ-POLLINATED SPECIES / TESTANDO A FUNÇÃO DOS APÊNDICES ESTAMINAIS NO SISTEMA DE POLINIZAÇÃO DE *HUBERIA INSIGNIS* (MELASTOMATACEAE), UMA ESPÉCIE POLINIZADA POR VIBRAÇÃO **29**

ABSTRACT 31
INTRODUCTION 32
MATERIALS AND METHODS 34
Study area *34*
Model Species *35*
Floral biology and mating system *36*
Pollen production and release *37*
Flower visitors, behavior, and frequency *38*
Reflectance of floral parts and staminal appendages removal test *38*
Morphoanatomy of anthers and staminal appendages *39*
Data analysis *40*
RESULTS 41
Floral biology and mating system *41*
Pollen production and release *42*
Flower visitors, behavior, and frequency *42*
Reflectance of floral parts and staminal appendages removal tests *43*
Morphoanatomy of anthers and staminal appendages *44*
DISCUSSION 45
Mating systems, pollinators, and reproductive success *45*
Staminal appendages: an adaptive or vestigial structure? *47*
REFERENCES 51
TABLES 58
FIGURES 59

CAPÍTULO 2: *"POLLEN TUBE SHOWER"* IN *BERTOLONIA* RADDI (MELASTOMATACEAE): A NEW DELAYED SELFING MECHANISM IN FLOWERS WITH PORICIDAL ANTHERS / "*POLLEN TUBE SHOWER*" EM *BERTOLONIA* RADDI (MELASTOMATACEAE): UM NOVO MECANISMO DE AUTOPOLINIZAÇÃO TARDIA EM FLORES COM ANTERAS PORICIDAS **65**

ABSTRACT 67
INTRODUCTION 68
MATERIALS AND METHODS 72
Study site *72*
Model species *72*

Floral biology, stigma, anther, and pollen traits *73*
Mating system, female and male success *74*
Flower visitors and behaviour........ *75*
Data analyses *76*
RESULTS 76
Floral biology, stigma, anther, and pollen traits *76*
Bertolonia paranaensis........ 76
Bertolonia mosenii 77
Mating system, female, and male success *79*
Bertolonia paranaensis........ 79
Bertolonia mosenii 80
Flower visitors and their behaviour *80*
Bertolonia paranaensis........ 80
Bertolonia mosenii 81
DISCUSSION 81
Pollen tube shower: a delayed selfing mechanism in flowers with poricidal anthers...... *81*
Floral traits and post-anthesis events favour delayed selfing in Bertolonia *82*
Mixed mating system, female, and male success in Bertolonia species........ *84*
LITERATURE CITED 87
TABLE 93
FIGURE LEGENDS........ 93

CAPÍTULO 3: IS *BERTOLONIA* (MELASTOMATACEAE) AN AUTOGAMOUS GENUS? NEW RECORDS OF POLLEN TUBE SHOWER ON SEVERAL SPECIES POINT TO THAT! / *BERTOLONIA* (MELASTOMATACEAE) É UM GÊNERO AUTOGÂMICO? NOVOS REGISTROS DE *POLLEN TUBE SHOWER* EM VÁRIAS ESPÉCIES APONTAM QUE SIM! 101

ABSTRACT........ 102
INTRODUCTION........103
MATERIALS AND METHODS 103
RESULTS AND DISCUSSION........104
REFERENCES........106
TABLE........108
FIGURE........109

CONSIDERAÇÕES FINAIS 111

REFERÊNCIAS........ 114

INTRODUÇÃO GERAL

Estratégias Reprodutivas em Melastomataceae

As estratégias reprodutivas expressadas pelas plantas são direcionadas por diferentes processos adaptativos (morfológicos, fisiológicos e/ou genéticos), todos relacionados com a aptidão do organismo no ambiente (Doust, 1989). A maioria desses processos está ligada a atributos florais, e a forma como estes interagem com os polinizadores, com consequências para o sucesso reprodutivo das plantas (Thompson, 1975; Mitchell *et al*., 2009; Schiestl & Johnson, 2013; Telles *et al*., 2020). O entendimento dos mecanismos que geram e mantêm esses processos adaptativos sempre atraíram a atenção na biologia evolutiva.

A família Melastomataceae Juss. é um grupo monofilético representada por cerca de 5750 espécies em 177 gêneros (Michelangeli *et al*., 2020) com ampla distribuição tropical, principalmente no Neotrópico, onde se encontra o centro de diversidade da família (Clausing & Renner, 2001). O grupo é representado por plantas de hábitos diversos, desde árvores a ervas, além de espécies epífitas e lianas. Esta diversidade de hábitos reflete diretamente na presença dessas espécies em diferentes formações fitogeográficas (Clausing & Renner, 2001). As flores de Melastomataceae são, em geral, radialmente simétricas, bissexuais e diplostêmones; com estames falciformes e deiscência geralmente poricida (Renner, 1989; 1993; Clausing & Renner 2001). Além disso, na maioria das espécies essas flores são conhecidas por fornecerem apenas pólen como recompensa aos seus visitantes, embora existam exceções (Varassin *et al*., 2008; Maia *et al*., 2016; Brito *et al*., 2016; Manrique, dados não publicados). O pólen enclausurado no interior das anteras poricidas pode ser considerado um mecanismo específico de distribuição do recurso, uma vez que o mesmo é removido, de maneira legítima, somente por movimentos gerados através da vibração dos músculos de voo de determinadas abelhas, num processo conhecido como polinização vibrátil ou *buzz pollination* (Buchmann, 1983; Luca *et al*., 2013). Entretanto, o uso do pólen como recompensa floral pode representar um conflito para a planta, uma vez que, além de ser um recurso oferecido aos visitantes, o pólen também contém os gametas masculinos, essenciais para a reprodução sexuada (Harder & Thomson, 1989; Westerkamp, 1997; Luo *et al*., 2009; Lunau *et al*., 2014).

Embora apresente um *bauplan* floral comum para quase todas as espécies, as estratégias reprodutivas que evoluíram dentro do grupo são múltiplas. Um exemplo é a variação contínua nos sistemas reprodutivos, desde espécies apomíticas até completamente dependente de polinizadores (Goldenberg & Shepherd, 1998; Melo *et al*., 1999, Goldenberg & Varassin, 2001; Fracasso & Sazima, 2004; Santos *et al*., 2012; Maia *et al*., 2016; Brito *et al*., 2017). Além disso, em muitas espécies da família, surgiram variações na morfologia das anteras ao longo da história evolutiva do grupo como forma de otimizar a liberação do pólen e assegurar a reprodução dessas plantas (Ferreira & Araújo, 2016; Brito *et al*., 2016; Maia *et al*., 2018; Velloso *et al*., 2018; Telles *et al*., 2020). Um exemplo foi o surgimento recorrente de estames de diferentes tamanhos (dimorfismo estaminal) em diversos clados de Melastomataceae (Cogniaux, 1891; Renner, 1989; Luo *et al*., 2008). Essa variação no tamanho dos conjuntos de estames é capaz de promover uma divisão de funções entre eles, separando o pólen funcionalmente em duas cargas: uma para ser utilizada como alimento para larvas de abelhas, e outra para ser usada na polinização. Essa adaptação ficou conhecida como "divisão de trabalho" (Müller, 1881; Müller, 1883; Vallejo-Marín *et al*., 2009; Luo *et al*., 2009), garantindo uma solução para o "dilema do pólen" em Melastomataceae. Outra estratégia reprodutiva associada à liberação dos grãos de pólen na família Melastomataceae é a variação do tamanho do poro das anteras em Miconieae (Goldenberg *et al*., 2008; Brito *et al*., 2016). Essa variação parece estar relacionada à generalização no sistema de polinização, já que anteras com poros grandes passam a incorporar moscas, vespas e abelhas não vibradoras como polinizadores, consequentemente diminuindo a importância da vibração na coleta do recurso e aumentando a guilda de visitantes capazes de polinizar estas espécies (Goldenberg *et al*., 2008, Kriebel & Zumbado, 2014; Brito *et al*., 2016; Gavrutenko *et al*., 2020).

Portanto, estas características florais aliadas à diversidade de espécies e aos diferentes contextos ambientais onde estão inseridas fazem da família Melastomataceae um excelente modelo para avaliar estratégias de reprodução nas angiospermas. Questões sobre como a grande variação de atributos florais refletem no sucesso reprodutivo de determinada espécie, quais estratégias aplicadas na otimização e alocação de recursos para a reprodução, assim como seus efeitos na interação com polinizadores, podem fornecer respostas ecológicas e evolutivas capazes de auxiliar na compreensão da gigantesca diversidade das plantas com flores.

Mata Atlântica: um exuberante e ameaçado cenário

A Mata Atlântica é uma das maiores florestas úmidas das Américas, com uma área original que ocupava cerca de 1,3 milhões km² e amplo gradiente latitudinal (3-30º S), estendendo-se por cerca de 3300 km ao longo da costa brasileira e alcançando, à oeste, países como Paraguai e Argentina (Carnaval *et al*., 2009; Tabarelli *et al*., 2010). A variação latitudinal associada a uma complexa topografia e regime variado de chuvas (maiores índices de precipitação da costa para o interior do continente; Câmara, 2003) fazem da Mata Atlântica um bioma de composição florística heterogênea, fator correlacionado à alta biodiversidade e alto grau de endemismo, sendo considerado um dos *hostspots* de biodiversidade do mundo (Myers *et al*., 2000). Entretanto, desde o início do século XVI, a Mata Atlântica vem sofrendo com contínuas perdas de habitats e outras perturbações de origem antrópica, visto que, aproximadamente 70% da população do Brasil vive ao longo da costa brasileira (Dean, 1997; Cincotta *et al*., 2000). Estudos apontam que, atualmente, restam cerca de 11,4-16% da área de cobertura do bioma (Ribeiro *et al*., 2009), embora esse número possa variar de acordo com a metodologia aplicada em cada estudo (e.g.: 28% de cobertura total; Rezende *et al*., 2018).

Cerca de 512 espécies de 40 gêneros da família Melastomataceae são conhecidas para a Mata Atlântica (Flora do Brasil 2020, em construção), entretanto, esse número tende a ser maior, considerando que ainda há áreas pouco amostradas (e.g.: Bacci *et al*., 2018). Sendo a riqueza de espécies subestimada, informações acerca de sistema reprodutivo, estratégias reprodutivas e polinização são extremamente limitadas nesse bioma (Goldenberg & Varassin, 2001; Franco *et al*., 2011; Brito & Sazima 2012; Maia *et al*., 2013; 2018; Malluceli *et al*. 2018; Telles *et al*., 2020). Estudos com esse enfoque são fundamentais para entender os processos ecológicos e evolutivos que atuam moldando as linhagens de Melastomataceae, assim como de seus polinizadores e a complexidade de suas interações na Mata Atlântica. Essas informações são uma excelente ferramenta para ações conservacionistas e tomadas de decisões políticas a fim de preservar e manter um bioma tão diverso e com alto grau de endemismo, mas tão ameaçado como a Mata Atlântica.

Estrutura da Dissertação

O principal objetivo desta dissertação é pesquisar, discutir e apresentar questões relacionadas às estratégias reprodutivas: traços florais, respostas dos visitantes florais a esses atributos e o efeito sobre o sucesso reprodutivo de espécies de Melastomataceae endêmicas da Mata Atlântica. Pontualmente, estudamos espécies pertencentes aos gêneros *Huberia* DC. e *Bertolonia* Raddi, reconhecidamente polinizados por abelhas (Passos, 2017; Passos, observação pessoal). Portanto, dividimos a dissertação em três capítulos. No primeiro, estudamos a biologia reprodutiva e da polinização de *Huberia insignis*, considerando sua morfologia floral para investigar a provável relação funcional dos apêndices dorsais das anteras (apêndices estaminais) com o sistema de polinização da espécie. No segundo, investigamos a potencial ligação entre a orientação do poro das anteras (introrsa/extrorsa) com o sistema de autopolinização espontânea de *Bertolonia paranaensis* e *B. mosenii* Cogniaux. Por fim, no terceiro capítulo desta dissertação, investigamos a capacidade de autopolinização em outras espécies de *Bertolonia* com base na recente filogenia proposta para o grupo (Bacci *et al.*, 2019), buscando reconhecer se o mecanismo de autopolinização descrito para as espécies de distribuição mais ao sul da Mata Atlântica (esta dissertação) está difundido entre as espécies do gênero e quais as implicações evolutivas disso para o grupo.

Os dois primeiros capítulos estão apresentados em forma de artigo científico e formatados segundo às normas dos respectivos periódicos: *Plant Biology* (ISSN 1438-8677) e *Annals of Botany* (ISSN 0305-7364). Por sua vez, o terceiro capítulo está formato como nota científica seguindo às normas do periódico *Plant Systematics and Evolution* (ISSN 0378-2697).

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CAPÍTULO 1

Testing the function of the staminal appendages on the pollination system of *Huberia insignis* (Melastomataceae), a buzz-pollinated species

Testando a função dos apêndices estaminais no sistema de polinização de *Huberia insignis* (Melastomataceae), uma espécie polinizada por vibração

* Manuscrito formatado nas normas do periódico *Plant Biology*

RESEARCH PAPER

Testing the function of the staminal appendages on the pollination system of *Huberia insignis* (Melastomataceae), a buzz-pollinated species

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Running head: The function of the staminal appendages in a buzz-pollinated species

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Keywords: *Bombus*; Cambessedesieae; mating systems; morphoanatomy; pollen removal; poricidal anthers; Reproductive success.

Abstract:

- Flowers are the main reproductive structures and sources of extensive morpho-physiological adaptations responsible for the transmission of characters and continuity of lineages in angiosperms. Morphological adaptations on the androecium related to the pollination system are evident in Melastomataceae flowers, e.g. the staminal appendages. However, little is known about the function of these structures and their potential impact on the reproductive success of these species. Here we investigated the effect of staminal appendages on the stamens attractiveness for pollinators and its biomechanical effect on the amount of pollen released on flowers of *Huberia insignis,* an endemic species of the Atlantic Forest.
- We conducted field experiments on flowers with/without staminal appendages to assess the pollinator preferences and the effect of pollen removal during a single visit. Also, we performed studies on floral biology, hand-pollination experiments, morphoanatomical analyzes and pollinator records for *H. insignis,* and associated these results with the staminal appendages removal tests.
- *Huberia insignis* is self-compatible and highly dependent on pollinators to set fruits and seeds. Buzzing-bees are the only pollinators, mainly two *Bombus* species (ca. 80% of visits). The staminal appendages showed neither a function on pollinator attractiveness nor an effect on pollen removal. Anthers and staminal appendages have neither secretory structures nor specialized biomechanical tissue.
- Staminal appendages play no role on the pollination system of *H. insignis*, suggesting that these structures are vestigial. However, ecological factors and intrinsic traits are fundamental to reproductive success. Also, morphoanatomical studies will be crucial for understanding the role of plant tissues on the vibration properties of buzz-pollinated species such as this.

INTRODUCTION

Several reproductive strategies have evolved throughout the evolutionary history of plants, ensuring the continuity of lineages and the transmission of traits to their descendants through morphological, physiological, and/or genetic adaptive processes (Doust 1989). In angiosperms, these adaptive processes generally occur in flowers, the structures responsible for the reception and dispersion of gametes, through modifications in floral traits to maximize individual reproductive success (Bell 1985; Barrett & Harder 1996; Barrett 2002; Ollerton & Dafni 2005; Bauer *et al*. 2017). In species of angiosperms where pollination is dependent on biotic vectors, the floral traits reflect strategies that may improve not only the release / deposition of resources, but also may keep the attraction and constancy of pollinators (Macior 1971; Herrera 1996; Fenster *et al.* 2004; Machado & Lopes 2004; Chittka & Raine 2006; Schiestl & Johnson 2013).

Many species of angiosperms offer pollen as the main or single resource to visitors (i.e., “pollen flowers”; *sensu* Vogel 1978). This trait is usually and strongly associated to poricidal anthers (Buchmann 1983; Vallejo-Marín *et al.* 2010). Pollen flowers are pollinated almost exclusively by bees capable of vibrating the anthers to access pollen (“buzz-pollination”; Buchmann 1983). The poricidal anthers and the consequent removal of the resource through vibration is a convergent floral trait found in more than 70 families of angiosperms (Buchmann 1983), and are very common among most species of Melastomataceae, where they occur in about 98% of the Neotropical species in the group (Renner 1989).

Morphological adaptations on the androecium that are directly related to pollination systems are very evident in some flowers of the group, such as heteranthery in tribes Microlicieae and Melastomateae (Luo *et al*. 2009; Ferreira & Araújo 2016; Maia *et al.* 2018; Velloso *et al.* 2018); changes in the size of the anther pores in Miconieae, leading to a generalization of the pollinator guild (Brito *et al*. 2016); the presence of nectaries in stamens of species pollinated by vertebrates (Varassin *et al*. 2008); and the diversity of traits related to stamen connective appendages with direct relation to pollination events (Dellinger *et al*. 2014; Telles *et al*. 2020; Bochorny *et al.* in press).

The Melastomataceae tribe Cambessedesieae includes three genera: *Cambessedesia* DC., *Huberia* DC. (including *Behuria* Cham. and *Dolichoura* Brade) and *Merianthera* Kuhlm. The last two stand out for the anthers with dorsal connective appendages (Goldenberg *et al*. 2012; Bochorny *et al.* 2019). These staminal dorsal appendages in *Huberia* are simple and descending, varying from linear to spiraled (Bochorny *et al*. 2019). Staminal appendages vary in color and size, but in most species they are shorter and present the same yellow color than the thecae (see the former genera *Behuria* Cham. and *Huberia sensu stricto* in Baumgratz 1997; 2000; Tavares 2005; Goldenberg *et al*. 2016). Striking exceptions to this pattern are the two species formerly placed in *Dolichoura* (*H. bradeana* Bochorny & R.Goldenb. and *H. kollmannii* (Brade) R.Goldenb. & R.Tav.; see Goldenberg & Tavares 2007), the first with purple anthers and staminal appendages, and the second with yellow stamens and reddish staminal appendages, both with staminal appendages that are longer than the thecae. Apart from their taxonomic importance, very little is known about the function of these staminal appendages. Some authors suggest that staminal appendages may be related to pollination (Morley 1953 *apud.* Martins 1984; Wilkinson 1978 *apud.* Martins 1984), acting on the pollinators' visual attraction (Velloso *et al.* 2018; Telles *et al.* 2020), or as a mechanical aid during landing and pollen gathering, as demonstrated in *H. bradeana* (Bochorny *et al.* in press). However, since in many species the staminal appendages are small and inconspicuous, there is some uncertainty on whether these staminal appendages actually have a functional role in the pollination system of *Huberia* species.

For instance, *Huberia insignis* (Cham.) Bochorny & R.Goldenb. (= *Behuria insignis* Cham.) is a shrub that is endemic to the Atlantic Forest; like in most species in the genus, its stamens have a filiform dorsal appendage (Tavares 2005; Goldenberg *et al.* 2016). Due to their small size, and because they apparently have a similar color pattern as the thecae (Baumgratz 1997; 2000; Tavares 2005; Goldenberg *et al.* 2016), we expect that these staminal appendages are visually and mechanically irrelevant to visitors. Therefore, we aimed to investigate if the staminal appendages of *H. insignis* play any role in the reproductive success of the species, either in the attraction (i.e., as a visual signal) or as a biomechanical support during the pollinator visits (i.e., as an auxiliary structure in anchoring and / or removing pollen). For that purpose, we studied the floral biology, mating system (through controlled pollination treatments), and the frequency and behavior of flower visitors. We also took a detailed account on the morphology of the staminal appendages. Finally we carried out floral experiments and focal observations on treatments with paired flowers (i.e., with and without appendages), in order to test (1) the effect of staminal appendages on the attractiveness of floral visitors, through preference tests, and (2) the possible biomechanical effect of staminal appendages on the amount of pollen released during a single visit.

MATERIALS AND METHODS

Study area

This study was conducted in the Environmental Protection Area of Pico Paraná, located in the municipality of Antonina, Paraná, Brazil. The study area belongs to the “Usina Hidroelétrica Govenador Viriato Parigot de Souza” (25°15'22.85 "S, 48°46'42.16 "W; 650-800 m above sea level), which in turn belongs to the “Companhia Paranaense de Energia” (COPEL). The climate is subtropical, humid, and lacking extreme events such as dry seasons or frost (Alvares *et al.* 2014). The average annual rainfall is 2521.6 mm, with summer being the rainiest season with 993.2 mm (Vanhoni & Mendonça 2008). The site is inside the Atlantic Forest biome, and predominantly covered with Montane to High-montane Rainforests, with relicts of subtropical montane grasslands (respectively "Floresta Ombrófila Densa" and "Campos de altitude" in the official Brazilian classification - Veloso *et al.* 1991).

Fieldwork was performed in two different periods. Between January and March/2017 we collected data for floral biology, mating system, and species visiting the flowers, and between January and February/2020 we collected data on the functionality of the appendages and pollinator frequency.

Model Species

Huberia insignis is endemic to Brazil, and it can be found in the states of São Paulo and Paraná; though for the latter, apparently only one population is known (Tavares 2005; Goldenberg *et al.* 2016), precisely the one studied here. The plants in this species are shrubs about 1.5-3 m tall, growing on hillsides (Goldenberg *et al.* 2016). The flowers are organized in thyrsoid, 6-15-flowered inflorescences, hexamerous, and with a white corolla. The androecium has 12 yellow stamens, each with a poricidal anther and a dorsal filiform appendage in the connective (Tavares 2005; Goldenberg *et al*. 2016; Fig. 1A, B). The size of the antesepalous anthers do not differ significantly from the antepetalous ones (6.37 mm ± 0.58 and 6.23 mm ± 0.59, respectively). The anthers are larger than their respective appendages (3.77 mm ± 0.42 and 3.63 mm ± 0.42). Samples were collected and deposited in the herbarium of the Botany Department, Federal University of Paraná (UPCB; vouchers UPCB84630, UPCB84631 and UPCB84632).

Floral biology and mating system

To describe anthesis and post-anthesis events, we performed focal observations and experiments on individuals *in situ*. To determine stigma receptivity, we tested ten flowers (n = 10 individuals) with hydrogen peroxide (H_2O_2; Kearns & Inouye 1993) when the corolla was opening (considered as the beginning of anthesis). To evaluate the viability of the pollen grains, we randomly collected two flowers in pre-anthesis from 10 individuals. From each flower, we selected two anthers. The anthers were fixed in a solution of 70% formaldehyde-acid-alcohol (FAA) and then macerated on a slide to release the pollen. We used the staining technique with 1% acetocarmine solution and analyzed the grains under an optical microscope. We considered viable only stained grains; uncolored or malformed grains were regarded as unviable (Kearns & Inouye 1993). We counted 200 pollen grains per slide (Maia *et al.* 2016), and then calculated the pollen viability rate, as the ratio between the number of viable grains and the total of grains counted. To verify the presence of osmophores (Dafni 1992), we tested ten flowers with neutral red, in the first hours of anthesis (7:00 - 9:00 a.m.; n = 10 individuals).

We described the mating system through controlled pollination treatments. We isolated floral buds in 33 individuals and applied five treatments: Apomixis (AX), emasculation of floral buds; Cross-pollination (CP), manual pollen deposition on the stigma of flowers from individuals at least 10 m apart from each other; Manual self-pollination (SP), pollen deposition on the stigma of the same flower; Autonomous self-pollination (ASP), with the isolation of the flower; Control (C), flower exposed to visitation. Flowers were identified according to the treatment received and, except for the control treatment, kept isolated inside *voile* bags after manipulation. The number of flowers per treatment is shown in Supporting Information - Table S1. After 30 days we recorded the fruit set. We calculated the self-incompatibility index (ISI; Bullock 1985), represented by the ratio between the fruit sets from

SP and CP. We considered that values above 0.25 indicate a self-compatible mating system. We also calculated the pollen limitation index (PL) to estimate the rate of reduction in reproductive success due to insufficient pollen deposition using the formula $PL = 1 - C_r/CP_r$, where C_r and CP_r are the rates of fruits formed in treatments C and CP, respectively (Larson & Barrett 2000; Freitas *et al.* 2010). We considered $PL \leq 0.3$ as indicating an absence of pollen limitation and $PL > 0.8$ as indicating extreme pollen limitation (Freitas *et al.* 2010).

To assess female reproductive success, we evaluated the quantity and viability of seeds formed in each treatment. For this, we randomly collected ten fruits from each treatment. From these fruits, we counted the total number of seeds/fruit. After that, we randomly selected 50 seeds from each fruit for germination test in an incubator (EL 202/3; Eletrolab, Brazil). In the incubator, all seeds were kept inside gerboxes on filter paper, and treated under the same temperature (25ºC ± 3°C) and photoperiod (12h), and wet once a day with a garden sprayer. We considered viable the seeds that germinated (radicle emission) within 30 days.

Pollen production and release

We estimated the average number of pollen grains in the anthers from flowers in bud stage and at post-anthesis, besides the amount of ovules per flower in the population. Our purpose was to associate these values with reproductive and ecological factors (e.g., seed set, effectiveness and frequency of pollinators). To estimate the number of pollen grains in the population we used 59 buds from 17 individuals following Telles *et al.* (2020); we macerated the anthers to extract the pollen, and then counted it under optical microscopy (40x; FWL 1000; Feldmann Wild Leitz, Brazil), using a hemocytometer. To relate the frequency of visits to the amount of pollen removed at the end of the anthesis, we collected post-anthesis flowers ($n = 12$ flowers in 12 individuals) and estimated the number of remaining pollen grains following the same method. We recorded the average amount of ovule produced per flower (*n* = 10 flowers in 10 individuals) with the aid of a stereoscopic microscope (ZEISS Stemi 305; Germany).

Flower visitors, behavior, and frequency

We performed focal observations and photographic records during February/2017 to determine the identity of floral visitors of *H. insignis* (*n* = 33 individuals). According to the behavior, we classified the visitors into effective pollinators, when they vibrated all anthers and touched the stigma simultaneously; occasional pollinators, when, due to their body size, they vibrated few anthers at a time and occasionally touched the stigma while moving between the anthers; thieves, when they accessed the pollen without damaging anthers but did not touch the stigmatic surface; and, robbers, when they caused damage when accessing the resource illegitimately (Inouye 1980). Visitors were identified in the field during visits or through photographs. The individuals were collected and deposited in the Entomology Museum “Padre Jesus S. Moure” (DZUP), from “Universidade Federal do Paraná” (UFPR).

Focal observations were made in order to determine the frequency and behavior of visitors, from 6:00 a.m. to 1:00 p.m., totaling 35 h. Plant individuals were randomly selected, and each individual was observed for 20 min; at the end of this period we moved to another individual. For each individual we recorded the number of open flowers per day, the number of visits received, and bee species. With these data, we calculated the frequency of visits per flower and hour in the population. We also calculated the frequency of visits per bee species.

Reflectance of floral parts and staminal appendages removal test

To determine the color of the floral parts, we measured spectral reflectance of petals, anthers and appendages of 16 flowers (eight individuals). We used a spectrophotometer (JAZ, Ocean Optics, USA), calibrated with a standard white (DH 2000-CAL; Ocean Optics; USA) and the absence of light as standard black. To infer if the bees were able to perceive any visual difference between the color of the staminal appendages and anthers, we used the visual system of *Bombus terrestris* (Linnaeus 1758) as a surrogate, and the hexagon visual model (Chittka 1992). We assumed a threshold of 0.09 hexagon distances for discrimination, considering empirical evidence for *Bombus terrestris* (Dyer 2006). The cleaning and analysis of the reflectance curves were performed using the *pavo* package (Maia *et al.* 2013).

During focal observations, previously isolated flowers were exposed to visitors for preference tests (n = 74 flowers in 37 pairs). We also estimated the pollen grains removal in anthers with and without staminal appendages (n = 46 and 44 flowers, respectively). We removed the staminal appendages with the aid of tweezers (Fig. 1C). The manipulation was performed at least two hours before the observations to avoid interference on pollinator attraction. For each visitor, we recorded (1) the approach events (when the bee hovered in front of the flowers), (2) landing or not, and in case of landing, (3) the first choice (flower without appendages or control).

To test the effect of the presence / absence of staminal appendages and the identity of the visitor on pollen removal, after a single visit we removed the visited flower (or both flowers if the visit occurred in sequence), and stored them individually in plastic vials with 1 mL of 70% FAA for subsequent counting of the remaining pollen in the anthers. For the pollen counting, we follow the method described in *Production and Pollen Release*.

Morphoanatomy of anthers and staminal appendages

We fixed flowers at anthesis in a 70% FAA solution in order to investigate a potential biomechanical and / or secretory role played by anthers and appendages through micromorphological and anatomical analyses. For scanning electron microscopy (SEM), anthers and appendages were dehydrated in an ethyl series, submitted to critical point drying, coated with gold, and observed in electron microscope JSM 6360-LV (JEOL, Japan).

Stamen samples were embedded in Leica historesin according to the manufacturer's instructions. Transverse and longitudinal sections were stained with toluidine blue (O'Brien *et al.* 1965). The presence of synthesized or secreted substances (indicative of a possible function in attracting visitors) by the appendages were verified through histochemical tests, i.e., ferric chloride to test for phenolic compounds (Johansen 1940), Lugol for starch (Johansen 1940) and periodic acid-Schiff's staining (PAS; Jensen 1962) for total polysaccharides. We analyzed the samples using an Olympus BX41 optical microscope (Olympus; Japan) attached to a digital camera.

Data analysis

We analyzed the data fitting different statistical models, depending on the distribution of the response variable. We used a generalized linear mixed model (GLMM) to analyze the possible effect of pollination treatments (predictor variable) on the proportion of fruit set (response variable), assuming a binomial distribution (with N being the number of flowers and P the probability of a flower to develop into fruit), logit function, and the identity of plants as the random term. We performed the analysis of seed set (response variable) in each treatment (predictor variable) using a linear model (LM). For the analysis of the proportion of germinated seeds (response variable) from different pollination treatments (predictor variable), we fitted a generalized linear model (GLM) with a binomial distribution.

We calculate the frequency of visits through the total of visits/total of flowers observed/hour, the result was expressed in visits.flower^{-1}.h^{-1}. To evaluate if the frequency of visits (response variable) was dependent on the time and number of flowers produced per individual (predictor variables), we fitted a GLMM, assuming a Poisson distribution, and the identity of each individual as a random term.

We verified if there was a preference of the visitors for flowers with or without appendages applying a GLMM with a binomial distribution, presence/absence of appendages and identity of the floral visitor as predictor variables, and the identity of the plants as a random term. We verified if there were differences in the pollen removal rates in flowers with and without appendages fitting a linear mixed model (LMM), considering the treatment and pollinator species as predictor variables and the identity of each plant as a random term.

All analyses were performed on the R 3.6.0 platform (R Core Team 2013). For the GLMM and LMM analyses, we used the *glmer* and *lmer* function of the *lme4* package (Bates *et al.* 2015). For the analysis of LM and GLM we use the functions *lm* and *glm* of the default R *stats* package. We apply *post-hoc* tests whenever necessary, with Tukey adjustment, assuming $\alpha \leq 0.05$, using the function *glht* of the package *multcomp* (Hothorn *et al.* 2008).

RESULTS

Floral biology and mating system

Huberia insignis flowered between January and February, with a few individuals with flowers in March. One or two flowers open in each inflorescence per day. The anthesis starts at 5:00 a.m., with the flowers totally opened around 7:00 a.m., and it lasts until 4:00 p.m., when the petals start to close, which is resumed by 5:30 p.m. Around 24 h after the beginning of the anthesis, the petals and stamens fall, leaving only the hypanthium and gynoecium. During anthesis, the stamens are grouped in a zygomorphic arrangement below the stigma, which means that there is a clear separation between male and female functions through herkogamy. The stigma is receptive since the flower opening (checked through the peroxidase test) and seems to remain like that until the petals close (evidenced by the wet aspect of the stigmatic surface). The only resource offered to floral visitors was pollen, with 95.5% ± 2.00 of viable grains. The flowers seem to emit no odor. The flowers and staminal appendages did not react to the neutral red test.

The population studied is self-compatible (ISI = 0.52), but its flowers need vectors for the pollen to be transferred. No fruits were formed in the apomixis treatment. Fruiting rates are higher in cross-pollinated (CP) than in self-pollinated (SP) flowers ($\chi^2 = 9.46$; $df = 2$; $P < 0.01$; Table S1; Fig. 2A). Although the flowers depend on pollinators for setting fruit and they slightly tend to produce more fruits with cross-pollination (CP treatment), when compared to pollination under natural conditions (Control treatment), pollen limitation was low (PL = 0.31). The number of seeds per fruit varied between treatments ($F = 3.99$; $df = 2$; $P = 0.03$; Table S1; Fig. 2B). Fruits resulting from cross-pollination produced more seeds than those from manual self-pollination ($t = 2.82$; $P = 0.02$; Table S1). However, the seed germination rate from the control treatment was higher than in cross- and self-pollination ($\chi^2 = 44.26$; $df = 2$; $P < 0.001$; Table S1), and did not differ between cross-pollination and manual self-pollination ($t = 0.7$; $P = 0.75$; Fig. 2C).

Pollen production and release

Each flower produced an average 1,537,692.31 ± 167,882.46 pollen grains, with a significant reduction in flowers exposed to pollinators at the end of anthesis (average of 330,208.33 ± 307,838.87 pollen grains / flower), indicating that about 78.5% of the pollen grains are removed. The average number of ovules per flower was 318 ± 34.

Flower visitors, behavior, and frequency

Bees were the only floral visitors observed in the flowers of *H. insignis* (Table S2), with 4.57 visits.flower^{-1}.h^{-1} (Table 1). Legitimate visitors vibrated the anthers, and had the pollen deposited in the ventral portion of their bodies. The main pollinators were two species of *Bombus* (Latreille 1802; Fig. S1), *Bombus* (*Fervidobombus*) *brasiliensis* (Lepeletier 1836) and *Bombus* (*Fervidobombus*) *morio* (Swederus 1787), that together represented about 80% of all visits (Table 1). In addition, their behavior confirms that both are effective pollinators, since they vibrated all the anthers at once and contacted the stigma simultaneously; bees from both species visited all available flowers in the same individual during each approach. Other medium to large species, such as *Euglossa* (*Glossura*) *annectans* (Dressler 1982), *Xylocopa* sp. and *Trichocerapis* sp., also vibrated all the anthers and contacted the stigma in the same visit; however, we recorded only few visits from these bees (Table 1), and we seldom observed them keeping a route or visiting all the flowers available in one individual. *Paratetrapedia fervida* (Smith 1879), *Trigonopedia* sp., *Augochlora* sp. and two other species from subfamily Halictinae were occasional pollinators (Table S2). The entire pollen collection process by these species required more time than by the larger ones, resulting in long visits to one or two flowers from the same individual. *Plebeia emerina* (Friese 1900) and *Trigona spinipes* (Fabricius 1793; Table S2) were considered pollen robbers, because they damaged the flowers by chewing the anthers to access pollen. In addition to anther destruction, these bees did not touch the stigma and remained in the same flower for a long time, repelling visits from other bees. Visits peaked at 10:00 am (Fig. S2; $\chi^2 = 28.30$; $df = 7$; $P < 0.001$). We did not find an effect of the number of flowers available per individual on the frequency of visits ($\chi^2 = 1.46$; $df = 1$; $P = 0.22$).

Reflectance of floral parts and staminal appendages removal tests

The petals of *Huberia insignis* are white, UV-absorbing. Its anthers and appendages present the same yellow, UV-absorbing pattern (Fig. S3). Thus, anthers and dorsal appendages of the connective are not discriminated from each other according to the established threshold (hexagon distance = 0.04) but are easily discriminated from petals (hexagon distance ≥ 0.50).

We found no preference by the bees to flowers with or without appendages (respectively 47% and 49% of choices; $\chi^2 = 0.0004$; *df* = 1; *P* = 0.98; Fig. 3A), nor a preference depending on the identity of visitor ($\chi^2 = 0.07$; *df* = 7; *P* = 1); this shows that the staminal appendages have no role in the attractiveness of flowers of *H. insignis* to pollinators. Pollen removal did not differ between flowers with and without appendages (*F* = 0.25; *df* = 1; *P* = 0.62; Fig. 3B), nor there is an effect of the pollinator's identity on its removal (*F* = 1.13; *df* = 3; *P* = 0.34).

Regarding the behavior of bees when visiting flowers with or without staminal appendages, they seem not to care about them neither visually nor mechanically. Exceptions were some visits by *Bombus* bees that occasionally touched the staminal appendages with their abdomen or with their hind legs during the vibration process; *Bombus brasiliensis* sometimes anchored its posterior and/or middle legs between the filament and the appendages, or below the thecae. The species that chewed the anthers left the staminal appendages untouched (Fig. S1).

Morphoanatomy of anthers and staminal appendages

The appendages and the anthers have epidermal cells covered with a rough and striated cuticle (Fig. 4). However, we noticed that these cells are longer in the appendages when compared to the ones from the anthers. We did not detect secretory structures (e.g., glandular trichomes or stomata).

The connective of the anthers and appendages (Fig. 5A) are filled mainly by cortical parenchyma cells (Fig. 5B, C). The epidermal cells from the proximal region of the appendages have dense cytoplasmic content (Fig. 5C) that reacts with ferric chloride, indicating the presence of phenolic compounds (Fig. 5D). In the most distal region (towards the appendages apex), the cytoplasmic content is less dense in the epidermis than in the subepidermal cells (Fig. 5E). In this region, the epidermal cells did not react with ferric chloride, but the subepidermal cells did (Fig. 5F). The appendages of *H. insignis* are vascularized (Fig. 5C, E, F, G). The Lugol test indicated the presence of starch grains in the proximal region of the appendages. Close to the connective, the reaction with PAS indicated the presence of polysaccharides in the epidermal cells and around the vascular bundles (Fig. 5H).

DISCUSSION

Huberia insignis is a self-compatible species that is totally dependent on pollinators for seed production. Due to its floral characteristics (e.g., few flowers per inflorescence and herkogamy), it tends to optimize its reproductive success through xenogamy. In addition, the high frequency of visits by two *Bombus* species and the foraging pattern displayed by these bees tend to increase cross-pollination rates. The staminal appendages of *H. insignis* seem to play no role in its pollination system, which in turn may corroborate the hypothesis that these structures may be vestigial in *H. insignis.*

Mating systems, pollinators, and reproductive success

Despite being self-compatible, *Huberia insignis* is totally dependent on pollinators for its reproductive success. Both its floral traits and the behavior of the most frequent pollinators favor xenogamy, with positive impacts on the reproductive success of the species, considering that more fruits and seeds are produced through cross-pollination. The low number of open flowers per individual in one day increases xenogamy rates and decreases geitonogamy events, as the low density of flowers makes pollinators visit several flowering individuals to supply their pollen needs (Arroyo 1976; Otárola & Rocca 2014). Moreover, the spatial separation between the stigma and the anther pore (herkogamy) shows that, when the flower is approached by effective pollinators, the stigmatic surface is the first structure to come into contact with the pollen-laden abdomen of the pollinator, favoring the deposition of xenogamic pollen (Webb & Lloyd 1986; Oliveira & Maruyama 2014).

The frequency of pollinator visits may be associated with the low pollen limitation in *H. insignis*; since 50% flowers produced fruits and seed set was higher under natural conditions. This frequency was effective in fruit and seed production under natural conditions. The frequency of visits can be positively associated with the large amount of pollen that is produced by the flowers and its quality since bees are highly dependent on floral resources (e.g., pollen) to feed their larvae and adults (Kevan & Baker 1983). Moreover, the main pollinators of *H. insignis* are two species of bumblebees (*B. brasiliensis* and *B. morio*), together showed a frequency of 3.69 visits.flower^{-1}.h^{-1} (i.e., ca. 80% of the total visits). The plant-pollinator interaction between Melastomataceae and bumblebees (*Bombus* species) is common in the Atlantic Forest (Laroca 1970; Franco *et al.* 2011; Brito & Sazima 2012; Maia *et al.* 2018; Malucelli *et al.* 2018). Bumblebees prefer flowers with viable pollen (i.e., with cytoplasmic content) capable of providing their nutritional needs (Robertson *et al.* 1999), and tend to visit flowers with more pollen available (Maia *et al.* 2016), so that they spend more time foraging inflorescences of the same plant, and increasing their frequency of visits (Harder 1990). Furthermore, self-compatibility, the average amount of removed pollen and the number of ovules produced per flower indicates that few visits are necessary to achieve maximum fertilization (success of the female component; Bell 1985), contributing to low pollen limitation in *H. insignis*.

Staminal appendages: an adaptive or vestigial structure?

As perceived by the flower visitors, appendages and anthers have a similar yellow UV-absorbent color display. This UV-absorbent pattern probably resulting from flavonoids (Lunau 2000), as we found phenolic compounds in the epidermal and subepidermal cells of the staminal appendages. This color pattern may act as a signal to pollinators, considering the innate preference of some bees for this color, usually found in pollen grains (pollen mimicry; Vogel 1978; Lunau *et al.* 2017; Lunau & Wester 2017). While the evident contrast of the white corolla with the background would assist in the detection of long distances by bees, the anther-appendage staminal set would facilitate the recognition of the flower during approach and the proper positioning of the bees during landing (Dyer & Chittka 2004). However, from a functional standing point, the appendages apparently do not have any direct role on pollinator attraction, considering that there was no preference by the bees between flowers with or without appendages, regardless of pollinator species. The small size of the appendages in *H. insignis* may not represent a significant increase in the visual display of the stamens, as suggested for other Melastomataceae species (Velloso *et al*. 2018; Telles *et al.* 2020).

In buzz pollinated species, the male structure seems to play a dual role regarding the pollinator approach: attraction of pollinator and landing / anchoring site. Also, several structural or biomechanical traits may influence both the attractiveness of flowers and the vibration properties of poricidal anthers (Michelsen *et al.* 1982; Arroyo-Correa *et al.* 2019). The microstructure of the epidermal cells of the petals in many species is associated to several factors linked to pollination, from color pattern (Noda *et al.* 1994) to pollinator adherence (Whitney *et al.* 2009a; 2009b) and exclusion of antagonistic visitors (Papiorek *et al.* 2014); however, there is still an information gap about the role of the microstructure of the androecium epidermal cells and its relation to pollination events. Therefore, we suggest that the striated surface of the epidermal cells of *H. insignis* anthers may influence the time spent by the bees when landing or vibrating the anthers since this rough surface may improve the adherence of the small claws and hairs on the legs of these insects (Voigt *et al.* 2012; Bräuer *et al.* 2017). Although we did not find any specialized tissue or anatomical traits in the staminal appendages that would indicate a biomechanical role in supporting pollinators, the many layers of parenchyma cells in the anther connective could provide support for the bee during landing and influence the biomechanical traits transmitted by their vibrations (King & Buchmann 1996; Arroyo-Correa *et al.* 2019).

We did not identify secretory structures in the anthers and appendages of *H. insignis*. The presence of starch in the appendages is apparently not related to pollinator attraction, considering that pollinators do not prefer flowers either with or without appendages. Even though we could speculate on the possibility that this starch could be broken into sugar or in any way made available as a resource for the pollinators, no visitor seemed to have been influenced by this content. If these sugars were in fact explored as a resource, visitors would be expected to contact the appendages to access it. Moreover, starch is the best-described polysaccharide used as reserve nutrient in anthers of several species (Bhandari 1984; Clément *et al.* 1994). Generally, the starch accumulation works as a source of metabolites for the microspore vacuolation phase and during the development and maturation of the pollen grains (Reznickova 1983).

The vascular bundles in the appendages of *H. insignis* have already been reported by Wilson (1950) in his anatomical study of stamens in species of Melastomataceae. According to this author, the vestigial vascular bundles in the appendages branch from the main vascular bundle in the connective and would be remnants of telomatic branching systems, this in turn based on the teloma theory by Zimmerman (1930; Wilson 1950). He also defended that the staminal appendages would be a “primitive” character in the family (Wilson 1950), which has been consistently rejected by other authors (see Morley 1953; Wilkinson 1978; Martins 1984), and nowadays remains utterly anachronistic, since it does not reflect the phylogeny of the family (Clausing & Renner 2001). Indeed, staminal appendages in Melastomataceae have appeared in different tribes and are very varied as to position, shape, colors and vascularization (Renner 1993; Clausing & Renner 2001; Goldenberg *et al.* 2012; Michelangeli *et al.* 2013; Bochorny *et al.* 2019), raising the hypothesis that such a trait may have emerged several times in the evolutionary history of the family. At least for *Huberia* species, staminal appendages probably can be considered plesiomorphic, since the common ancestor of the genus had appendages in its anthers (Bochorny *et al.* 2019).

Apart from some bees touching the staminal appendages during vibration or even grasping the stamens between the structure and the filament with their hind legs, we did not find any direct function of the appendages during visits. Moreover, there was no difference in the amount of pollen removed from stamens with or without appendages. This contrasts with the results for the congeneric *Huberia bradeana*, where a similar methodology showed that the amount of pollen released from intact flowers was significantly bigger than in flowers with removed appendages (Bochorny *et al.* in press). The difference here is that the staminal appendages of *H. insignis* are on average 1.5 times shorter than the anthers, while the ones in *H. bradeana* are about 2-3 times longer than the anthers (4.1-6.2 mm and 2.1-2.5 mm, respectively; *fide* Goldenberg & Tavares 2007). In *H. bradeana*, the big, coiled appendages provide a greater contact area between the flower and the pollinator body, and in turn may absorb more energy while the stamens are vibrated and, consequently, may release more pollen (Bochorny *et al.* in press). In this case, the appendages could be an evolutionary response to the short duration of the flower and the low frequency of pollinator visits (two visits.flower^{-1}.hour^{-1}, according to Bochorny *et al.* in press), as their presence increases the quantity of pollen removed in a single visit, and consequently optimizes the flower's male success (Bochorny *et al.* in press).

The lower frequency of visits in *H. bradeana*, when compared to *H. insignis*, could be related to its main pollinators, bees of the genus *Xylocopa* (*X. brasilianorum* Linnaeus 1767 and *X. frontalis* Olivier 1789; Bochorny *et al*. in press). *Xylocopa* bees are generally solitary or facultatively social (Marchi & Alves-dos-Santos 2013) and present a differential foraging performance when compared to social bees, as species of the genus *Bombus*. Under experimental conditions, social bees have higher rates of learning than solitary bees, indicating that solitary bees learn more slowly about types or sites of flowers with higher amounts of resources (Dukas & Real 1991). Furthermore, social bees may specialize only in the collection of food, resulting in higher visit rates, and different from solitary bees which are responsible for several other tasks (e.g., such as building and defending the nest; Pyke 1984); moreover, social bees can specialize in one type of food, such as only pollen or nectar; whereas solitary bees need to alternate between sources of pollen and nectar (Dukas & Real 1991). These characteristics may explain the higher frequency of visits in *H. insignis*, pollinated mainly by social bees, when compared to *H. bradeana*, pollinated by solitary bees.

We found no evidence that the connective appendages in anthers of *H. insignis* play a role in its pollination process, and this suggests that such structures may be vestigial for this species. The color pattern found in *H. insignis* flowers (i.e., visual cues: UV patterns, contrast between corolla and androecium, the yellow anthers mimicking pollen; Lunau 2000 and references therein) and the high production and quality of pollen grains can be positively related to flower constancy (Michener 2007; Telles *et al.* 2020). Moreover, the self-compatible system gives the species a broader opportunity for ovule fertilization, considering that viable seeds will be produced even in cases of autogamy or geitonogamy. Thus, the sum of these factors results in both female (ovule fertilization) and male (pollen removal and deposition) reproductive success in *H. insignis*. Therefore, both ecological (i.e., pollinator availability) and plant intrinsic factors (such as color pattern and mating system) may have allowed the evolution, or permanence of vestigial appendages, these without adaptive value at all in the pollination system of *H. insignis*.

ACKNOWLEDGMENTS

We thank the UHE (Copel) teamwork for the support and permission to access the study area; the CTAF/UFPR (Centro de Tecnologias Avançadas de Fluorescência) and CME/UFPR (Centro de Microscopia Eletrônica) for the fluorescence and SEM images. We also thank Stephanie Bianco for the preparation of the anatomical sections, Isabela Galarda Varassin from LINTER/UFPR (Laboratório de Interação e Biologia Reprodutiva) for support and permission to use the laboratory, and Thuane Bochorny for their contributions to the manuscript. Finally, we are also grateful to Leticia Graf and Rodrigo Gonçalves, both from the "Laboratório de Abelhas" (UFPR), for assistance with bee species identification. This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (masters grant 88882.382585/2019-01 to LSP, and postdoctoral grant 1659767 to FJT); Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil ("produtividade em pesquisa", 300865/2017-4 to RG).

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TABLES

TABLE 1. Number and frequency of visits (visits.flower-1.h-1) in flowers (n=265 flowers; during 35 h) of *Huberia insignis* from Antonina, Paraná, Brazil.

Species	**Number of visits**	**Visits/flower**	**Frequency of visits**
Ariphanarthra palpalis	11	0.042	0.125
Augochlora sp.	31	0.117	0.351
Bombus brasiliensis	115	0.434	1.302
Bombus morio	211	0.796	2.389
Euglossa annectans	12	0.045	0.136
Halictinae1	4	0.015	0.045
Halictinae 2	4	0.015	0.045
Trichocerapis sp.	5	0.019	0.057
Trigona spinipes	5	0.019	0.057
Xylocopa sp.	6	0.023	0.068
Total	**404**	**1.52**	**4.57**

TABLE S1. Results of controlled pollination treatments: Fruit set, production and germination of seeds of *Huberia insignis* (Melastomataceae) from Antonina, Paraná, Brazil.

			Seed	
Treatments	***n***	**Fruit set**	**Production**	**Germination**
Control	52	50% (26)	291.1 ± 28.96	21.8% (109/500)
Cross-pollination	43	72.09% (31)	323.1 ± 47.04	1.8% (9/500)
Manual self-pollination	56	32.5% (21)	257.9 ± 64.36	5.8% (29/500)
Autonomous self-pollination	50	0	0	-
Apomixis	31	0	0	-

TABLE S2. Floral visitors observed in *Huberia insignis* (Melastomataceae) from Antonina, Paraná, Brazil.

Species	**Behavior**
APIDAE	
Apinae	
Bombus (Fervidobombus) brasiliensis (Lepeletier, 1836)	Effective pollinator
*Bombus (Fervidobombus) morio (*Swederus, 1787)	Effective pollinator
Euglossa (Glossura) annectans (Dressler, 1982)	Effective pollinator
Paratetrapedia fervida (Smith, 1879)	Occasional pollinator
Plebeia emerina (Friese, 1900)	Pollen robber
Trichocerapis sp.	Effective pollinator
Trigona spinipes (Fabricius, 1793)	Pollen robber
Trigonopedia sp.	Occasional pollinator
Xylocopa sp.	Effective pollinator
Halictinae	
Ariphanarthra palpalis (Moure, 1951)	Effective pollinator
Augochlora sp.	Occasional pollinator
Augochloropsis sp.	Effective pollinator
Halictinae 1	Occasional pollinator
Halictinae 2	Occasional pollinator

FIGURES

Figure 1. (A) Flower of *Huberia insignis*. (B) Detail of anthers with the arrows indicating the filiform appendages. (C) Flower with appendages removed for preference and pollen removal tests.

Figure 2. Results of controlled pollination treatments in flowers of *Huberia insignis*. (A) Fruit set from each pollination treatment; (B) Distribution of the quantity of seeds set in each pollination treatment; (C) Germination rate of seeds from each pollination treatments. Treatments: Manual self-pollination (SP), Control (C) and Cross-pollination (CP). Letters indicate differences or similarities among treatments. Bars represent 95% of confidence intervals.

Figure 3. Results of staminal appendages experiments of *Huberia insignis*. (A) Proportion of visits in flowers with (Control) and without staminal appendages (Treatment). (B) Quantity of pollen removed in flowers with (Control) and without staminal appendages (Treatment).

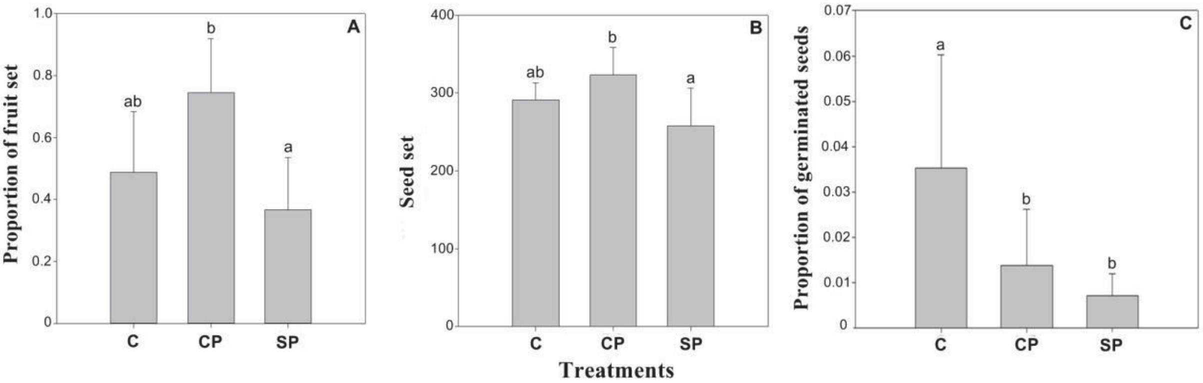
Figure 4. Scanning electron microscopy images of *Huberia insignis* anthers. (A) Detail of the rough cuticle on anther epidermal cells. (B) Apex of staminal appendages. (C) Elongated cells that in the appendages. (D) Detail of the anther cells, shorter than the cells of the appendages.

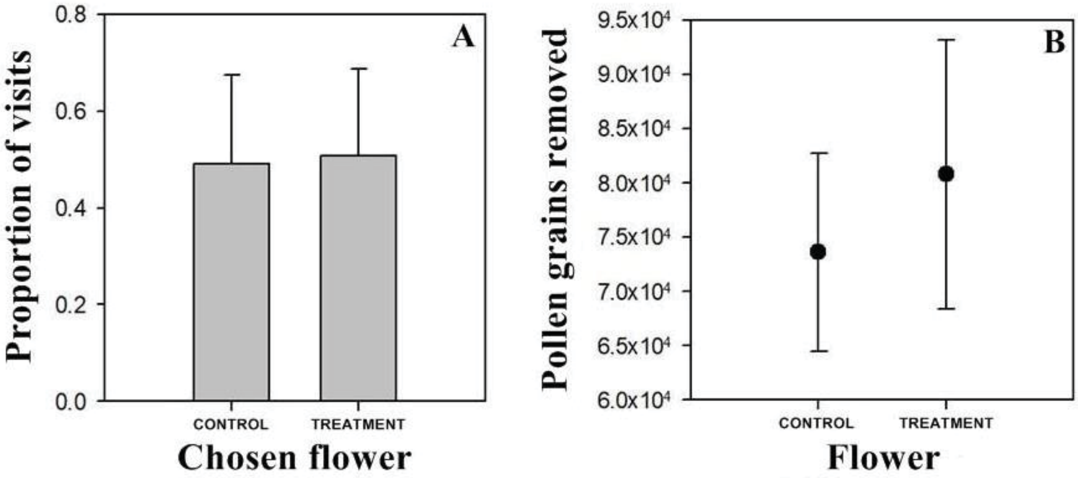
Figure 5. (A) Details of antesepalous (left) and antepetalous stamens (right) of *Huberia insignis*. (B) Anatomical structure of the stamen in a longitudinal section; connective with several layers of parenchyma cells. (C) Transverse section of the proximal portion of the appendage, with the epidermis intensely stained. The arrow indicates the vascular bundles in a central position. (D) Longitudinal section of the proximal portion of the appendages indicating a positive reaction with ferric chloride in the epidermal cells. (E) Transverse section of the distal portion of the appendage subepidermal cells with dense cytoplasmic content. (F) Longitudinal section of the distal portion of the appendages; arrows indicate subepidermal cells that reacted positively to ferric chloride. (G) Longitudinal section; the highlighted area indicates vascular tissue. (H) Positive reaction with PAS in the proximal region of the appendages; starch grains densely stained. Appendages (ap), connective (cn), epidermis (ep) and vascular bundles (vb).

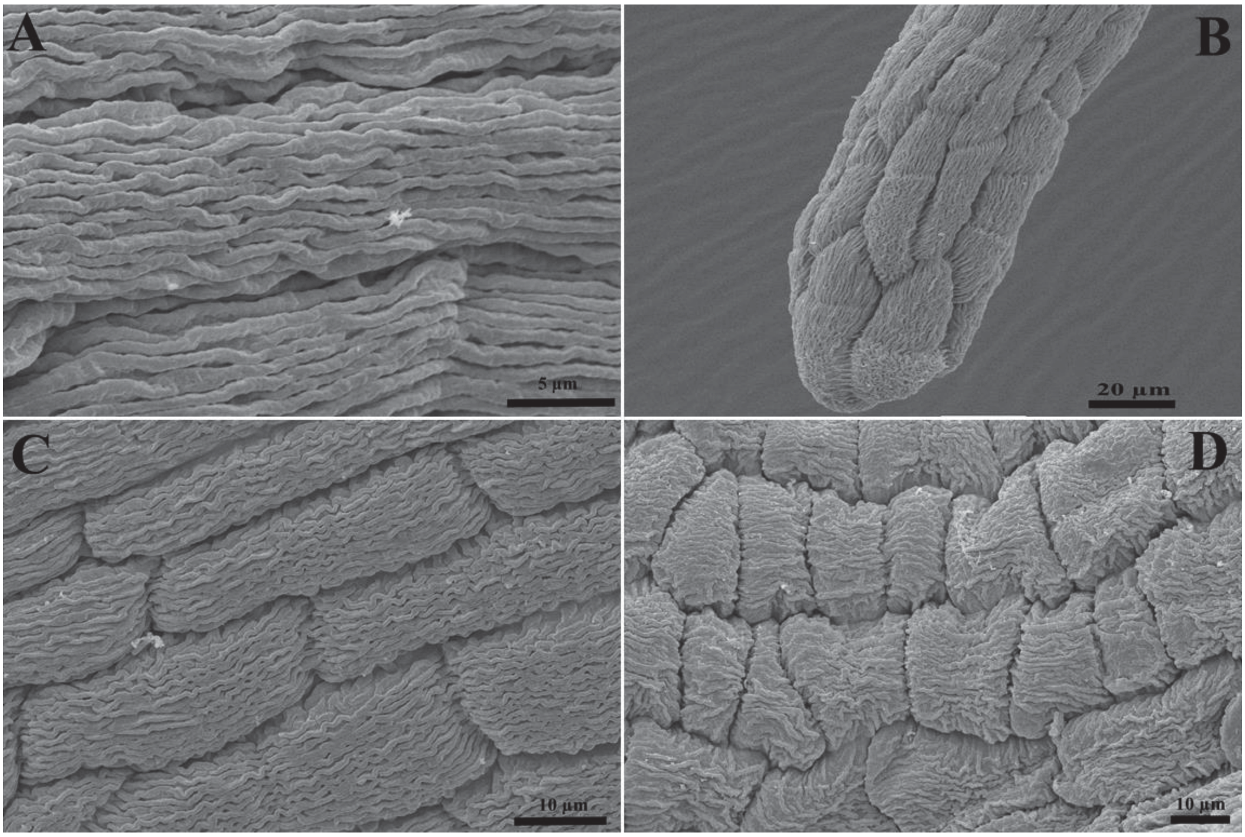
Figure S1. (A) *Bombus brasiliensis* and (B) *B. morio* buzz pollinating *H. insignis*. (C) Anthers chewed by *Trigona spinipes* to steal pollen; arrows indicate intact appendages.

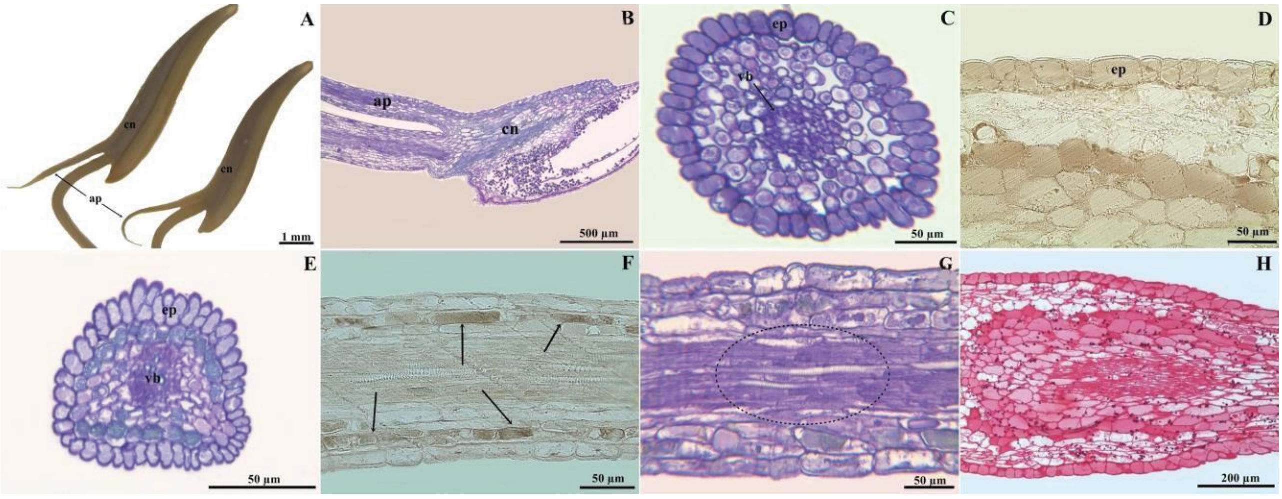
Figure S2. Frequency of visits (visits.flower^{-1}.h^{-1}) in *Huberia insignis* flowers. Letters indicate differences or similarities among treatments.

Figure S3. Wavelength (in nm) reflected by the anthers (dashed line), appendages (dotted line) and petals (whole line) of *Huberia insignis*.

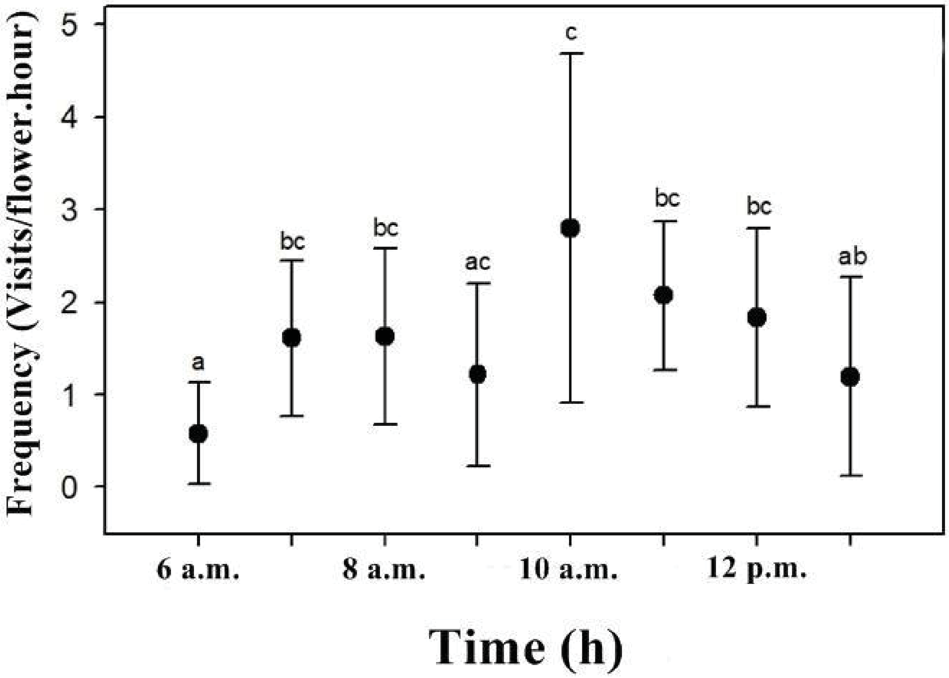

A
Proportion of fruit set
1.0
0.8
0.6
0.4
0.2
0
ab
b
a
C
CP
SP
B
Seed set
400
300
200
100
0
ab
b
a
C
CP
SP
Treatments
C
Proportion of germinated seeds
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
b
b
C
CP
SP

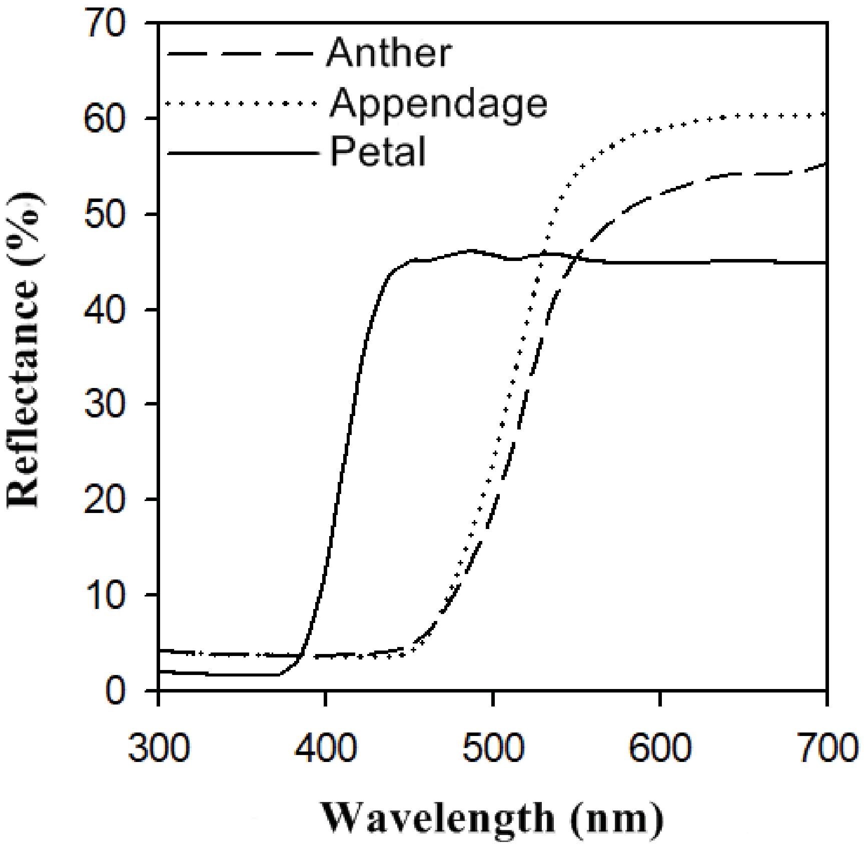

A
Proportion of visits
0.8
0.6
0.4
0.2
0.0
CONTROL
TREATMENT
Chosen flower
B
Pollen grains removed
9.5x10⁴
9.0x10⁴
8.5x10⁴
8.0x10⁴
7.5x10⁴
7.0x10⁴
6.5x10⁴
6.0x10⁴
CONTROL
TREATMENT
Flower


A
5 µm
B
20 µm
C
10 µm
D
10 µm


A
cn
cn
ap
1 mm
B
ap
cn
500 µm
C
ep
vb
50 µm
D
ep
50 µm
E
ep
vb
50 µm
F
50 µm
G
50 µm
H
200 µm


A
B
C


Frequency (Visits/flower.hour)
5
4
3
2
1
0
a
bc
bc
ac
c
bc
bc
ab
6 a.m.
8 a.m.
10 a.m.
12 p.m.
Time (h)


Anther
Appendage
Petal
Reflectance (%)
0
10
20
30
40
50
60
70
300
400
500
600
700
Wavelength (nm)

CAPÍTULO 2

"Pollen Tube Shower" in *Bertolonia* Raddi (Melastomataceae): a new delayed selfing mechanism in flowers with poricidal anthers

"*Pollen Tube Shower*" em *Bertolonia* Raddi (Melastomataceae): um novo mecanismo de autopolinização tardia em flores com anteras poricidas

* Manuscrito formatado nas normas do periódico *Annals of Botany*

ORIGINAL ARTICLE

"Pollen Tube Shower" in *Bertolonia* Raddi (Melastomataceae): a new delayed selfing mechanism in flowers with poricidal anthers

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Running Title: Pollen tube shower: new selfing mechanism in flowers with poricidal anthers

ABSTRACT

Background and aims: About one-fifth of angiosperms reproduce predominantly through self-fertilization, which is influenced by morphological and functional flower traits. Autonomous selfing is rare in Melastomataceae, a megadiverse family with most species presenting poricidal anthers and herkogamy. However, previous studies indicated that some species of the genus *Bertolonia* were capable of autonomous selfing. Our aim is to investigate whether there is indeed autonomous selfing in the group, as well as to understand how and at what stage in the flower's development this event occurs, and assess the impact of these events on the mating system of *Bertolonia* species. We also investigated if the autonomous selfing may be influenced by the anther's pore orientation.

Methods: We conducted field studies on floral biology, pollination experiments and observed the presence and rate of pollinator visitation for *Bertolonia paranaensis* (introrse pores) and *B. mosenii* (extrorse pores).

Key Results: The transient absence of herkogamy and dynamics of flower parts during post-anthesis events promoted a delayed selfing in the two species that were studied here. In post-anthesis flowers of both species, pollen grains germinate inside the anthers, and pollen tubes come out through the anther pores and reach the stigma, penetrating the transmission tissue and fertilizing the ovules. Pore orientation did not affected selfing. Both species received visits from buzzing bees and have mixed mating system, i.e. the flowers may be cross-pollinated during anthesis, and selfed during post-anthesis. This is the first register of autonomous selfing for plants with poricidal anthers, and we called this novel mechanism "pollen tube shower".

Conclusions: Pollen tube shower promotes reproductive assurance to flowers that have not been pollinated and ensures the reproductive success of individuals even in the absence of pollinators.

Keywords: Bertolonieae; buzz-pollination; female success; herkogamy; male success; mixed mating system; reproductive assurance; outcrossing.

INTRODUCTION

Most angiosperms have hermaphroditic flowers (Barrett, 2002). In the absence of self-incompatibility barriers, this morphological condition can lead to self-fertilization (Richards, 1997; Rea and Nasrallah, 2004). Mechanisms of self-fertilization, hereafter "selfing", are favoured in approximately 20% of angiosperms species (Barrett, 2002; Barrett, 2010). Its selection can be due to many different factors (Goodwillie *et al.,* 2005), but especially because it may increase the transmission of alleles when compared to cross-pollination (automatic selection hypothesis; Fischer, 1941; Lloyd, 1979; Richards, 1997), and promote reproductive assurance in environments or founding events with low density or viability of reproductive partners or pollinators (Baker, 1955; Lloyd, 1992; Morgan *et al*., 2005). Despite these benefits, in a long term, selfing can result in the production of genetically identical offspring, reducing the genetic diversity, which has the potential to negatively affect the dynamics of populations under unpredictable environments (Stebbins, 1957; Wright *et al.,* 2013).

Self-pollination may occur within the same flower (autogamy) or between flowers of the same individual (geitonogamy). Although both selfing types result in similar genetic consequences, there are usually differences in pollen removal success, e.g., geitonogamy is generally facilitated by pollinating agents (De Jong *et al.,* 1993), ultimately affecting the reproductive success (Lloyd, 1979; 1992). Autonomous self-pollination, i.e. the one performed without vectors, can be divided into three types, modulated by potential cross-pollination events: prior selfing, competitive selfing and delayed selfing (Lloyd, 1979). Prior selfing occurs before a potential cross-pollination event (e.g., Abdelaziz *et al.,* 2019), and may happen even before floral anthesis, i.e., during the bud stage (Lloyd, 1979). Competitive selfing occurs concomitantly with cross-pollination events, generating competition for ovule fertilization (Lloyd, 1979; Brys and Jacquemyn, 2011). Finally, delayed selfing occurs after potential cross-pollination events (Lloyd, 1979). Delayed selfing may provide reproductive assurance, without gamete discounting, and it is favoured in cases of unreliable pollinators, or even in their absence (Stebbins, 1974; Lloyd, 1979; 1992; Fenster and Martén-Rodríguez, 2007). Competing and even prior selfing may offer a selective advantage, compared to delayed selfing, by reducing investment in traits associated with pollinator attraction, such as flower size and longevity, and reward production (Ornduff, 1969; Elle and Carney, 2003; Carleial *et al.,* 2017).

The occurrence of autonomous selfing is generally associated with morphological and functional adaptations in flowers. For instance, variations in the spatial (herkogamy) and/or temporal (dichogamy) separation between the male (anther) and female (stigma) functions can increase self-pollination rates (Richards, 1997). The floral trait that is possibly the strongest one associated with autonomous selfing is cleistogamy (Lord, 1981; Culley and Klooster, 2007). Cleistogamous flowers do not open, therefore they must produce seeds by autogamy (Lord, 1981), either by direct deposition of pollen in the stigma, or by pollen germination inside the anthers, making their way to reach the stigma and fertilizing the ovules (Lord, 1981; Mayers and Lord, 1983). In chasmogamous (i.e., non-cleistogamous) species, autogamy may also occur with *in situ* germination of pollen grains, or with early germination inside the anthers, which has been reported in at least a dozen of angiosperm families (see Pacini and Franchi, 1982; Koul *et al.*, 1985; Sahai *et al.,* 2016; Mann *et al.,* 2020), but never in species with poricidal anthers.

Poricidal anthers have appeared several times throughout the evolutionary history of at least 72 families of angiosperms (Buchmann, 1983). For these species, pollen usually acts as both reproductive and attractive resources. Thus, plants benefit from pollen restriction, only accessed by visitors presenting the proper behaviour and size (Michener, 1962; Buchmann, 1983; De Luca and Vallejo-Marín, 2013). This adaptation in anthers may favour herkogamy, and consequently hinder or even make autonomous self-pollination impossible, although it does not prevent geitonogamy in self-compatible species, especially in species flowering synchronously (De Jong *et al.*, 2013).

Melastomataceae is a megadiverse plant family with pantropical distribution (Renner *et al*., 2019), in which most species have flowers with poricidal anthers (Renner, 1989). This floral trait, together with the expression of herkogamy, is believed to favour outcrossing in the group (Renner, 1989). In addition, at the floral bud stage the stamens are folded downwards, with the pores facing the hypanthium, ovary or the bottom of the flower, depending on the pore orientation, which keeps the pollen grains away from the stigma, and consequently preventing prior selfing events (Renner, 1989). There are a few records of autonomous selfing in the family (see Santos *et al*., 2010). However, for most of these records, the autonomous selfing mechanism is unclear.

Bertolonia is the only genus in tribe Bertolonieae (Bacci *et al.*, 2019) and it is represented by 34 species (Baumgratz, 1990; Bacci *et al*., 2018; Bisewski *et al*., 2020), all endemic to the Brazilian Atlantic Forest (Bacci *et al*., 2019). It is monophyletic (Bacci *et al.*, 2020), and its plants are small, mostly terrestrial herbs that usually grow in the shaded understory of dense, humid forests (Baumgratz, 1990). Its flowers may have white to pink petals, and white or yellow stamens (Bacci *et al.*, 2020). The stamens may show some variation in pore orientation: in some species they have extrorse pores (i.e., the pores facing outwards), and in others the pores are introrse (i.e., facing the centre of the flower), which was tentatively explained by Bacci *et al.* (2020) as related to selfing rates: they proposed that higher selfing rates would be favoured in species with introrse pores, while species with extrorse pores could be associated to higher outcrossing rates.

Despite these theoretical speculations, the only information about reproductive strategies and mating systems for the genus is the record of self-compatibility in *B. marmorata* (Naudin) Naudin (Ziegler, 1925 *apud.* Renner, 1989), and also the ability to set fruits without pollinators in *Bertolonia paranaensis* (Wurdack) Baumgratz (LSP, personal observation). In species of *Bertolonia,* herkogamy seems to be transient: during anthesis, the stamens are positioned in a way that the pores are kept distant from the stigma; after anthesis, the petals close (instead of just falling out), and the stamens form a bundle around the style, with anthers' pores at the same level of the stigma (LSP, pers. obs.). Apart from this delayed lack of herkogamy, selfing may also be favoured by the orientation of the anther pore (Bacci *et al.,* 2020). Curiously, the two above cited species, *B. marmorata* and *B. paranaensis* have this transient lack of herkogamy and present anthers with introrse pores (Baumgratz,1990; Bacci *et al.,* 2020), suggesting a relationship between these two floral traits and the potential capacity of self-pollination (Lloyd, 1965, Busch, 2005; Toräng *et al.*, 2017).

In this study, we selected two species of *Bertolonia*, with distinct pore dehiscence orientations, to test the relationship between floral traits and mating strategy under experimental pollination conditions. We assessed the potential existence of autonomous selfing on the genus, and compared the reproductive success of *B. paranaensis*, presenting introrse pores, and *B. mosenii* Cogn., with extrorse pores, to check whether introrse pores favour selfing in *B. paranaensis*. Specifically, we raised the following questions: (1) Is there, in fact, autonomous selfing in *Bertolonia* species? If confirmed, (2) are selfing events influenced by pore orientation? (3) How and at what stage of the anthesis does pollen self-deposition occur? (4) What is the specific contribution of outcrossing and selfing to the female and male success of individuals?

MATERIALS AND METHODS

Study site

Field work was performed during Dec. 2018 for *B mosenii*, Dec. 2018, Dec. 2019 and Jan. 2020 for *B. paranaensis*, at the Usina Hidrelétrica Parigot de Souza (UHE; 25°15'22.85 "S, 48°46'42.16 "W; between 650-800 m above sea level). UHE belongs to the “Environmental Protection Area of Pico Paraná” (Portuguese acronym: APAPP), in the “Serra do Mar”, in the municipality of Antonina, Paraná, Brazil. The site is within the Atlantic Rainforest biome, and predominantly covered with Montane to High-montane Ombrophilous Dense Forests (Veloso *et al*., 1991). The climate of the area is subtropical, humid, and lacking both a dry and frost seasons (Alvares *et al*., 2014).

Model species

Bertolonia paranaensis is an endangered herb species (CNCFlora, 2012). Its flowers present white petals and yellow stamens with introrse poricidal anthers (i.e., the anther pores are ventral and facing the stigmatic surface; Goldenberg *et al*., 2016; Fig. 1A, C). *Bertolonia mosenii* is an herb with flowers presenting white petals and stamens, with extrorse poricidal anthers (i.e., the anther pores are dorsal and facing outwards at beginning of anthesis; Baumgratz, 1990; Goldenberg *et al*., 2016; Fig. 1B, D). *Bertolonia mosenii* presents anthers with a slightly bifid apex and a double pore, a unique trait in the genus (Baumgratz, 1990; Goldenberg *et al*., 2016). Flowers of *B. paranaensis* (flower: 13 ± 4.2 mm, *fide* Baumgratz, 1990) are slightly bigger than those of *B. mosenii* (flower: 9.5 ± 2.5 mm, *fide* Baumgratz, 1990). The distribution of the two species are distinct: while *B. paranaensis* is microendemic, occurring only in the southern portion of São Paulo and in Paraná, *B. mosenii* exhibits the largest geographical distribution among the species in the genus, from Espírito Santo to Santa Catarina (Baumgratz, 1990; Bacci *et al*., 2018; Bacci *et al*., 2019). Samples of both species are deposited in the Universidade Federal do Paraná herbarium (vouchers UPCB93779, UPCB94777 for *B. paranaensis*; UPCB93763 for *B. mosenii*).

Floral biology, stigma, anther, and pollen traits

To describe the floral biology, we registered the events from pre- to post-anthesis for both species. We considered as anthesis the period of time when flowers were available for visits, i.e., the time elapsed since the start of floral aperture until its closure. To determine stigma receptivity, we tested 10 stigmas (n=10 individuals) with hydrogen peroxide (H_2O_2) at the beginning of the anthesis (Kearns and Inouye, 1993). To understand the dynamics of floral parts and whether it facilitates selfing events, we described the arrangement and morphology of the floral structures during and after the anthesis.

Since pollen viability can be related to mating system (Goldenberg and Shepherd, 1998; Goldenberg and Varassin, 2001; Caetano *et al.,* 2013), we tested the viability of pollen grains. For that, we selected two pre-anthesis buds per individual (n = 10 individuals). From each bud, we randomly selected two anthers, totalling 40 anthers per species. Anthers were fixed in 70% formalin–acetic acid-alcohol (FAA) solution, and later macerated on a blade. Pollen grains were stained with acetocarmine solution (1%) and counted under a light microscope (40x; FWL 1000; Feldmann Wild Leitz, Manaus, Brazil). We counted the first 200 grains in each sample (following Maia *et al.*, 2016), considering as viable the stained grains; malformed or uncoloured grains were considered as unviable (Kearns and Inouye, 1993). We calculated the percentage of pollen viability as the number of viable grains/total grains counted in a sample, multiplied by 100.

Because we noticed a variation in stigma size during anthesis we speculated that an increase in the stigmatic area may favour selfing, we took pictures of the stigmas of 30 flowers in pre-anthesis, and 30 in post-anthesis (60 flowers for species), under a stereoscopic microscope. Later we measured them using the *ImageJ* software (Rasband, 2004). To evaluate the occurrence of autonomous pollen deposition on the stigmas, we obtained images of stamens and pistils through scanning electron microscopy (SEM; JSM 6360-LV, JEOL, Tokyo Japan). We submitted post-anthesis flowers (six flowers per species) previously fixed in 70% FAA to dehydration through ethanol series (70, 80, 90 and 100%). After fixation in aluminium stubs, the samples went through critical point drying with CO_2, and were metallised with gold for about two minutes.

Mating system, female and male success

The mating system of the two species of *Bertolonia* were investigated through controlled pollinations in two flowering seasons for *B. paranaensis* (Dec. 2018 and Dec. 2019) and one season for *B. mosenii* (Dec. 2018). We used previously isolated flowers to apply the following manual pollination treatments, always paired with a control on the same inflorescence: Apomixis (AX: with emasculation of floral buds); manual cross-pollination (MCP; performed in emasculated flowers); autonomous self-pollination (ASP; floral buds and then flowers remained untouched and isolated); and control (C; we left flowers open for visitation). The *voile* bags were removed after two days in all treatments except ASP, for which they were kept for three days; right after the removal of the bags, the flowers were marked and followed till the fruits became ripe.

To determine the roles of crossing and selfing to the female success, and to check whether individuals suffer from pollen limitation, we followed the treated flowers until fruit set. We then randomly collected ten fruits per treatment from each species, in order to estimate seed set, and to evaluate seed viability. Due to lack of information on physiological characteristics of seed germination, we applied a qualitative assessment of seed formation using 30 seeds from each of the 10 fruits per species. The seeds were bleached in sodium hypochlorite for 2 h. We considered as perfect seeds the ones that contained embryos, while those without an embryo or with an atrophied one were considered unviable (*sensu* Baumgratz, 1983-1985).

To estimate the male success after manual outcrossing and selfing treatments, using the control treatment as a parameter, we selected four flowers to evaluate pollen tube growth 24 and 48 h after pollination under fluorescence microscopy (following Martin, 1959; see adaptations to the method in Maia *et al.,* 2016; Nikon A1R MP+, Tokyo, Japan). We assessed the existence of self-incompatibility sites preventing the development of pollen tubes (i.e., callose deposition) and observed in which period the pollen tubes reached the ovules.

Flower visitors and behaviour

We recorded the guild of floral visitors during the flowering peak of each species to infer whether the visitation rates and diversity of pollinators influenced the breeding strategies of both species. We made 30 h of focal observation for *B. paranaensis* and 28 h for *B. mosenii*. Prior to focal observations, we selected groups of individuals presenting the higher number of opened flowers in the day. We observed each group for 30 min (*B. paranaensis* = five groups in three days, from 0600 h to 1600h; *B. mosenii* = six groups in four days, from 0700 h to 1400 h). We recorded the frequency of visits and the behaviour of each visitor throughout the anthesis. Visit rates were calculated considering the frequency of a species/total number of flowers observed.

According to their behaviour, we characterised visitors as pollinators, when they vibrated all or most all of the anthers and simultaneously touched the stigma; thieves, when they vibrated one or a few anthers, without touching the stigma; and robbers, when they accessed the resource illegitimately, damaging floral tissues, and without making contact to stigma (Inouye, 1980). Visitors were collected and deposited in the Museum of Entomology Pe. Jesus S. Moure (DZUP), at the Universidade Federal do Paraná.

Data analyses

Data were analysed by fitting different models depending on the distribution of the response variable. To evaluate whether there was a difference in the diameter of the stigma between pre- and post-anthesis periods, we used the paired Student *t*-test. We assessed the effect of pollination treatments on the proportion of fruit set for each species (with N being the number of flowers and P the probability of a flower to develop into a fruit) using a generalised linear mixed model (GLMM), assuming a binomial distribution and logit function, using the identity of plants (individuals) as the random factor. For the analysis of seed set and its relationship with treatments, we used a linear model (LM). All analyses were performed in the software *R* 3.6.0 (R Core Team, 2013). For the analyses of Student *t*-test and LM, we used the *t.test* and *lm* functions, from the *stats* package (R Core Team, 2013). For the GLMM analyses, we used the *glmer* function, from *lme4* package (Bates *et al*., 2015). We apply post-hoc tests whenever necessary, with Tukey adjustment, assuming $\alpha \leq 0.05$, using the function *glht* from the *multcomp* package (Hothorn *et al*., 2008).

RESULTS

Floral biology, stigma, anther, and pollen traits

Bertolonia paranaensis

Bertolonia paranaensis bloomed for approximately 20 days, during December. There is a considerable variation in the number of flowers per inflorescence, ranging from 7 to 43, although a maximum of five flowers opened in each inflorescence in the same day. Before anthesis, the stamens remain folded down in the space between the ovary and hypanthium, with the pores facing the hypanthium (**Supplementary Data Fig. S1**). Anthesis begins at 0400 h, with the corolla fully opened between 0600 and 0630 h. When the petals are fully opened, the stamens begin to stretch out, being positioned slightly below the gynoecium, without touching it, until approximately 1000 h. After that, the stamens start to rise, but still not contacting the stigma, until 1500 and 1700 h. At about 1700 h the stigma starts the secretion of a viscous exudate (Fig. 5A), which happens concomitantly to the closure of the corolla. The flowers started closing the petals at 1630 h and were fully closed at 1830 h. Around 1900 h, we removed the petals from some post-anthesis flowers to check the arrangement of the structures. We observed that at this stage a few anthers (usually two) contacted the stigma through their pores (Fig. 5B). Petals and stamens remained attached to the flowers for up to 48 hours after the petals closed.

The stigmas from all tested flowers were receptive at the beginning of anthesis. Pollen viability was high (98.05% ± 1.21) and it is the only resource for pollinators. By the end of the anthesis, there was a mean increase of 0.15 mm in the diameter of the stigmatic surface (*t*-test = 8.74; df = 29; $P < 0.001$; Fig. 2). Forty-eight hours after the beginning of anthesis anther apices were held together and connected to the stigma by a thick tangle of pollen tubes coming out from the anther pore (Fig. 6). The pollen grains apparently germinate inside the anther and the pollen tubes exit through the pore and reach the stigma, penetrating the transmission tissue inside the style. This event results in the structures (stamens and pistil) joined by a weft of pollen tubes, observed only in post-anthesis flowers.

Bertolonia mosenii

Bertolonia mosenii flowered from late November to January, with a peak in the first two weeks of December. Inflorescences vary widely in the number of flowers, with 1-24 flowers per inflorescence, with one to four flowers open per day. There is an intense movement by the stamens, from bud stage to the end of the anthesis (**Supplementary Data Fig. S1**). Before anthesis, the stamens are folded downwards, with the anther pores facing the ovary wall. The anthesis is shorter than in *B. paranaensis*, with the petals starting to open between 0600 and 0630 h, being fully open around 0700 h. Right after the petals open, the stamens stretch out, positioning themselves around the stigma, in a nearly radial arrangement. In this arrangement, even though the anther pores are extrorse, the pores were always pointed in the same direction (outwards). About 0830 h, the stamens are more distant from to the style. From 1000 h, the distance between the anthers and the pistil decreases when the stamens, now grouped in two bundles, arrange themselves laterally to the style. At this moment, the filaments of the stamens positioned below the style twist a little bit, and the anthers consequently present introrse pores, i.e., facing in the same direction as the stigma. The stamens move themselves again into an almost radial position at around 1200 h, with the pores closer to the stigma than previously, but without contact it. After that, the stamens get even closer, surrounding the style, which is more evident by 1400 h. Around 1530 h the flowers are closed. During anthesis, there is no contact between anther pores and stigma. Nevertheless, after anthesis (around 1800 h), we removed the petals from some flowers to check the disposition of the stamens; by then, we observed the production of an exudate by the stigma, and also a direct contact between the pore of some (usually two) anthers and the stigma (Fig. 5C-D). The petals and stamens remain attached to the receptacle for up to 72 h after anthesis.

The stigma was receptive at the beginning of the anthesis in all tested flowers. The only resource offered for the visitors is pollen, with high viability (97.71% ± 3.55). The stigmas in post-anthesis flowers are, on average 0.11 mm larger than in pre-anthesis flowers (*t*-test = 4.85; df = 29; $P < 0.01$; Fig. 2). Twenty-four hours after anthesis, anther apices were connected to the stigma by a thick wad of pollen tubes, in the same way as observed for *B.*

paranaensis (Fig. 5E; Fig. 6). The pollen grains apparently germinate inside the anther and the pollen tubes exit through the pore and reach the stigma, penetrating the transmission tissue inside the style.

Mating system, female, and male success

Bertolonia paranaensis

Fruiting rates did not vary between years ($\chi^2 = 0.82$; df = 1; $P = 0.36$), and therefore we gathered the data from both years during the analyses. *Bertolonia paranaensis* did not set apomictic fruits. Fruiting rates differed between pollination treatments ($\chi^2 = 10.89$; df = 2; $P < 0.01$), being higher in the control treatment (C; 42/54 - 77.8%). Autonomous self-pollination (ASP; 22/63 - 34.9%) and manual cross-pollination (MCP; 17/52 - 34.7%) treatments did not differ from each other ($Z = -0.11$; $P = 0.99$; Fig. 3A). The same pattern was found in seed production ($F = 7.31$; df = 2; $P < 0.01$; Fig. 4A). Control fruits (589 ± 72.5 seeds) set more seeds than autonomous self-pollination (470.2 ± 79.17 seeds; $t = 3.74$; $P < 0.01$) and manual cross-pollination (508.8 ± 59.6 seeds; $t = -2.53$; $P = 0.04$). The amount of seeds formed in fruits from manual cross-pollination did not differ from autonomous self-pollination ($t = 1.22$; $P = 0.45$). The proportion of perfect seeds in all treatments was over 90%.

We did not find self-incompatibility reactions in the pollen tubes from any of the treatments. Twenty-four hours after anthesis, the pollen tubes from treatments C and MCP had already contacted the seminal rudiments. For ASP, pollen tubes reached the seminal rudiments only within 48 hours after the anthesis (Fig. 5F-G), suggesting a delayed selfing event.

Bertolonia mosenii

Bertolonia mosenii produced fruits by autonomous self-pollination (ASP; 21/39 - 53.8%) and also in treatments MCP (18/25 - 72%) and C (16/27 - 59.2%), but did not produce apomictic fruits. Although there was a tendency of the MCP treatment to produce more fruits, it was not significant ($\chi^2 = 0.50$; df = 2; $P = 0.78$; Fig. 3B). The fruits produced through autonomous self-pollination (489.49 ± 92 seeds) had more seeds than the fruits from the other treatments (C = 347.1 ± 71 seeds; MCP = 369.6 ± 131.75 seeds; $F = 11.52$; df = 2; $P < 0.01$; Fig. 4B). The proportion of perfect seeds in all treatments was over 90%.

There was no evidence of self-incompatibility reactions, such as callose deposition, along the transmission tissue. From the ASP treatment, we recorded pollen tubes in contact with the ovules only 48 h after the beginning of anthesis (Fig. 5H-I). On the other hand, pollen tubes from MCP and C had already reached the ovules 24 h after anthesis.

Flower visitors and their behaviour

Bertolonia paranaensis

We recorded three species of bees visiting the flowers of *B. paranaensis* during Dec. 2019 (Table 1). *Ariphanarthra palpalis* was the most frequent visitor (1.38 ± 0.02 visits/flower), followed by *Trichocerapis* sp. (0.79 ± 0.04), while *Trigonopedia* sp. was seen only once (0.03). The three species presented size and behaviour of true pollinators: in each visit they vibrated all anthers at the same time and contacted the stigma. While *Trichocerapis* sp. (Fig. 5J) collected the pollen following a route, distributing the pollen among the individuals within a group, the other species visited one or two flowers and left to other groups of plants. The visits were concentrated in the mornings (from 0600 to 1140 h), with a peak between 0800 and 1000 h (ca. 65% of visits). Although anthesis starts at dawn, there were no visits at this time.

Bertolonia mosenii

The flowers of *B. mosenii* were visited by the same three species of bees that visited *B. paranaensis* (Table 1). The bee species with highest visitation rate was *Ariphanarthra palpalis* (0.58 ± 0.02; Fig. 5K), followed by *Trigonopedia* sp. (0.31 ± 0.02; Fig. 5L) and *Trichocerapis* sp. (0.27 ± 0.02). During the visits, individuals from the three species acted as true pollinators since they vibrated all the anthers and contacted the stigma (Fig. 4G). The visits were restricted to the morning period, between 0700 and 1200 h. About 60% of the visits took place between 0800 and 1000 h.

DISCUSSION

Pollen tube shower, a delayed selfing mechanism, enables *Bertolonia paranaensis* and *B. mosenii* to present a mixed mating system, providing reproductive assurance for both species in the absence or ineffectiveness of cross-pollination events. The delayed selfing mechanism does not present a direct relation with the orientation of the anther's pore in flowers of *Bertolonia*. Other floral traits, such as the transient absence of herkogamy due to the dynamics of stamen movement, the enlargement of stigmatic surface, and the production of exudate on the stigma during post-anthesis events assure the occurrence of the delayed selfing, even on the species with extrorse pores. Furthermore, during the anthesis, visits of buzzing bees in both species maintain levels of outcrossing.

Pollen tube shower: a delayed selfing mechanism in flowers with poricidal anthers

Germination of pollen grains inside the anthers is a frequent phenomenon in cleistogamous plants (Lord, 1981), but less common in chasmogamous flowers (Sahai *et al.,* 2016; Mann *et al.,* 2020). Our study is an unprecedented record of autonomous self-pollination through *in situ* germination of pollen grains in plants with poricidal anthers.

Poricidal anthers tend to avoid pollen waste by flower visitors, since they are associated with a specialised pollination system (buzz-pollination; Buchmann, 1983; Renner, 1989; but see Brito *et al.,* 2016). Poricidal anthers are also associated to herkogamy, decreasing the probability of self-pollination. Both floral traits are commonly present in Melastomataceae (Renner, 1989). However, for the species of *Bertolonia* studied here, the anther pores apparently favour self-pollination in a peculiar way, by restricting the area of the anthers that may contact the stigma. This contact is very precise, and it allows (1) the entry of the exudate produced by the stigma, which seems to be important for pollen germination inside the anthers (see below) and, (2) it directs the pollen tubes germinated inside the anthers trough the only exit available, which is precisely the part of the anther that is in contact with the stigma. This peculiarity led us to call this mechanism "pollen tube shower", when the pollen grains germinate inside the anthers, and a thick waft of pollen tubes exit anthers through the pore to reach the stigma. Moreover, the pollen tube shower occurs only after anthesis, i.e., after potential cross-pollination events, characterising the reproductive assurance mechanism by delayed selfing.

Floral traits and post-anthesis events favour delayed selfing in *Bertolonia*

In *B. paranaensis* and *B. mosenii*, the dynamics of the stamens position (resulting in the lack of herkogamy), short flower lifespan and the late production of exudate favoured delayed selfing after anthesis. It is relevant to highlight the dual role (dual adaptive significance; Ruan & Silva, 2011) that the dynamics and position of stamens provide during anthesis and post-anthesis, and their impacts on the reproductive success of both species: i. during anthesis, the arrangement of stamens provides a functional/temporal herkogamy, especially during the peak of pollinator visits, spatially separating the reproductive structures, and consequently decreasing the chances of selfing and promoting outcrossing; ii. in post-anthesis flowers, the final position of anthers and stigma enable the occurrence of the pollen tube shower, providing reproductive assurance in *Bertolonia* species.

Another trait that may be related to the selfing strategy in *Bertolonia* is the fact that the flowers close their petals, which is not common in Melastomataceae. In most species in the family, the flowers usually last one day, and petals and stamens fall right after anthesis (Renner, 1989). The flower/corolla closure may have two consequences. The first is that it may provide a microenvironment capable of maintaining an optimum level of humidity, and consequently helping to keep the viability of pollen, and also limiting the entrance of pathogenic microorganisms (van Doorn & van Meeteren, 2003). The second is a mechanical help from the petals: since during the anthesis the pores do not touch the stigma, it is reasonable to suppose that the petals push the stamens to the style while closing.

The increase in size of the stigmatic surface during the anthesis provides a broader area facilitating contact with the anthers. Together with the late production of exudate in the stigma, those are traits related to the selfing process in *Bertolonia*. This happens near to the petals closing time, and may be associated with the stimulation of pollen germination inside the anthers. Exudates produced by the stigma serve as a source of water, sugars, secondary compounds and other substances that enable the germination of pollen grains, acting in the formation of a biochemically active environment for successful pollination (Dafni, 1992; Rejón *et al.*, 2014). Exudates may also contain phenolic compounds that act on gametophytic recognition through interaction with growth regulators or compounds related to this process (Martin, 1969). In summary, these exudates may help pollen grains to germinate, and also provide for the pollen tube development and orientation towards the stigma.

Mixed mating system, female, and male success in *Bertolonia* species

Bertolonia paranaensis and *B. mosenii* are not apomictic, as suggested by high pollen viability (Goldenberg & Shepherd, 1998; Goldenberg & Varassin, 2001; Caetano *et al.,* 2013) and lack of fruits produced from emasculated and isolated flowers. Both species produce fruits and seeds by cross- and self-pollination, with the former taking precedence over the later. Although both species present a mixed mating system (Goodwillie *et al.*, 2005), we recorded variations in fruit and/or seed produced in controlled pollination treatments. In *B. paranaensis*, both fruit set and number of seeds produced were higher in the control treatment than in crossing and selfing, and there was no difference between the last two. However, considering the fruit set in the control group, we suspect that this result may be due to a methodological bias. After manipulations, flowers of autonomous self-pollination and manual cross-pollination were immediately isolated with bags, which may have affected the normal post-anthesis process, such as petal closure, interfering in pollen tube growth in both treatments, or resulting in flower abortion (Dafni, 1992; Young & Young, 1992). Although fruit set did not differ between treatments in *B. mosenii*, the number of seed formed increased in the absence of pollinators, suggesting that the species might actually invest more in production of seeds from selfing (female success) than in pollinator attraction (male success). One evidence might be the small size of flowers and the lower number of opened flowers per day, which negatively affect visitor attraction (Ashman & Schoen, 1997; Goodwillie *et al.,* 2010). Therefore, pollen from non-visited flowers can be recruited to self-fertilize the ovules, ensuring seed production.

As maternal tissues may allocate more resources to the first fertilized ovules (Havens and Delph, 1996; Delph *et al.*, 1998), and the growth of outcrossing pollen tubes in *Bertolonia* occurs before selfing events, outcrossing pollen might be favoured when pollinators are available in both species. Conversely, delayed selfing promotes female success in cases of limitation or absence of partners and/or pollinators (Lloyd, 1979). An extreme consequence of the investment concentrated in seed production (female success) through selfing is cleistogamy. There is one evidence for this in the genus: when describing *B. sanguinea* Sald. ex Cogn. var. *sanguinea*, Baumgratz (1990) mentioned that the petals were kept almost closed during the whole flower lifespan. The same author proposed that the pollinator should be very small in order to access the pollen, but another explanation could be that these flowers do not really open and would not be visited at all, i.e. they may be cleistogamous, and fruits and seed would be produced autonomously.

Since outcrossing levels are directly related with pollinator effectiveness in *Bertolonia* species (control treatment), higher outcrossing rates are consequence of higher pollen removal and deposition rates (male success). Visitation rates were higher in *B. paranaensis* than *B. mosenii*, which may indicate an increase in the outcrossing level in the former. The bigger size and higher quantity of flowers per inflorescence (when compared to *B. mosenii*), may explain a higher rate of pollinator visits, and consequently higher pollen removal and deposition (Bell, 1985; Stanton and Preston, 1988; Moré *et al.,* 2012; Toledo *et al*., 2020), increasing both male and female reproductive success. Even with higher resource allocation in selfing events, *B. mosenii* still received visits from legitimate pollinators, a factor that indicates a balance in autogamy rates.

We emphasize that we have not confirmed that the extrorse pore orientation in *Bertolonia* species is actually associated with higher outcrossing rates as proposed by Bacci *et al.* (2020b). The establishment of a mixed mating system in both *Bertolonia* species is certainly due to the consistent advantages of outcrossing (Darwin, 1876; Hamrick & Godt, 1989; Holsinger, 2000; Wright *et al.,* 2008) and reproductive assurance by delayed selfing events (Morgan & Wilson, 2005; Busch & Delph, 2012). Small-sized populations like the ones usually found in species of *Bertolonia* (Bacci *et al.,* 2018), are less attractive to pollinators (Dauber *et al.,* 2010; Marques *et al.,* 2014) and, consequently, reduce the amount of pollen deposited on the stigmas. Therefore, a mixed mating system is advantageous and may be an evolutionary consequence to avoid pollen limitation (Lloyd, 1992; Ashman *et al*., 2004; Goodwillie *et al.,* 2005; Busch & Delph, 2012). Moreover, small flowers also lead to a decrease in pollen supply due to lower recruitment rate of pollinators (Elle & Carney, 2003; Brys & Jacquemyn, 2011; Kennedy & Elle, 2008), which can result in reduced anther/stigma distance and, consequently, in autonomous selfing events (Ornduff, 1969; Eckhart & Geber, 1999), as found in species of the genus *Bertolonia.*

SUPPLEMENTARY DATA

Figure S1. Dynamics of stamens in flowers of (A-E) *Bertolonia paranaensis* and (F-J) *B. mosenii* during anthesis. (A) Flower of *B. paranaensis* at the beginning of anthesis; (B) at 0800 h, (C) 1000 h, (D) 1500 h, and (E) at 1700 h. (F) Flower of *B. mosenii* at the beginning of anthesis, (G) at 0830 h, (H) 1200 h, (I) 1300 h, and (J) at 1500 h.

FUNDING

This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (masters grant 88882.382585/2019-01 to L.S.P., and postdoctoral grant 1659767 to F.J.T.); Conselho Nacional da Pesquisa Científica, Brazil (“produtividade em pesquisa 300865/2017-4 to R.G.).

ACKNOWLEDGMENTS

We thank the UHE (Copel) teamwork for the support and research permits; the CTAF/UFPR (Centro de Tecnologias Avançadas de Fluorescência) and CME/UFPR (Centro de Microscopia Eletrônica) for the fluorescence and SEM images; Isabela Galarda Varassin from LINTER/UFPR (Laboratório de Interação e Biologia Reprodutiva) for support and permission to use the laboratory; Leticia Graf and Rodrigo Gonçalves, both from the "Laboratório de Abelhas" (UFPR), for assistance with bees identification.

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TABLE

Table 1. Abundance, visitation rates (abundance/total number of flowers observed) and behaviour of visitors of *Bertolonia paranaensis* (n = 29 flowers) and *B. mosenii* (n = 26 flowers).

Species	Abundance	Rate ± sd	Behavior
Bertolonia paranaensis			
HYMENOPTERA			
APIDAE			
Apinae			
Trichocerapis sp.	23	0.79 ± 0.04	Pollinator
Trigonopedia sp.	1	0.03	Pollinator
Halictinae			
Ariphanarthra palpalis (Moure, 1951)	40	1.38 ± 0.02	Pollinator
TOTAL	**64**	**2.20**	
Bertolonia mosenii			
HYMENOPTERA			
APIDAE			
Apinae			
Trichocerapis sp.	7	0.27 ± 0.02	Pollinator
Trigonopedia sp.	8	0.31 ± 0.02	Pollinator
Halictinae			
Ariphanarthra palpalis (Moure, 1951)	15	0.58 ± 0.02	Pollinator
TOTAL	**30**	**1.16**	

FIGURE LEGENDS

Figure 1. Flowers and stamens of (A) *Bertolonia paranaensis* and (B) *B. mosenii*. (C) Illustration of lateral view and introrse pore detail of a stamen of *B. paranaensis*. (D) Stamen of *B. mosenii*, dorsal view and detail of extrorse double pore.

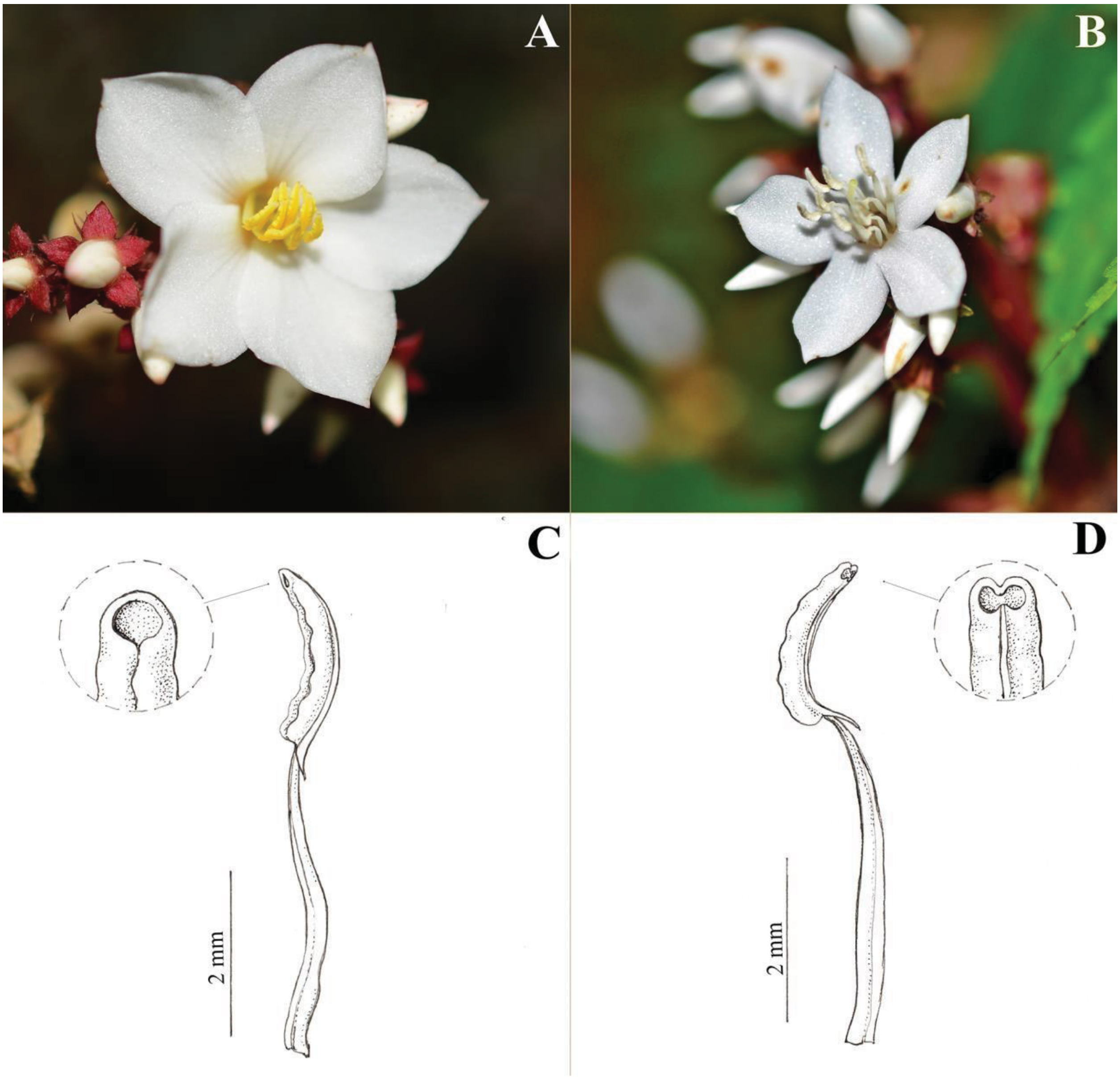
Figure 2. Stigma diameter (mm) of *Bertolonia paranaensis* (white circles) and *B. mosenii* (black circles), in pre- and post-anthesis. * represents statistical differences between stages.

Figure 3. Fruit-set resulting from the manual pollination experiments in (A) *Bertolonia paranaensis* and (B) *B. mosenii*. Letters indicate differences or similarities among treatments. Ns = non-significant differences among treatments. Control (C), manual cross-pollination (MCP) and autonomous self- pollination (ASP).

Figure 4. Seed-set resulting from the manual pollination experiments in (A) *Bertolonia paranaensis* and (B) *B. mosenii*. Letters indicate differences or similarities among treatments. Control (C), manual cross-pollination (MCP) and autonomous self-pollination (ASP).

Figure 5. (A) Exudate on the stigmatic surface in a flower of *B. paranaensis.* (B) Flowers of *Bertolonia paranaensis,* details of a flower in anthesis and another one in post-anthesis (in the background) with the corolla removed, showing the stamens positioned around the style. (C) Stigmatic exudate in a post-anthesis flower with the corolla removed of *B. mosenii.* (D) Anthers in contact with the stigma of *B. mosenii*. (E) Mass of pollen tubes leaving the anther and penetrating the stigma in *B. mosenii*. (F) Selfing pollen tubes growing in the transmitting tissue of *B. paranaensis* and (G) penetrating the ovules. (H) Selfing pollen tubes reaching the ovary and (I) penetrating ovules of *B. mosenii*. (J) *Trichocerapis* sp. pollinating *B. paranaensis.* (K) *Ariphanarthra palpalis* and (L) *Trigonopedia* sp. pollinating *B. mosenii*. Anthers (a), style (s), pollen tubes (pt), transmitting tissue (tt) and ovules (o).

Figure 6. Scanning electron microscopy images of reproductive whorls of (A-C) *Bertolonia paranaensis* and (D-F) *B. mosenii*. (A) Pollen grains germinating inside anthers and pollen tubes penetrating the stigma in *B. paranaensis*; (B) detail of pollen tube exiting the anther pore and (C) penetrating the stigma. (D) Pollen grains germinating inside anthers and pollen tube penetrating the stigma in *B. mosenii*; (E) pollen tubes contacting the dilated stigma. (F) Detail of stigma with unicellular papillae receiving pollen tubes in *B. mosenii*. Anthers (a), style (s) and pollen tubes (pt).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2 mm
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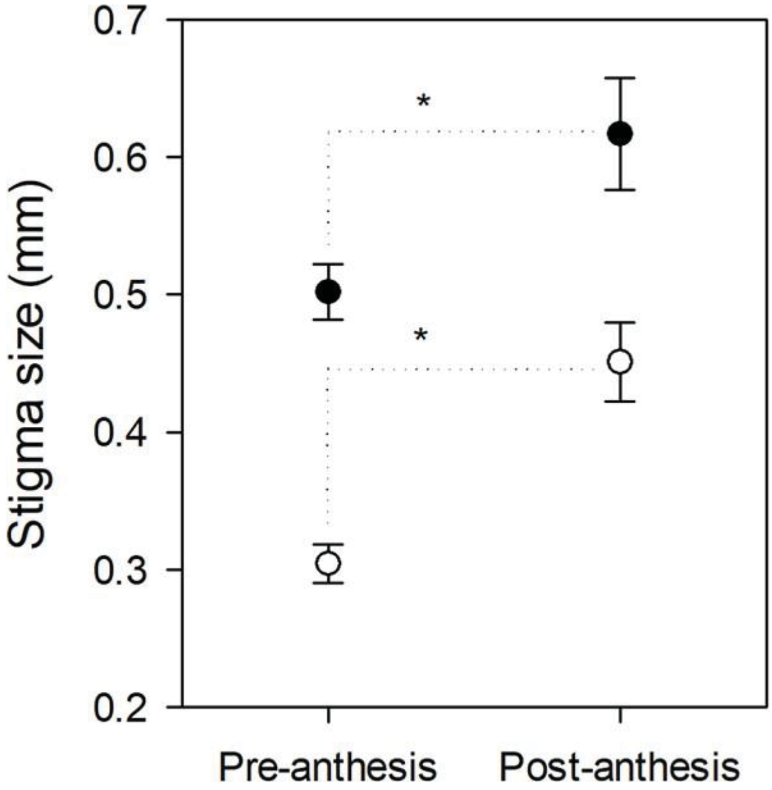
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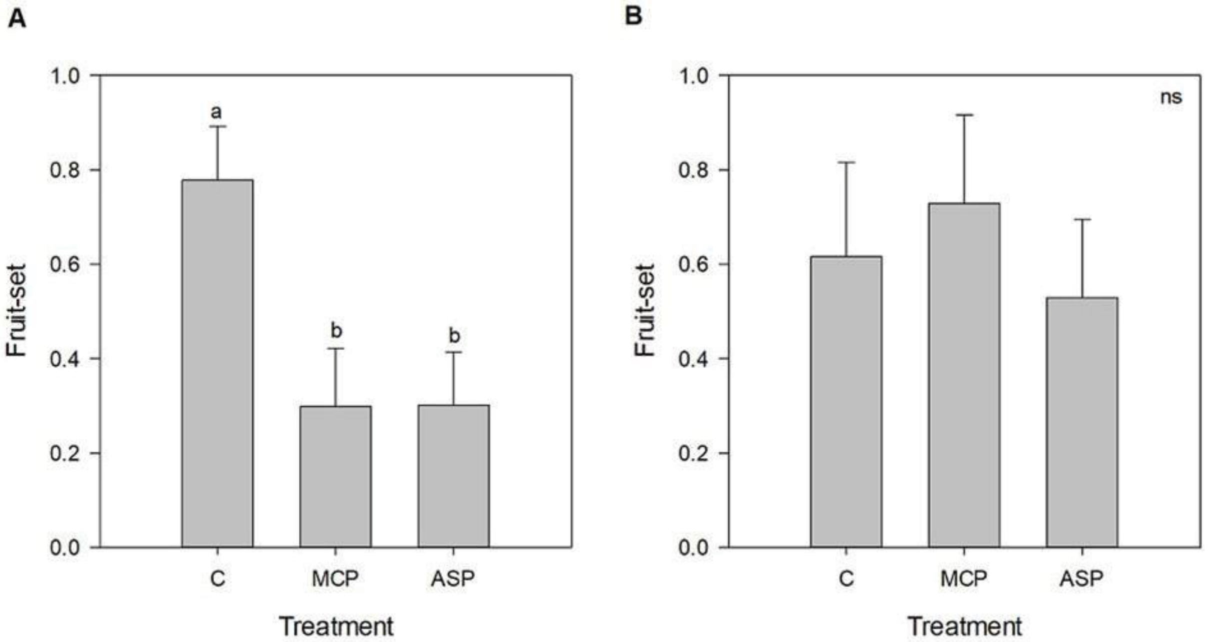
Post-anthesis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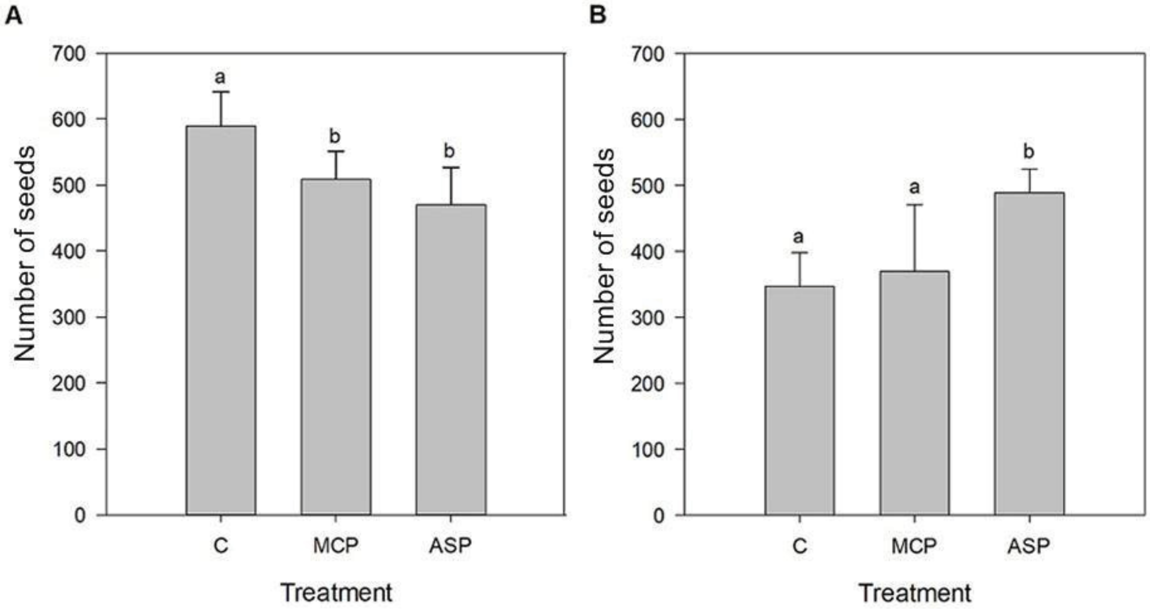
Treatment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Number of seeds
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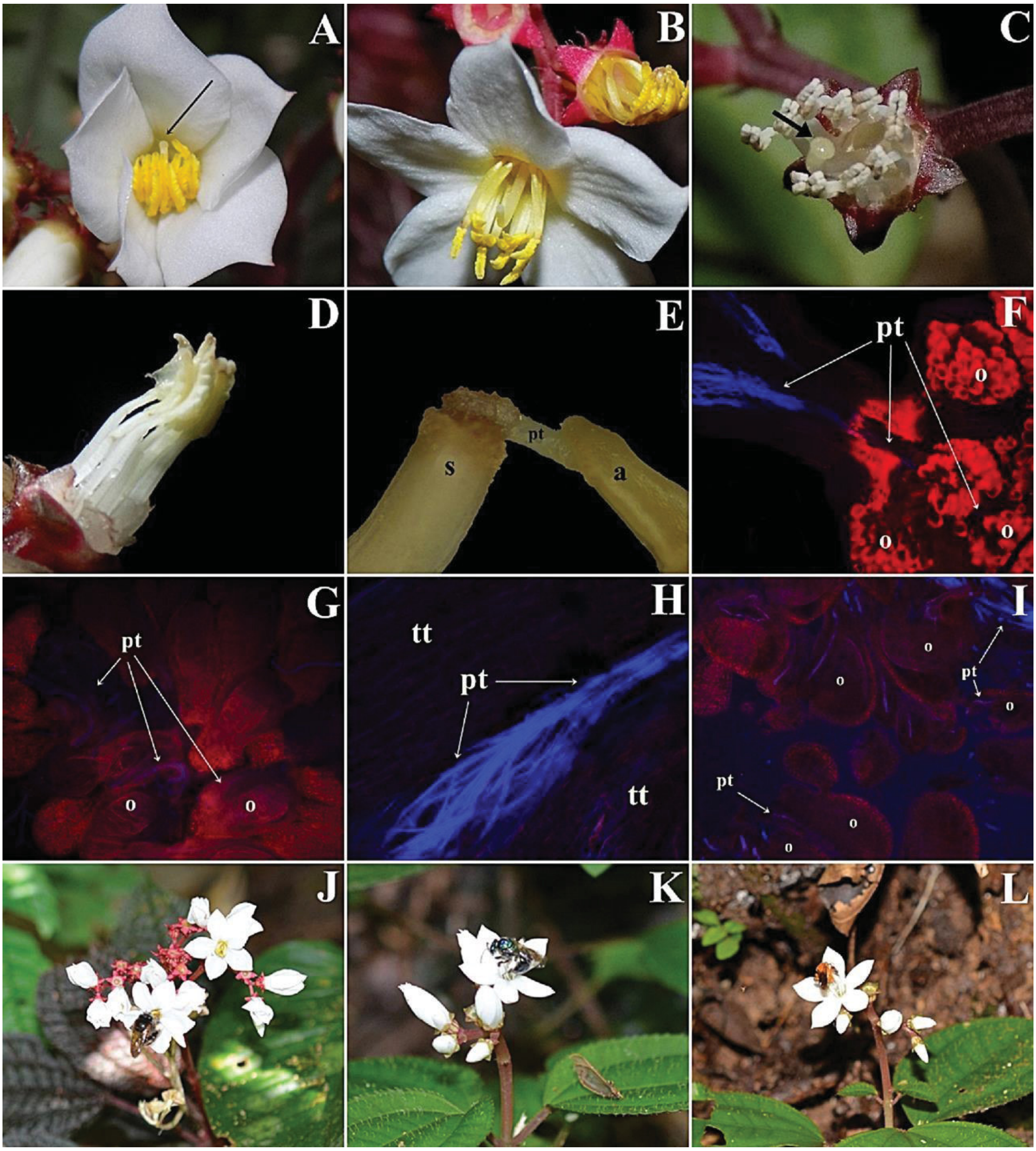
ASP
Treatment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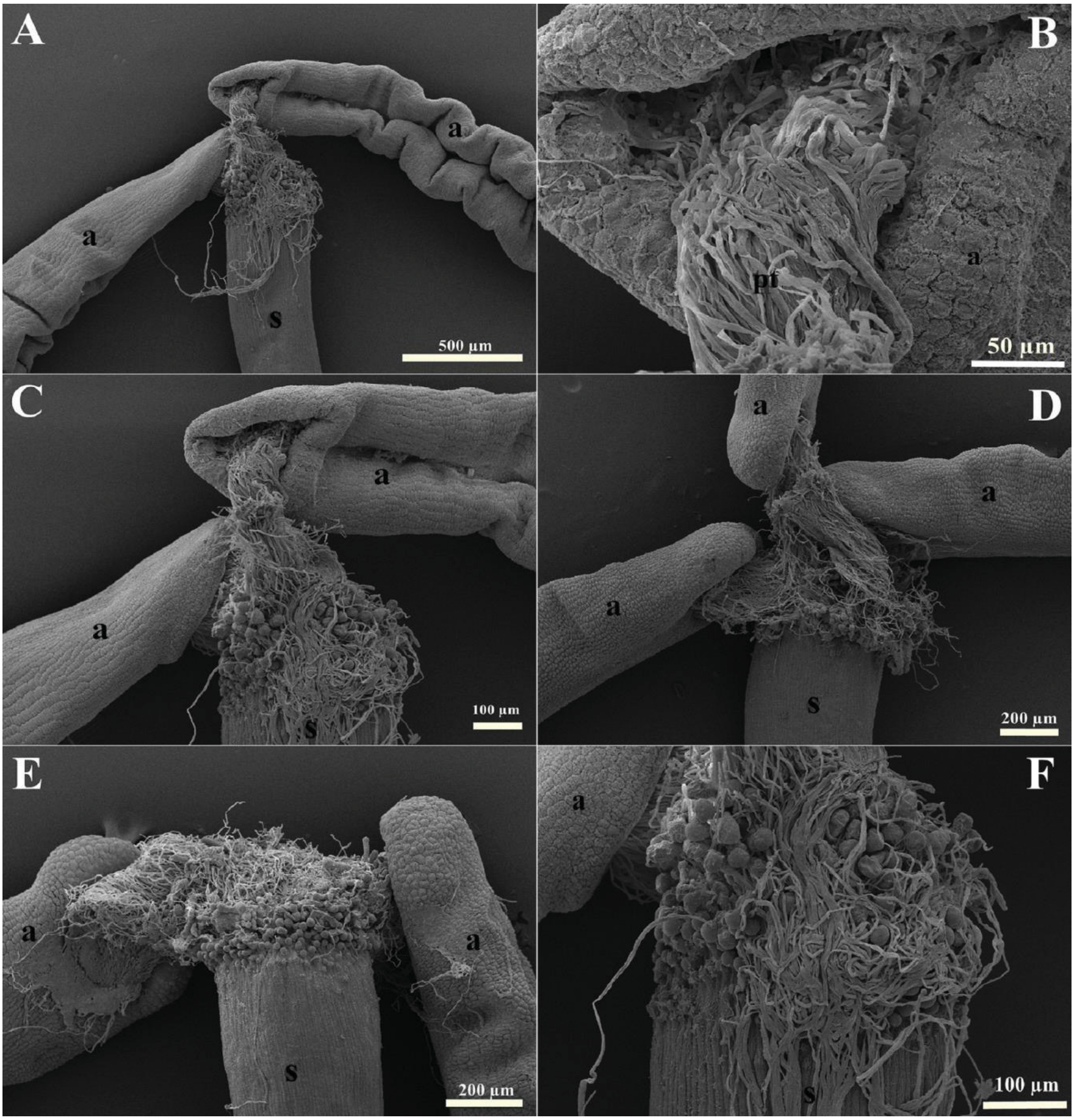
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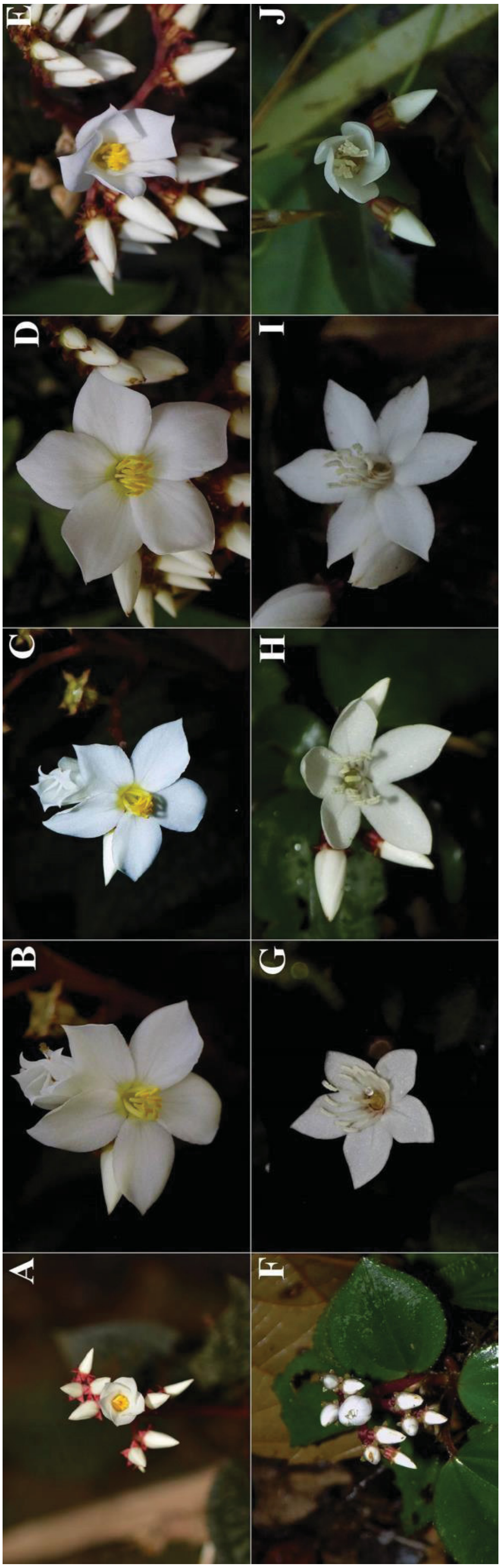
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CAPÍTULO 3

Is *Bertolonia* (Melastomataceae) an autogamous genus? New records of pollen tube shower on several species point to that!

Bertolonia (Melastomataceae) é um gênero autogâmico? Novos registros de *pollen tube shower* em várias espécies apontam que sim!

* Manuscrito formatado nas normas do periódico *Plant Systematics and Evolution*

Is *Bertolonia* (Melastomataceae) an autogamous genus? New records of pollen tube shower on several species point to that!

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ABSTRACT *Bertolonia* is a Brazilian genus of Melastomataceae and endemic to the Atlantic Forest (AF). A recently study based on molecular phylogenetic evidence shows a strong correlation between species distribution patterns and floral traits, being the genus informally divided into three clades: marmorata (northern AF), formosa (central AF) and nymphaeifolia (southern AF). A recent study with two species of the genus (both of nymphaeifolia clade) revealed a curious delayed selfing mechanism, the pollen tube shower (PTS), which ensures the reproductive assurance of these species. We investigate a potential occurrence of PTS in other species of the genus: *B. violacea* (marmorata clade) and *B. acuminata* (nymphaeifolia clade), as well as we search in exsiccates and taxonomic works evidence of traits that indicated the possibility of selfing in other species. Both *B. violacea* and *B. acuminata* produce seeds through the PTS. We also observed the occurrence of PTS in flowers of an exsiccate of *B. maculata*. Taxonomic records indicate that in all clades of *Bertolonia,* there are a lacking herkogamy, closure and retention of the corolla in the floral receptacle in post-anthesis, traits associated with the occurrence of PTS. We present in our work empirical evidence and descriptions of flower traits that indicate that the genus *Bertolonia* is autogamous, something rare in Melastomataceae.

Keywords Autonomous self-pollination; Bertolonieae; Delayed selfing; Poricidal anthers; Reproductive assurance.

Introduction

Bertolonia is a genus represented by 35 herbaceous species and two varieties (Baumgratz 1990; Bisewski et al. 2020; Bacci et al. 2020*a*). Its distribution is restricted to eastern Brazil, and species are endemic to the Atlantic Forest (AF) biome (Bacci et al. 2019). Based on molecular evidence, Bacci et al. (2020b) recovered *Bertolonia* alone within the Bertolonieae tribe. Moreover, the genus was divided informally in three clades: "marmorata", "formosa" and "nymphaeifolia" (Bacci et al. 2020*b*). These clades present a strong correlation with geographical distribution of their species, with the members of the "marmorata" clade occurring more often in northern AF, the members of the "formosa" clade in central AF and also with species of the “nymphaeifolia” clade (together with the type of the genus: *B. nymphaeifolia* Raddi) distributed in southern AF. Furthermore, together with this geographic congruence, several morphological characters, mainly related with the flowers, such as color patterns, were also somewhat conserved among the clades recovered (Bacci et al. 2020*b*).

In a recent survey, a new selfing mechanism called *pollen tube shower* (PTS) was described, where pollen tube germinates from poricidal anthers reaching the stigmas, and then fertilizes the ovules, resulting in perfect seeds (Passos et al. in prep.). Pollen tube shower was registered for only two species, *B. paranaensis* (Wurdack) Baumgratz and *B. mosenii* Cogniaux, both members of "nymphaeifolia" clade, thus ensuring the reproductive assurance of these species through delayed selfing (Passos et al. in prep.).

In this study, we aim to investigate if this selfing mechanism also occurs in species of the other clades within the genus. We investigated in living and herborized specimens of the three clades of *Bertolonia* the possibility of PTS events. Moreover, we searched in taxonomic works for features that could indicate the possibility of the occurrence of self-pollination mechanism in other species of the genus.

Materials and Methods

We performed autonomous self-pollination experiments by isolating the flower with *voile* bags to avoid visits from potential pollinators on two species of *Bertolonia*, *B. violacea* Bisewski, Bacci & R. Goldenberg (Fig. 1A), and *B. acuminata* Gardner (Fig. 1B-C), to investigate the capacity of fruit set through the PTS mechanism. We conducted the pollination experiments in the greenhouse (n = 27 flowers; plants from the Instituo Nacional da Mata Atlântica, Santa Teresa, Espírito Santo) for the first species and a natural population for the second (n = 12 flowers; Rio Mãe Catira, Serra da Graciosa, Morretes, Paraná, Brazil – 25°21’49.04”S, 48°52’37.75”W). We also realized observations of floral biology for *B. violacea*, evaluating the time of anthesis and post-anthesis, and the arrangement and dynamics of the floral whorls during these events.

We investigated the ocurrence of self-pollination events in other *Bertolonia* species in the UPCB (Thiers 2020) collection, through the analysis of flowers in post-anthesis. Since when the PTS occurs anthers and pistil of post-anthesis flowers are joined by a weft of pollen tubes (Passos et al. in prep.), flowers with this condition were considered a very relevant evidence of the occurrence of self-pollination events. Therefore, we removed flowers from the exsiccates, rehydrated in heated water and observed them under a stereoscopic microscope (ZEISS Stemi 305, Jena, Germany). During the observation, we also evaluated if there was pollen deposited or adhered to the stigma of the respective flowers.

We obtained in taxonomics descriptions and monographs of *Bertolonia* species (references in Table 1) data and field information (e.g., flower traits, time of anthesis) that provided us with evidence of the existence of features of selfing species, such as brief floral longeivity and retention of floral pieces in post-anthesis as occurs in *B. paranaensis* and *B. mosenii* (Passos et al. in prep.). We employed this approach to expand our knowledge about other species which we did not have access to the flowers (living, fixed in solution or even herborized ones).

Results and Discussion

We registered that both *B. violacea* (14/27 - 51.9%) and *B. acuminata* (6/12 - 50%) can form fruits and seeds through PTS. We observed that the reproductive structures in flowers of post-anthesis of *B. violacea* (Fig. 1D) and *B. acuminata* (Fig. 1E) were joined by filaments coming out of the pore of the anthers that entered through the stigmatic surface of the respective flowers, as it occurs in *B. paranaensis* and *B. mosenii* (Passos et al in prep.). *Bertolonia violacea* presents a short flower lifespan, with anthesis beginning at 5 a.m. and the corolla fully opening between 6 a.m. and 6:30 a.m., and the beginning of the corolla closure around 11:30 a.m., fully closing at 2 p.m. During the anthesis, the stamens are arranged radially and away from the stigma. With the beginning of the petal closure, the androecium is organized in the center of the flower (Fig.1F-G), with the anther pores approaching the stigma. The post-anthesis flowers (Fig. 1H) remain in the floral receptacle until 48h after the anthesis. Also, we registered the production of exudate on the stigmatic surface in flowers in post-anthesis. These same floral characteristics (i.e. brief period of anthesis, transitory herkogamy, and flower retention in the receptacle, and exudate formation in post-anthesis) were described in *B. paranaensis* and *B. mosenii* (Passos et al. in prep.).

Most exsiccates of *Bertolonia* species analyzed present only fruits or floral buds, exsiccates with post-anthesis flowers are rare, which seems to be a pattern in several *Bertolonia* collections (Bacci et al. 2020*b*). However, we observed in an exsiccate of *B. maculata* DC. (voucher UPCB79510) that some anthers were "connected" to the stigma by pollen tubes that came out of the anthers, a possible evidence from PTS. We also noticed that the stigma of other flowers was impregnated with pollen between the stigmatic papillae (Fig. 1).

Bertolonia species generally do not present herkogamy (Table 1). When there is a significant difference in size between the style and stamens, the latter is larger (e.g. *Bertolonia sanguinea* Sald. ex Cogniaux var. *sanguinea* and *Bertolonia valenteana* Baumgratz; Table 1), and possibly at the end of the anthesis they towards the stigma, as previously observed in *B. paranaensis* and *B. mosenii* (Passos et al. in prep.), and also evidenced *B. violacea*.

Some species have been described as having cupuliform corolla, such as *B. bullata* Baumgratz, Amorim & A. B. Jardim, *B. sanguinea* var. *sanguinea* and *B. nymphaeifolia* Raddi (Baumgratz 1990; Baumgratz et al. 2011). Also, for *B. sanguinea* var. *sanguinea*, Baumgratz (1990) propose that the pollinator should be too small to access the pollen; however, it may be that the flowers are not visited at all, and this species presents some degree of cleistogamy (Passos et al. in prep.). However, considering the brief period of anthesis of *B. violacea* and *B. mosenii* (Passos et al. in prep.), possibly the species mentioned above were found in the field at the end of the anthesis, when the petals return, in a disarranged way, to the initial position, as in floral bud phase (Passos et al. in prep.; Bacci et al. 2020*a*). This behavior of returning the petals to the initial position and their retention seems to be common in *Bertolonia* and can be associate to autonomous self-pollination (Passos et al. in prep.); *Bertolonia lucernula* Bacci, D.T. Iglesias & R. Goldenb., a species recently described, also presents these characteristics (Bacci et al. 2020*a*). Although they are excluding hypotheses, both the potential cleistogamy in *B. sanguinea* var. *sanguinea* and the brief period of anthesis have already been recorded for *Bertolonia* species (Passos et al. in prep.; this paper) and could be strategies that favor selfing. Cleistogamous flowers are necessarily autogamous (Lord 1981; Culley and Klooster 2007), while flowers with a brief period of anthesis would represent a trade-off between investing resources in seed production through selfing and investing in pollinator attraction (Darwin 1876; Lloyd 1987; Richards 1997).

The five species of which there is evidence of PTS occurrence and, consequently, the occurrence of selfing, are distributed in the two opposite clades on the phylogeny; *B. maculata* and *B. violacea* to the north ("marmorata" clade), and, *B. paranaensis*, *B. mosenii* and *B. acuminata* to the south ("nymphaeifolia" clade). Although we have not found evidence of selfing in species of the "formosa" clade due to the lack of herborized material with flowers or of plants in vivo for studies of floral biology, taxonomic records such as the absence of herkogamy have been found in almost all species (Table 1). Moreover, cupuliform corolla in *B. bullata* which indicates a certain level of cleistogamy or short period of anthesis, both related to the possibility of autonomous self-pollination, besides the register of closing and permanence of the petals in the flowers of *B. lucernula* (Bacci et al. 2020*a*), are indications that the autonomous self-pollination also occurs in this clade and, therefore, in the genus as a whole. Moreover, other groups that present similar floral traits (similar sizes between the reproductive structures and the closure and retention of the petals in post-anthesis, facilitating the meeting between anther and style) may be also capable of delayed self-pollination, as occurs in *Monolena trichopoda* R. H. Warner (Warner 2002). The genera *Monolena* Triana ex Benth. & Hook, *Triolena* Naudin, *Macrocentrum* Hook f., *Salpinga* Mart. ex DC. and *Boyania* Wurdack , that once belonged to Bertolonieae s.l., could potentially perform autonomous self-pollination due to homoplastic floral traits very similar to *Bertolonia* (Cogniaux 1891; Renner 1993; Clausing & Renner 2001; Goldenberg et al. 2012; Bacci et al. 2019).

Finally, our work points out an important finding, considering that at least five species of *Bertolonia* (14.2% of all species) are capable of producing fruits by selfing. The morphological and functional traits that involve the flowers of the genus are an excellent indication of the existence of a Melastomataceae group capable of reproducing sexually and without the requirement for pollen vectors. Thus, these species can ensure the formation of new offspring resulting from a few visits or absence of pollinators, something rare in Melastomataceae.

Acknowledgments We thank Isabela Galarda Varassin from LINTER/UFPR (Laboratório de Interação e Biologia Reprodutiva) for support and permission to use the laboratory. We also thank Lucas Bacci for his contributions to the manuscript, and Lucas Katsumi for supplying seedlings of *Bertolonia violacea.*

Funding This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (masters grant 88882.382585/2019-01 to LSP, and postdoctoral grant 1659767 to FJT); Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (“produtividade em pesquisa”, 300865/2017-4 to RG).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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Table

Table 1. Orientation of pore dehiscence in anthers and length (in mm) of stamens and style in *Bertolonia* Raddi species.

Species	Pore orientation	Length (mm)		References
		Stamens	**Style**	
Bertolonia acuminata Gardner	Introrse	3.6-5.5	4-6.5	2
Bertolonia alternifolia Baumgratz, Amorim & A. B. Jardim	Extrorse	6.6-9.9	7.5-11.4	7
Bertolonia angustifolia Cogniaux	Introrse	No data	No data	1
Bertolonia angustipetala Bacci & R. Goldenb.*	Introrse	6-7.5	5-6	5
Bertolonia bullata Baumgratz, Amorim & A. B. Jardim	Introrse	3.7-4.5	3.8-4.5	7
Bertolonia carmoi Baumgratz	Introrse	8.2-9.5	6-11	1
Bertolonia cuspidata Bacci & Amorim	Introrse	4.4-5.9	3.6-5.9	7
Bertolonia duasbocaensis Bacci & R.Goldenb	Introrse	6-7	5	4
Bertolonia formosa Brade	Introrse	8.0-9.7	7.5-8.0	1
Bertolonia foveolata Brade	Introrse	5.5-6	5-5.5	1
Bertolonia grazielae Baumgratz	Introrse	6.2-7.2	6	1
Bertolonia hirsutissima Bacci, Michelang. & R. Goldenb.	Extrorse	6-7	5	3
Bertolonia hoehneana Brade	Introrse	5.5-6	6-6.5	1
Bertolonia igrapiuna Bisewki, Bacci & R. Goldenb.	Extrorse	4.5-6.5	4.3-6.4	7
Bertolonia kollmannii Bacci & R. Goldenb*	Extrorse	4.5-5.5	6	5
Bertolonia leuzeana (Bonpl.) DC.	Introrse	5-5.6	5	1
Bertolonia linearifolia Bacci & Michelang.*	Extrorse	5-7	7	5
Bertolonia lucernula Bacci, D.T. Iglesias & R. Goldenb.	Introrse	5.5-8	4.5-5.5	8
Bertolonia macrocalyx Bacci & R.Goldenb.	Introrse	6.3	5	4
Bertolonia maculata DC.	Apical	5.8-8.7	7-7.6	1
Bertolonia margaritacea Naudin	Extrorse	6.5-7**	3-6	2
Bertolonia marmorata (Naud.) Naudin	Introrse	4-6	4.8-6	1
Bertolonia michelangeliana Bacci & R.Goldenb.	Extrorse	10.9–11.5	12	4
Bertolonia mosenii Cogniaux	Extrorse (two pore)	3.6-8.8	6.5-8.2	1
Bertolonia nymphaeifolia Raddi	Introrse	7 -10.5	8-8.5	1
Bertolonia organensis Baumgratz, Gonçalves-Silva & Nunes-Freitas	Introrse	5.5-7.5	6	6
Bertolonia paranaensis (Wurd.) Baumgratz**	Introrse	5.8-7	5.6-5.8	1
Bertolonia riocontensis Bisewski, Bacci & R. Goldenb.	Extrorse	7-8	8	7

Bertolonia reginatoi Bacci & Michelang. *	Introrse	5-7.5	8-10	5
Bertolonia ruschiana Bacci & R.Goldenb.	Introrse	6-7.5	6	4
Bertolonia sanguinea Safd. ex Cogniaux var. *sanguinea*	Introrse	18-19	12.5-13	1
Bertolonia sanguinea var. *santos-limae* (Brade) Baumgratz	Introrse	18-19	12.5-13	1
Bertolonia valenteana Baumgratz	Introrse	12.1	9.7	1
Bertolonia violacea Bisewki, Bacci & R. Goldenb.	Extrorse	5.7-5.9	5.7-7.3	7
Bertolonia vitoriana Bacci & Amorim*	Introrse	6.3	5.1	5
Bertolonia wurdackiana Baumgratz	Introrse	5.6-6.6	5.3-5.5	1

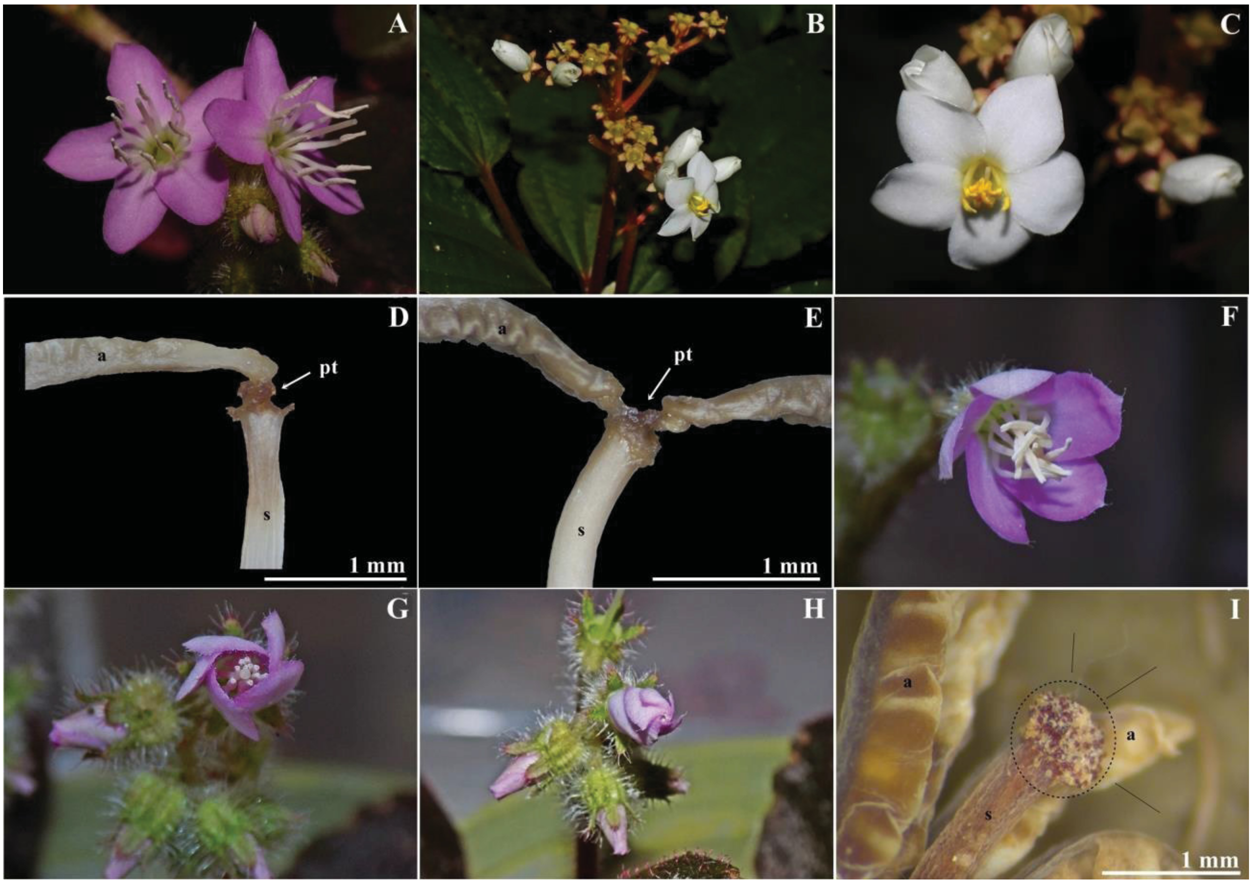
[1]Baumgratz (1990); [2]Goldenberg et al. (2016); [3]Bacci et al. (2016*a*); [4]Bacci et al. (2016*b*); [5]Bacci et al. (2018); [6]Silva-Gonçalves et al. (2016), [7]Bisewski et al. (2020) and [8]Bacci et al. (2020*a*).

*Length of stamens results of filaments length plus anther length (Goldenberg et al. 2016; Bacci et al. 2018).

*******Bertolonia paranaensis* was described, in Goldenberg et al. (2016), with anthers dehiscing through an extrorse pore, although, we observed introrse pore; confirmed by Bacci (pers. comm.).

Figure

Fig. 1 (A) Flowers of *Bertolonia violacea*. (B) *Bertolonia acuminata*. (C) Detail of *B. acuminata* flower. Mass of pollen tubes leaving the anther and penetrating the stigma in (D) *B. violacea* and (E) *B. acuminata*. Flower of *B. violacea* at the end of the anthesis: (F) stamens gathering in the center of the flower, around the style; (G) petals closing with stamens and style included, and (H) flower in post-anthesis with reproductive structures included. (I) Pollen on stigma of *B. maculata*, anther and stigma were connected through pollen tubes but separate during handling. Subtitle: (a) anthers, (s) style and (pt) pollen tubes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CONSIDERAÇÕES FINAIS

Os dados obtidos nesta dissertação nos permitiram discutir sobre diferentes estratégias reprodutivas adotadas por espécies endêmicas da Mata Atlântica pertencentes a dois gêneros distintos de Melastomataceae. Embora ocupem uma mesma região geográfica e estejam submetidas às mesmas condições climáticas, estas espécies desenvolveram diferentes estratégias de reprodução. Tais diferenças podem ser reflexo de fatores como a diferença na estrutura da vegetação, micro-habitat e disponibilidade de luz; influenciando, por exemplo, na disponibilidade de recursos e polinizadores.

No primeiro capítulo, trabalhamos com *Huberia insignis*, a fim de investigar uma possível função dos apêndices estaminais, uma estrutura peculiar e presente nas espécies do gênero *Huberia*, no sistema de polinização da espécie. Para tal, utilizamos diferentes abordagens, como testes de presença/ausência dos apêndices para averiguar a atratividade de polinizadores e taxas de remoção de pólen, reflectância espectral dos apêndices e também análises morfo-anatômicas, com a finalidade de identificar possíveis estruturas ou tecidos que pudessem influenciar na atração de polinizadores ou mesmo na biomecânica da vibração das flores. Não encontramos nenhuma função dos apêndices estaminais que afetassem a atração de polinizadores ou na biomecânica floral durante a vibração das abelhas para a retirada de pólen. Portanto, concluímos que estas estruturas sejam vestigiais para *H. insignis*, utilizando também como base, além dos experimentos, o tamanho reduzido dos apêndices quando comparados a outras espécies do mesmo gênero. *Huberia insignis* é uma espécie que ocupa áreas montanas à altomontanas. Nessas áreas, a estrutura vegetacional é mais baixa e por ocorrerem em encostas, os indivíduos de *H. insignis* são mais conspícuos em meio à vegetação, principalmente durante a floração. Esta característica é muito importante, considerando que esta espécie, apesar de ser autocompatível, é totalmente dependente de polinizadores para a frutificação e formação de sementes, principalmente através da polinização cruzada. Suas flores apresentam hercogamia espacial e atraem principalmente abelhas do gênero *Bombus*, que mantém rotas de visitação, ambos os fatores contribuem positivamente para o aumento das taxas de xenogamia em *H. insignis*. Neste capítulo destacamos também a importância da aplicação de análises morfo-anatômicas nos estudos que envolvem a polinização por vibração, considerando que o substrato a ser vibrado (sejam as anteras ou estames como um todo) tem influência direta na propriedade biomecânica de vibração, sendo uma excelente ferramenta capaz de elucidar padrões e processos evolutivos que envolvem este sistema de polinização tão extraordinário.

No segundo e terceiro capítulos, trabalhamos com aspectos reprodutivos de espécies do gênero *Bertolonia*, endêmico da Mata Atlântica. Este grupo reúne espécies que geralmente ocupam o estrato herbáceo de florestas úmidas e sombreadas. Muitas espécies do gênero apresentam um padrão de distribuição microendêmico, além de estarem sob algum grau de ameaça. Recentemente, foram propostos três clados intragenéricos para *Bertolonia*, sendo que estes clados apresentam uma forte correlação com traços florais e a distribuição geográfica ao longo da Mata Atlântica.

Mais especificamente, no segundo capítulo desta dissertação, buscamos investigar se, de fato, algumas espécies do gênero eram aptas para realizar a autopolinização espontânea, assim como verificamos se essa potencial capacidade de autopolinização estava relacionada com a orientação da deiscência dos poros das anteras. Conduzimos esta investigação com as espécies *B. paranaensis* e *B. mosenii*, inseridas no clado de *Bertolonia* de distribuição mais ao sul da Mata Atlântica (a saber, clado nymphaeifolia). De maneira surpreendente, constatamos que ambas as espécies são capazes de se autopolinizar e que a orientação do poro não parece influenciar nessa capacidade, mas outras características florais, sim. Aspectos morfo-estruturais e fisiológicos das flores das espécies estudadas configuram estratégias que possibilitam a formação de frutos e sementes de forma autônoma, sem a necessidade de vetores de pólen. Além disso, a forma como a autopolinização ocorre nestas espécies, a qual denominamos "*pollen tube shower*" ("banho de tubo polínico", uma tradução literal para o português), é um mecanismo de autopolinização curioso e inédito para Melastomataceae e outros grupos que apresentem anteras de deiscência poricida. Neste mecanismo, observamos que os grãos de pólen germinam ainda no interior das anteras, emitindo tubos polínicos que saem pelos poros, que por sua vez, direcionam estes tubos polínicos até o estigma da respectiva flor. Além disso, o *pollen tube shower* confere a essas espécies segurança reprodutiva na ausência de polinizadores ou em caso de poucos parceiros reprodutivos, considerando que a autopolinização é tardia, ou seja, ocorre apenas durante a pós-antese, favorecendo a polinização cruzada quando esta ocorre.

A partir dos resultados obtidos no segundo capítulo, levantamos a hipótese de que o *pollen tube shower* pudesse ocorrer em mais espécies do gênero *Bertolonia*, até mesmo para o gênero como um todo. Para isso, em um terceiro capítulo, investigamos em outras duas espécies: *B. violacea*, com distribuição mais ao norte da Mata Atlântica (clado marmorata) e *B. acuminata* (clado nymphaeifolia), a ocorrência desse novo mecanismo de autopolinização. Além disso, considerando a dificuldade de obter plantas ou flores de outras espécies de *Bertolonia*, buscamos em exsicatas com flores (algo raro para o grupo) e em trabalhos taxonômicos para o gênero, informações que nos permitissem inferir sobre a capacidade de autopolinização em outras espécies. Constatamos que tanto *B. violacea* e *B. acuminata* apresentaram evidências de *pollen tube shower*, assim como encontramos evidência em uma exsicata de *B. maculata.* Registros taxonômicos de outras espécies também apontam características de autogamia para o gênero. Portanto, concluímos que *Bertolonia* pode ser um gênero autogâmico, algo raro em Melastomataceae.

Nossos resultados levantam a possibilidade de que outras abordagens e metodologias seriam muito importantes para obtermos conclusões mais robustas nos três capítulos, como monitorar o tempo gasto por polinizadores em flores com e sem apêndices de *H. insignis* (Capítulo 1), técnicas moleculares para avaliar a contribuição da polinização cruzada e autogamia nas espécies de *Bertolonia* (Capítulo 2) e acesso a espécimes floridos dos três clados de *Bertolonia* (Capítulo 3). Neste sentido, nossos dados trazem o *insight* para que novas perguntas possam ser respondidas acerca de estratégias reprodutivas, biologia floral e polinização em Melastomataceae, ou mesmo em outros grupos polinizados por vibração.

Por fim, interessante ressaltar que, mesmo que muitos caracteres florais sejam, de maneira geral, conservados em Melastomataceae (e.g.: flores hermafroditas, diplostêmones e anteras tubulares de deiscência poricida), as estratégias reprodutivas podem divergir muito entre as espécies. Revelando as múltiplas estratégias reprodutivas existentes em Melastomataceae, uma família amplamente distribuída em ambientes tropicais e protagonista de diversas interações complexas que compõem padrões e processos evolutivos capazes de moldar o contexto que habitam, principalmente em biomas tão antigos e severamente ameaçados como o Cerrado e a Mata Atlântica.

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