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BIOLOGIA FLORAL DE *ZEYHERIA TUBERCULOSA* (BIGNONIACEAE)

CAMILA VAZ DE SOUZA

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Ciências Biológicas (Botânica), Área de concentração: Morfologia e Diversidade Vegetal.

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SUMÁRIO

VAZ, C. **BIOLOGIA FLORAL DE *ZEYHERIA TUBERCULOSA* (BIGNONIACEAE).** 2015. 79p. DISSERTAÇÃO (MESTRADO) – INSTITUTO DE BIOCIÊNCIAS, UNESP UNIVERSIDADE ESTADUAL PAULISTA, BOTUCATU.

Resumo - A família Bignoniaceae é um bom modelo dos tipos de diversificação evolutiva que deram origem à diversidade de comunidades de plantas tropicais, sendo sua interação com os polinizadores um fator determinante para tal. *Zeyheria tuberculosa* é uma espécie ameaçada de extinção e apresenta características relacionadas à síndrome de melitofilia. Informações sobre a reprodução desta espécie poderão auxiliar na compreensão dos processos ecológicos que podem ter levado esta espécie a ser incluída na lista vermelha das espécies ameaçadas de extinção. Assim, este estudo teve como objetivo central investigar a biologia floral de *Z. tuberculosa*, enfocando o padrão de secreção de néctar e as características funcionais e estruturais do nectário floral, responsável pela produção do recurso essencial à manutenção da interação com os visitantes florais. Os resultados obtidos foram divididos em dois capítulos que abordam questões específicas e complementares. O primeiro contempla informações sobre os eventos ocorridos durante a antese floral, a ecologia da polinização e o padrão de secreção de néctar em plantas de *Z. tuberculosa* situadas em fragmentos de floresta estacional semidecídua, imersos em uma paisagem alterada. O segundo aborda a estrutura e o funcionamento do nectário floral desta espécie, com enfoque nas características relacionadas ao tipo de secreção. Para o Capítulo 1, monitoramos flores durante todo o período de antese; fizemos observações focais dos visitantes e os capturamos para examinar a área de deposição de pólen; registramos a porcentagem de grãos de pólen viáveis e verificamos o período de receptividade do estigma; obtivemos dados relativos à morfometria das flores e das abelhas polinizadoras; fizemos testes de autoincompatibilidade para avaliar a dependência de vetores de pólen para a fecundação cruzada; avaliamos a dinâmica de secreção de néctar através dos parâmetros volume, concentração e miligramas totais de açúcares, utilizando este último parâmetro para calcular a oferta calórica média disponível aos polinizadores por flor e por planta e realizamos análises da composição química de açúcares do néctar floral. Para o Capítulo 2, realizamos análises estruturais utilizando métodos convencionais de anatomia vegetal e de microscopia eletrônica, além de realizarmos testes histoquímicos para verificar as principais classes de compostos presentes nos tecidos secretores do nectário. *Zeyheria tuberculosa* mostrou-se uma espécie melitófila, dependente de abelhas médias para a transferência de pólen entre plantas por ser autoincompatível. O néctar foi o principal atrativo primário e fonte de energia floral para seus polinizadores, sendo rico em hexoses, com predomínio dos monossacarídeos glicose e frutose, o que corresponde às preferências

alimentares de abelhas. Registramos um aumento na concentração de açúcares ao longo da antese, o que possivelmente ocorreu por evaporação, já que as flores campanuladas de *Z. tuberculosa* aparentemente não conferem uma barreira tão efetiva contra a perda d'água, quanto as flores de Bignoniaceae que possuem tubos cilíndricos e longos. De modo geral, houve variação significativa nos miligramas totais de açúcar produzidos ao longo da vida flor, ocorrendo um aumento progressivo do primeiro para o segundo dia de antese. Verificamos também variação significativa entre plantas nos miligramas totais de açúcar produzidos por flor, indicando uma oferta intraespecífica variável deste recurso. O volume e a concentração do néctar foram baixos quando comparados a outras espécies melitófilas de Bignoniaceae, e isso somado ao fato da redução dos ecossistemas naturais em que a espécie ocorre, pode estar relacionado às baixas taxas de visitação e de frutificação natural registradas para a espécie neste estudo. O nectário floral de *Z. tuberculosa* mostrou-se estruturalmente complexo sendo composto pelo disco nectarífero reduzido, pelo estipe, pela axila das pétalas e pela base do tubo floral, recoberta por tricomas capitados e papilas esparsas. Análises histoquímicas mostraram que todos os tecidos da câmara nectarífera estão envolvidos na produção de néctar e todos eles apresentaram ultraestruturalmente características subcelulares de secreção mista, lipofílica e hidrofílica. Os resultados sugerem que o néctar de *Z. tuberculosa* além dos açúcares glicose, frutose e sacarose, seja possivelmente enriquecido com óleos, aminoácidos/ proteínas e flavonoides durante a antese, o que poderia conferir um valor nutricional e energético adicional para a manutenção das abelhas polinizadoras. Assim, os resultados obtidos neste estudo indicam certa especialização em relação às dimensões e formato das flores, ao padrão de secreção de néctar e à estrutura e funcionamento do nectário floral que levam à polinização de *Z. tuberculosa* por um único grupo funcional de abelhas.

Palavras chave: composição química de açúcares, histoquímica, fecundação cruzada, melitofilia, nectário floral, padrão de secreção do néctar floral, ultraestrutura.

Abstract - The Bignoniaceae family is a good model for studies about evolutionary diversification that gave rise to the diversity of tropical plant communities, and the interaction with pollinators is a determining factor. *Zeyheria tuberculosa* is an endangered species and has characteristics related to melittophily syndrome. Information on the reproduction of this species may help to understand the ecological processes that may have led this species to be included in the Red List of endangered species. This goal of this study was to investigate the floral biology of *Z. tuberculosa*, focusing on the pattern of nectar secretion and the functional and structural characteristics of the floral nectary, which is the responsible for producing the essential resource for maintaining the interaction with pollinators. The results were divided into two sections that deal with specific and complementary issues. The first includes information about the events of floral anthesis, the pollination ecology and the pattern of nectar secretion in plants of *Z. tuberculosa* located in semi-deciduous forest fragments, immersed in a modified landscape. The second deals with the structure and functioning of the floral nectary of this species, focusing on the characteristics related to the type of secretion. For Chapter 1, we monitored flowers throughout anthesis; we performed focal observations of floral visitors and captured them to examine the pollen deposition area; recorded the percentage of viable pollen grains and the period of stigma receptivity; obtained data on the morphometry of flowers and pollinator bees; made self-incompatibility tests to assess the dependence of pollen vectors for cross-fertilization; evaluated the dynamics of nectar secretion through the volume, concentration and total milligrams of sugars parameters, using the latter parameter to calculate the caloric supply available to pollinators by flower and plant, and we carried out analyzes on the chemical composition of the floral nectar sugars. For the Chapter 2, we made structural analysis using conventional methods of plant anatomy and electron microscopy, and accomplished histochemical tests to verify the main classes of compounds present in the secretory tissues of the floral nectary. *Zeyheria tuberculosa* showed mellitophylous, dependent on bees for pollen transfer between plants. Nectar was the main primary source of floral energy supply to pollinators, being hexose-rich, predominantly the monosaccharides glucose and fructose, which corresponds to the preferences of bees. We recorded an increase in the sugar concentration during anthesis, which possibly occurred by water evaporation, since the campanulate flowers *Z. tuberculosa* apparently does not provide an effective barrier to water loss, as provided by flowers of Bignoniaceae possessing cylindrical and long tubes. Overall, there was significant variation in the total milligrams of sugar produced during flower life, with a gradual increase from the first to the second day of

anthesis. We also found significant variation between plants in the total milligrams of sugar produced per flower, indicating an intraspecific variability. The volume and concentration of nectar were low as to compared to other melittophilous species of Bignoniaceae. This, associated to the reduction of natural ecosystems where the species occurs, may be related to low visitation rates and low natural fruit set recorded for the species in this study. The floral nectary of *Z. tuberculosa* was considered as structurally complex being composed by the reduced disk, stipe, petals axil and the base of the floral tube, which was covered with capitate trichomes and scattered papillae. Histochemical analysis showed that all of the nectar chamber tissues are involved in the production of nectar. All the nectar chamber tissues showed subcellular characteristics of mixed secretion, lipophilic and hydrophilic. These results suggest that the nectar of *Z. tuberculosa*, in addition to the sugars glucose, fructose and sucrose, it is possibly enriched with oils, amino acids/ proteins and flavonoids during anthesis, which could confer an extra nutritional value and additional energy for maintaining the pollinators. Thus, the results obtained in this study indicated certain degree of specialization of *Z. tuberculosa* flowers regarding the floral shape and dimensions, the pattern of nectar secretion and the structure and functioning of the floral nectary, which together may lead to pollination by a single functional group of bees.

Keywords: cross-fertilization, floral nectar secretion pattern floral nectary, histochemistry, melittophily, nectar sugar composition, ultrastructure.

INTRODUÇÃO GERAL E REVISÃO DE LITERATURA

A família Bignoniaceae apresenta 827 espécies pertencentes a 82 gêneros de ocorrência pantropical, com 78% de suas espécies localizadas nos Neotrópicos, possuindo representantes que podem ser encontrados em diversos ambientes, desde savanas a floresta ombrófilas (Gentry, 1980; Lohmann & Ulloa 2007). Seu centro de diversidade encontra-se no Brasil, onde ocorrem 396 espécies que se distribuem em 32 gêneros (Gentry, 1980; Lohmann, 2010). Esta família é um componente de grande importância para as florestas neotropicais, sendo considerada como um bom modelo para se compreender os tipos de diversificação evolutiva que deram origem à incrível diversidade de comunidades de plantas tropicais (Gentry, 1980).

A família é predominantemente lenhosa, composta por árvores, arbustos e lianas, sendo a maioria de suas espécies caracterizada por possuir folhas compostas e opostas, sem estípulas e sementes aladas (Gentry, 1980; Judd, 2009). Possui flores geralmente vistosas, bissexuais, zigomorfas, pentâmeras, gamossépalas e gamopétalas, com corola bilabiada, tubular e com estreitamento em sua base, quatro estames férteis didínamos, com filetes adnatos à corola, e o quinto estame reduzido a estaminódio (Judd, 2009; Souza, 2012). O ovário é súpero e, geralmente, abaixo dele encontra-se um disco nectarífero conspícuo, o estigma é bilabiado e sensível ao toque, fechando-se quando sua superfície interna é tocada (Gentry, 1980; Judd, 2009; Souza, 2012). O estigma normalmente localiza-se anteriormente às anteras na entrada da corola, permitindo que o pólen trazido pelo polinizador contate justamente a porção interna, receptiva, dos lobos estigmáticos; o não fechamento dos lobos após a coleta de pólen por alguns visitantes florais é indicativo de que os mesmos não entraram em contato com a superfície do estigma e, portanto, não realizaram papel de polinizadores (Gentry, 1974).

Ao realizar estudos com Bignoniaceae na América Central, Gentry (1974) observou interações com herbívoros, frugívoros e polinizadores, ressaltando que os padrões de coevolução são importantes para a compreensão das estratégias adaptativas entre animais e plantas desta família. Além disso, posteriormente, Gentry (1990) verificou que o modo de interações com os polinizadores é um fator determinante da diversidade em Bignoniaceae. Nesta família a polinização é majoritariamente realizada por animais antófilos e suas flores atraem diversos grupos funcionais de visitantes tais como abelhas, vespas, borboletas, mariposas, beija-flores e morcegos (Gentry, 1974; Gentry, 1990; Bittencourt Jr & Semir, 2004; Yanagizawa & Maimoni-Rodella, 2007; Guimarães *et al.*, 2008; Galetto, 2009, Alcantara & Lohmann, 2010).

O processo de coevolução é um dos principais agentes na estruturação das comunidades e resulta de interações mutualistas entre táxons filogeneticamente distantes (Thompson, 1998). A polinização é baseada em uma interação em que ambas as partes aumentam sua aptidão, pois, normalmente, os animais obtêm alimento e material para cuidados com a prole e as plantas têm a possibilidade de fecundação cruzada (Wilmer, 2011; Torezan-Silingardi 2012). Esta relação é estabelecida por meio de atrativos primários tais como néctar, pólen, óleo, resina e fragrâncias que representam recursos essenciais aos animais, e de atrativos secundários que consistem na atração dos animais às flores, tais como formato da corola, cor e odor (Faegri & Van Der Pijl, 1979). A comunicação planta-animal é fundamental para a evolução floral visto que as classes de animais diferem muito em sua biologia sensorial, o que influencia em sua maneira de perceber, sabores, cores, odores e a qualidade do recurso (Schaefer & Ruxton, 2011).

Em Bignoniaceae o principal recurso floral é o néctar, que apresenta em sua composição açúcares e uma série de outros componentes tais como, aminoácidos, antioxidantes, lipídios, fenóis e alcaloides (Baker & Baker, 1975; Galetto & Bernardello,

2005; Nepi, 2007). Estudos com ênfase na composição química do néctar, bem como nos padrões de secreção, incluindo concentração e volume, são muito importantes para a compreensão das necessidades energéticas e preferências dos polinizadores (Baker & Baker 1975; Galetto & Bernadello, 2005; Galleto (2009).

Bignoniaceae é um grupo monofilético, com sua filogenia sustentada por caracteres morfológicos e moleculares obtidos a partir das sequências de cloroplasto *ndhF*, *rbcL* e *trnL-F* (Olmstead, 2009). Gentry (1980) baseou-se fundamentalmente no hábito e na deiscência dos frutos para dividir Bignoniaceae em oito tribos (Bignonieae, Coleeae, Crescentieae, Eccremocarpeae, Oroxyleae, Schlegelieae, Tourrettieae e Tecomeae), porém estudos mais recentes da filogenia desta família realizados por Olmstead e colaboradores (2009) evidenciaram a exclusão de *Paulownia* (agora elevado a família Paulowniaceae) e *Schlegelia* e *Exarata* (pertencentes agora a Schlegeliaceae), reconhecendo oito tribos, que diferem parcialmente daquelas propostas por Gentry (1980): Bignonieae, Catalpeae, Colleae, Crescentieae, Jacarandeae, Oroxyleae, Tourrettieae e Tecomae, incluindo a tribo Eccremocarpeae em Tourrettieae (Spangler & Olmstead, 1999; Olmstead, 2009).

O gênero *Zeyheria* inicialmente inserido na tribo Tecomeae por Gentry (1980), atualmente não se encontra inserido em nenhuma das tribos descritas, pertencendo ao clado *Tabebuia alliance*, que tem sua monofilia baseada na morfologia de suas folhas. Todos os componentes desse amplo grupo apresentam folhas compostas palmadas (Grose & Olmstead, 2007) e a maior parte de seus integrantes eram reconhecidos por Bentham & Hooker (1876) como 'Digitifoliae', um grupo informal dentro da tribo Tecomeae. O gênero *Zeyheria* apresenta duas espécies, *Zeyheria tuberculosa*, espécie arbórea cuja ocorrência se dá em florestas ombrófilas e florestas estacionais semidecíduas, e seu par vicariante *Zeyheria montana*, espécie arbustiva típica de fisionomias savânicas de cerrado (Gentry, 1992; Rizzini, 1997).

Vicariância é o fenômeno mediante o qual, no curso da diferenciação ou especiação, certas espécies ou variedades morfologicamente muito afins ocupam áreas distintas e exclusivas, todavia, possuem a mesma origem, pois derivam de um ancestral comum (Rizzini, 1997; Romariz, 2008). Normalmente espécies vicariantes, sobretudo savana-mata, diferem por caracteres de natureza ecológica, ligados a diversos fatores ambientais, dentre eles a maior intensidade luminosa e dessecação atmosférica nas savanas, o que pode levar a divergências morfológicas tais como porte menor, ramificação mais basal e aberta, folhas com índice de esclerofilia maior, sendo mais secas e duras e com nervuras mais robustas (Rizzini, 1997). As duas espécies do gênero *Zeyheria*, apesar de muito próximas, apresentam divergências nas características acima citadas, bem como em suas síndromes de polinização. *Z. montana* possui flores com caraterísticas normalmente atribuídas à polinização por beija-flores, isto é, corola tubular cilíndrica, com ausência de odor e copiosa produção de néctar diluído acumulado em uma câmara nectarífera (Bittencourt & Semir, 2004; Guimarães *et al*., in prep). Já *Z. tuberculosa* apresenta características relacionadas a síndrome de melitofilia, tais como corola amarela, com guias de néctar vermelhas evidentes, tubos curtos e ausência de anteras extrorsas (Alcantara & Lohmann, 2010). Assim, estudos envolvendo as necessidades e preferências dos polinizadores relacionados aos principais atrativos florais são fundamentais para compreensão dos processos de diversificação floral em táxons filogeneticamente muito próximos, porém com polinizadores pertencentes a grupos funcionais distintos.

Além disso, é de extrema importância conhecer a biologia floral desta espécie devido a sua recente inclusão na lista de espécies em risco de extinção da flora brasileira (Martinelli, 2013) e na Lista Vermelha da União Internacional para a Conservação da Natureza e dos Recursos Naturais (IUCN) (World Conservation Monitoring Centre, 1998). Isso se deve à destruição de seus hábitats (Luz, 1985), bem como ao fato desta espécie ser explorada tanto por sua madeira nobre (Carvalho, 2005), quanto por suas propriedades medicinais (Bastos,

2009; Sarmento *et al.*, 2014). A fragmentação do habitat pode resultar em diminuição da diversidade genética das populações (Ellstrand & Elam, 1993), podendo levar à redução do sucesso reprodutivo devido a alterações em um ou ambos os organismos interagentes (Aguilar *et al.*, 2009; Murcia, 1995; Aguilar *et al*., 2009).

Neste contexto, estudos sobre a polinização de *Z. tuberculosa* no atual cenário, em que seu habitat natural foi modificado, pode nos ajudar a compreender os processos ecológicos que envolvidos nessa interação mutualista. Além disso, a integração de informações das áreas de ecologia e anatomia vegetal pode ajudar a entender os fatores que levaram *Z. tuberculosa* à especialização na polinização por abelhas médias. Com isso, o objetivo central de nosso estudo foi investigar a biologia floral de *Z. tuberculosa* enfocando o padrão de secreção de néctar e as características funcionais e estruturais do nectário floral, responsável pela produção do recurso essencial à manutenção da interação com os visitantes florais.

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Capítulo 1

Floral biology and nectar secretion pattern of a threatened Bignoniaceae tree from Brazilian tropical forest

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FLORAL BIOLOGY AND NECTAR SECRETION PATTERN OF A THREATENED BIGNONIACEAE TREE FROM BRAZILIAN TROPICAL FOREST

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Abstract

Pollination is among the most important interactions to guarantee the persistence of populations in large time scale. The relationship between plants and pollinators is established by means of attractants, being the food urges the primary cause of the majority of blossom visits. So, the aim of this study was to describe the floral biology of *Z. tuberculosa*, a threatened species of Brazilian tropical forests, with focus on secretion pattern of nectar, the main floral source of energy to pollinators. For this, we monitored the opening period of the flowers; we made focal observations of floral visitors and we captured them to identify the pollen deposition area; we tested the viability of pollen grains and stigma receptivity; we made the morphometry of flowers and bee pollinators; we made manual self-pollination to verify the dependence on a pollen transfer vector for outcrossing; we evaluated the nectar secretion pattern using volume, concentration and total mg of sugars as parameters, we calculated the caloric supply available to pollinators per flower and per tree and we conducted analysis of the nectar sugar composition. *Zeyheria tuberculosa* showed melittophylous, with floral morphology and dimensions compatible with *Bombus* cf. *pauloensis* a medium-sized bee pollinator, and it was self-incompatible. The hexose-rich nectar was the primary attractant and the main floral energy source for bee pollinators. We registered an increasing in nectar concentration throughout the day, which probably occurred due to water evaporation. In general, there was a significant variation in total mg of sugar (mgS) produced throughout flower lifespan, with general increasing from the first to the second day of anthesis. We also

verified a significant variation among plants in mgS. In the meantime, *Z. tuberculosa* showed low bee visitation frequency and low fruit set, what could be related to pollen/ pollinator limitation in this changing landscape.

Key words: *Zeyheria tuberculosa*, pollination, nectar sugar composition, nectar secretion pattern, self-incompatibility system, reproductive success.

INTRODUCTION

The relationship between plants and pollinators is established by means of attractives that usually fit on primary needs of the animals to be effective, like feeding and breeding (Faegri & Van Der Pijl, 1979). Animal pollinated flowers advertise themselves by various stimuli presented simultaneously, being this attractives classified into primary and secondary (Faegri & Van Der Pijl, 1979; Wilmer, 2011). Primary attractives are pollen, nectar, water, oils, and others that represent the resource for pollinators whereas the secondary ones, as corolla shape, color and scent, represent the signaling for pollinators (Faegri & Van Der Pijl, 1979).

Food urge is the main cause of the majority of blossom visits, being nectar the main floral energy source to pollinators (Wilmer, 2011). Studies focusing on nectar sugar composition may provide information about the requirements and preferences of pollinators (Baker & Baker 1975; Galetto & Bernadello, 2005). In addition, parameters such as nectar viscosity, volume and concentration are fundamental to understanding the behavior of floral visitors and the complexity of plant-animal relationships because animals of different functional groups require varying amounts of calories to compensate the energy expended during foraging (MacArthur and Pianka, 1966; Nicolson, 2007). Galetto (2009) conducted a comparative study about the nectar parameters related to melittophylous and ornithophilous species of Bignoniaceae and verified that melittophylous species tend to have more concentrated nectar, secreted in smaller amounts as compared to ornithophilous species, which secrete more diluted nectar in larger quantities.

Brazil is the diversity center of Bignoniaceae (Gentry, 1980; Lohmann & Ulloa 2007) and although plants of this family attract a large spectrum of pollinators, such as butterflies, moths, bats, (Gentry, 1990) and hummingbirds (Bittencourt Jr & Semir, 2004), there is a predominance of medium and large-sized bees pollination (Gentry, 1974; Gottsberger &

Silberbauer-Gottsberger, 2006; Yanagizawa & Maimoni-Rodella, 2007). The reproductive biology of *Zeyheria tuberculosa*, a tree Bignoniaceae that occurs in a broad latitudinal range in Brazilian forests (Gentry, 1992), is unknown up to date, although its flower syndrome fits on pollination by bees (Faegri & Van Der Pijl, 1979), such as campanulate yellow corolla with coloured nectar guides and introrse anthers (Gentry, 1992; Lohmann & Pirani, 1996; Alcantara & Lohmann, 2010). Knowledge about the floral biology of this species is very important because *Z. tubeculosa* is included in the red list of threatened species of Brazilian flora (Martinelli, 2013) and in the International Union for Conservation of Nature (IUCN) red list of threatened species as vulnerable, which means that this taxon is facing a high risk of extinction in the wild in the medium-term future (World Conservation Monitoring Center, 1998). Additionally, this species is explored by its hardwood (Carvalho, 1994) and its medicinal properties (Bastos, 2009; Lima et al., 2009; Sarmento et al, 2014).

The causes of the severe habitat loss are especially related to the human agricultural activities in the Southeast, Northeast and Midwest regions of Brazil, resulting in extensive destruction of natural ecosystems in which this species occurs and in its consequent decline (Luz, 1985; World Conservation Monitoring Centre, 1998). The habitat fragmentation may result in changes in species distribution and abundance, and the population isolation in landscape patches may result in higher levels of endogamy, low gene flow and events of genetic drift that may affect negatively the genetic diversity (Aguilar *et al*., 2009 and references therein). That way, we expect fitness reduction in successive generations, what may result in a higher susceptibility to biotic and abiotic changes, increasing the extinction probability of these populations, which may affect interacting organisms in this ecosystem (Ellstrand and Elam, 1993; Murcia, 1995; Aguilar et al., 2009). Interactions related to reproduction of plants are among the most critical for the maintenance of populations in long term (Kearns and Inouye, 1997, Aguilar et al., 2009), especially because they may result in

important bottom-up effects in the ecosystem (Chapin III et al.,). Hence, studies on pollination of *Z. tuberculosa* in the current modified landscape may help us understand, beyond the evolutionary processes, the ecological processes that involve this mutualistic plant-animal interaction. Besides, a detailed description about the floral biology may shed light on the mechanisms involved in the primary attraction of anthophilous animals. Thus, the aim of this study was to investigate the floral biology of *Zeyheria tuberculosa*, focusing on nectar that is the primary flower attractant, being responsible for the caloric offer for bee pollinators.

MATERIAL AND METHODS

We studied four reproductive individuals, varying from 10 to 20 meters high and 30 to 80 cm in stem diameter, occurring in patches of tropical seasonal forest located in Botucatu (22º53'S 48º29'O) and Pratânia (22º48'S 48º44'O) municipalities, São Paulo state, South-eastern Brazil. The climate of the region, according to the classification of Köppen, is Cwa, mesotermal with rains in the summer and drought in the winter (Cunha & Martins 2009).

Zeyheria tuberculosa (Vell.) Bureau ex Verl, occurs in seasonal and rainy forests from southeastern Brazil, São Paulo state, until northeastern Brazil, Ceará state. It is a deciduous species; reaching 20-30 m tall with thick vertically fissured bark; opposite leaves; terminal panicle inflorescence; campanulate flowers without a narrow basal tube; didynamous stamens with the anther thecae divaricate; ovary broadly obovoid, densely stellate-pilose disk not obviously differentiated from base of ovary; fruit a broadly obovoid to round capsule with the surface tannish stellate tomentose; seeds thin, with a distinctly verrucose surface surrounded by broad hyaline-membranaceous wing (Gentry, 1992; Lohmann & Pirani, 1996).

Vouchers of the studied materials were collected and deposited in the 'Irina Delanova Gemtchujnicov' Herbarium (BOTU) of the Biosciences Institute of the Universidade Estadual Paulista, Botucatu, SP, Brazil, under numbers 30818 and 30819.

Floral biology

We monitored flowers from five individuals throughout the day to detect the time of flower opening, the duration of anthesis, the color and the dimensions of the floral elements. We made focal observations, according to Dafni *et al* (2005) and we analyzed photographs and videos to check for the species of flower visitors, their behavior, and the time and frequency of visits to flowers of *Z. tuberculosa*. The records were made from 0500 h to 2200 h, totaling 174 hours of field observations, distributed over 15 non-consecutive days. Each day, plants were monitored for 6-12 consecutive hours. We observed each plant twice (n = 4 reproductive individuals) covering the entire daylight hours, totaling eight replications per hour. We captured floral visitors to examine the area of pollen deposition on their body and for taxonomic identification. Besides, we made tests for stigma receptivity using hydrogen peroxide (H_2O_2) according to Dafni *et al*. (2005) and we estimated the viability of pollen grains using acetic carmine as a vital dye (Radford *et al*., 1974) in five flowers of first and second day of anthesis, from three individuals (n= 15 flowers/ day of anthesis). To investigate the presence and location of osmophores we used the neutral red solution (Vogel, 1990).

We performed morphometric analysis on 70 flowers from three plants aiming to describe the floral morphology associated with the body dimensions of pollinators, and with the placement of pollen load in pollinator body. For this, we monitored the following traits: calyx length, corolla length, diameter at corolla mouth (horizontally to the soil), diameter of zygomorphic plan at corolla mouth (vertically to the soil), diameter of constriction region of the corolla, distance between the top of the longest stamen and the basis of nectar chamber,

length of pollen deposition area, distance between the upper portion of stigma and basis of nectar chamber, diameter and length of the nectar chamber.

We performed morphometric analysis on 10 bee-pollinator specimens aiming to associate with the floral morphology and to identify the adjustments between flower and bee dimensions. For this, we measured body length, proboscis length, thorax dorsiventral thickness, thorax latero-lateral thickness, head length, head lateral size.

Self-incompatibility test

To investigate the degree of dependence on pollinators to carry-out crosspollination in *Z. tuberculosa*, we performed tests for self-incompatibility. For that, in each individual we bagged five inflorescences with pre-anthesis buds, to avoid any contact with floral visitors, and we made tests for self-incompatibility in the following days, using functional flowers of first (newly opened) and second (24-hour flowers) days of anthesis. We transferred pollen from anthers to stigmas in three sets of flowers: a) pollen from newly opened flowers to the stigmas of 24-hour flowers, b) pollen from newly opened flowers to the stigmas of newly opened flowers c) pollen of 24-hour flowers to stigmas of 24-hour flowers. After hand pollination experiments, the inflorescences were re-bagged and observed until the development of fruit or flower abscission. Besides, we registered the rate of natural fruit set by the reason of fruits produced per tree/ flowers produced per tree during the entire blooming.

Pattern of floral nectar secretion during anthesis and nectar sugar composition

To evaluate the natural pattern of nectar production through anthesis we used three nectar parameters: volume (μL), concentration (%, weight/weight) and milligrams of total sugar per flower (mgS). For that, we took five inflorescences per individual (n = 4 individual),

in a non-systematic way, containing five pre anthesis buds each (n = 25 flowers/ individual), totaling 100 flowers per period. After this, we protected all the inflorescences with bridal veil bags to avoid nectar depletion by floral visitors. In the next day we collected five flowers per inflorescence and measured the volume (μL) using calibrated syringes, and the concentration of soluble solids (%) using manual refractometer. We performed these measurement at three times: 9:00 (recently opened flowers), 19:00 (flowers opened for approximately 12 hours) and at 19:00 of the next day (flowers opened for about 36 hours). The flowers were very difficult to access and handling because of the height of the canopy in this species, what did not allow us to work with the flowers attached to the inflorescence. So, we measured each flower just once, discarding it immediately after the nectar sampling. The assessment made in these three schedules allowed us to observe: (1) if there was nectar available to pollinators at the beginning of anthesis and how much nectar was available at this time, (2) how much nectar was produced throughout the light period in the first day of anthesis and (3) how much nectar the pollinators could find at the end of the second day of anthesis.

We compared the mean nectar volume, concentration and milligrams of total sugars per flower by two-way factorial ANOVA among four plants at two, 12 hours and 36 hours of anthesis. We performed post hoc comparison of means per time by Tukey HSD test for homogeneous groups.

We used data of nectar volume and concentration to estimate the caloric offer in terms of total sugars milligrams available per flower, in each of the sampling times, according to the exponential regression proposed by Bernadello and Galetto (2005). Additionally, we counted the total number of flowers per inflorescence and estimated the number of inflorescences per individual to estimate total amount of energy available in nectar per plant per day and per plant along the entire blooming period.

We determined the sugar composition of floral nectar in flowers of first day of anthesis (n= 72 flowers of three plants). We stored samples of nectar for sugar composition analysis in vials at -20^{o} C. Prior to the analysis, nectar samples were thawed to ambient temperature, diluted 1:25 with distilled water, and analyzed for sugar content by High Performance Liquid Chromatography (HPLC). The mobile phase used was water (MilliQ, pH 7). Sugars were separated in a Waters Sugar-Pack I (6.5–300 mm) column and identified by a refractive index detector (Waters 2410). Twenty milliliters of sample and standard solution were injected. The mobile phase flow rate was 0.5 mL/min, and column temperature was 85–90^{o}C.

RESULTS

Floral biology

Flowering of *Z. tuberculosa* occurred in January-February 2014 (Fig. 1A). The flowers were diurnal and started opening around 07:00 h. Although the beginning of anthesis was concentrated in the morning, we registered some few flowers opening throughout the day, until about 1700 h. The inflorescences presented 9.3 $\pm$ 2,69 first-day flowers per day, but the old flowers remained attached to the inflorescence, even when they were no longer receptive. The flowers were functional at least for two days and, on the first day the flowers had yellow color, but in the second day they became reddish (Fig. 1B, C). The flowers of *Z. tuberculosa* had a campanulate corolla (Fig. 2A, B) and the dimensions of the floral elements are shown in the Table 1.

Anther dehiscence and stigmatic lobe opening occurred in the pre-anthesis period, so that when the flower opened the pollen was totally exposed and the stigma lobes separated. The stigma remained receptive at least during the first two days of anthesis reacting positively to hydrogen peroxide test.

Table 1. Floral traits dimensions of *Zeyheria tuberculosa* (Bignoniaceae)

Floral traits	Mean ± SD (mm)
Calyx length	6.98 ± 0.63
Corolla length	15.26 ± 0.80
Diameter at corolla mouth (horizontally to the soil)	7.12 ± 0.78
Diameter of zygomorphic plan at corolla mouth (vertically to the soil)	5.09 ± 0.49
Diameter of constriction region of corolla	3.06 ± 0.46
Distance between the upper portion of stigma and basis of nectar chamber	12.60 ± 0.74
Distance between the top of the longest stamen and basis of nectar chamber	10.65 ± 0.44
Length of pollen deposition area	3.00 ± 0.38
Diameter of nectar chamber	1.82 ± 0.22
Length of nectar chamber	0.95 ± 0.098

The stigma was sensitive, and closed when touched, however it returned to open gradually after a while. The pollen grains were yellowish and remained clustered inside the anthers. The mean viability of pollen grains was 86 ± 17,25 %. Osmophores presence was revealed by reaction with the neutral red test and they were concentrated at the distal extremity of the three lower corolla lobes (Fig. 2C, D).

All visitors observed at *Z. tuberculosa* flowers belonged to Hymenoptera. We observed small, medium and large-sized bees from distinct functional group visiting the flowers (Fig. 1D, E) with variable frequency throughout the daylight (Fig. 3).

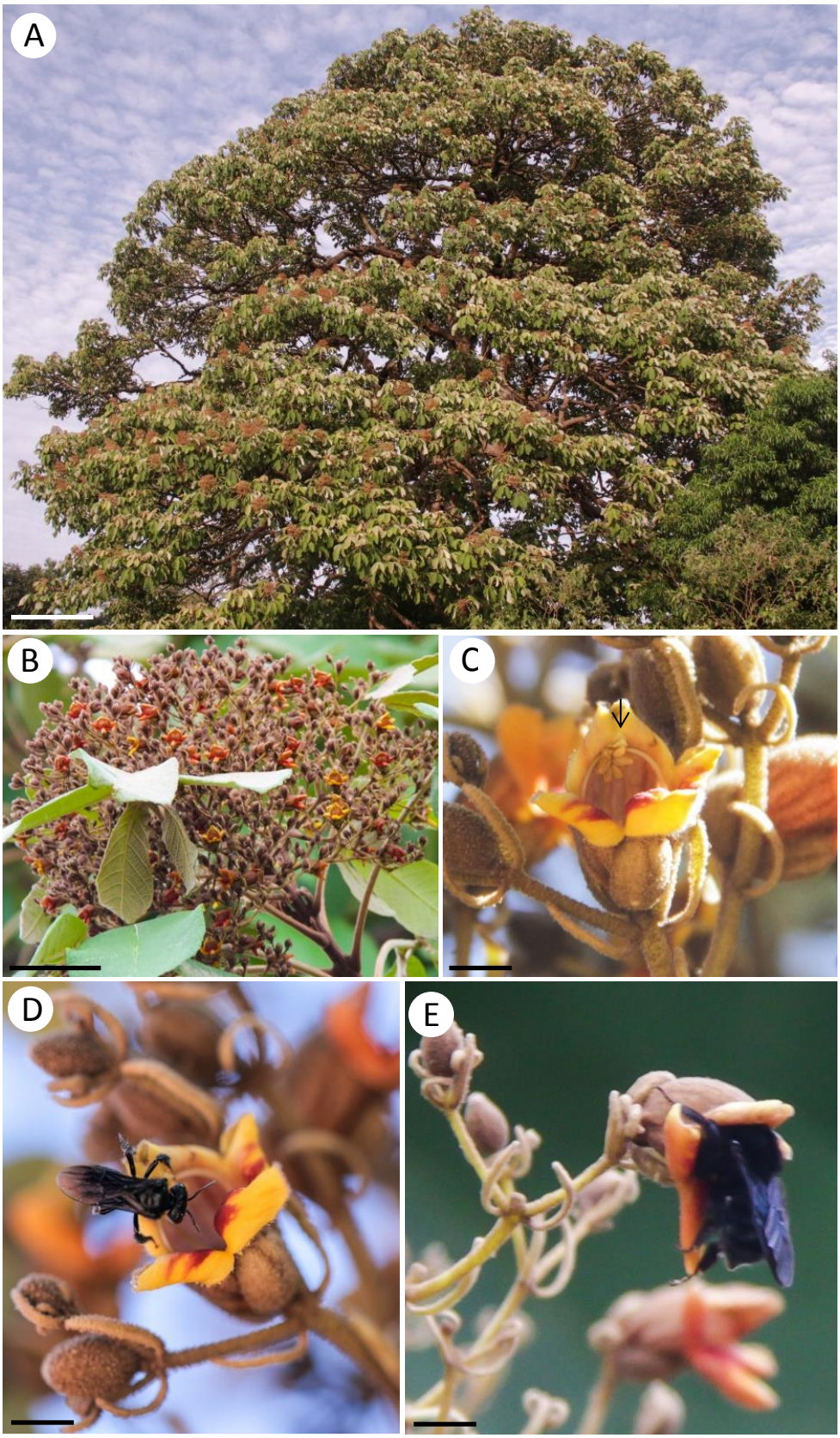

Figure 1. *Zeyheria tuberculosa* flowers and floral visitors. (A) Flowering canopy of a tree. Scale bar: 1 m. (B) Inflorescence with first (yellow) and second (reddish) day flowers. Scale bar: 4 cm. (C) Recently opened flower showing the hercogamy with separate stigma lobes (arrow), in an upper position, followed by the anthers, full of pollen. Scale bar: 5 mm (D) Robber bee collecting pollen directly from the anthers. Scale bar: 5 mm. (E) Medium-sized bee of the genus *Bombus* visiting flower in a legitimate way. Scale bar: 5 mm.

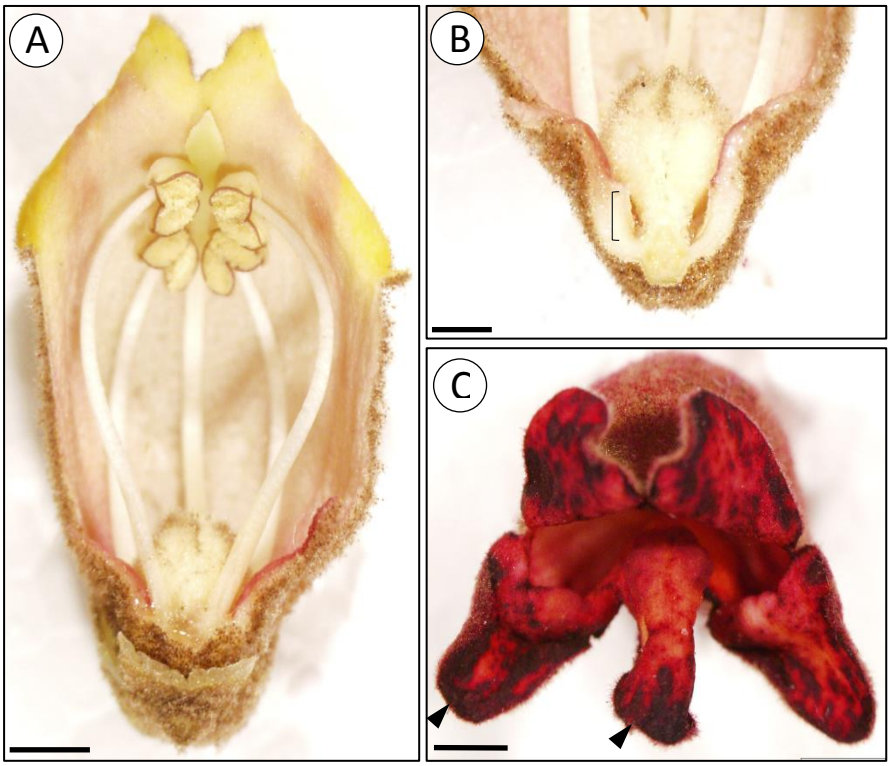

Figure 2. Flower of *Zeyheria tuberculosa*. (A) Longitudinal section showing the relative position of reproductive structures. Scale bar: 2 mm. (B) Longitudinal section evidencing the nectar chamber (bracket). Scale bar: 2 mm. (C) Corolla lobes stained with neutral red solution (arrows). Scale bar: 2 mm.

These bees entered deep into the corolla tube, sought the nectar chamber and apparently gathered nectar; however, they never contacted the stigma when entering the flower. Additionally, we did not observe stigma closure during the foraging of these bees at any time.

Medium-sized bees of the genus *Bombus* was considered the only legitimate visitor because they contacted male and female reproductive structures when searching for nectar (Fig. 1E). These bee visits were more frequent during the morning (Fig. 3C). *Bombus* cf *pauloensis* visited approximately 15 flowers at a time, visiting around five flowers per inflorescence. These bees entered the corolla deeply (Fig. 1E), apparently searching for nectar, spending an average of 10 seconds per flower.

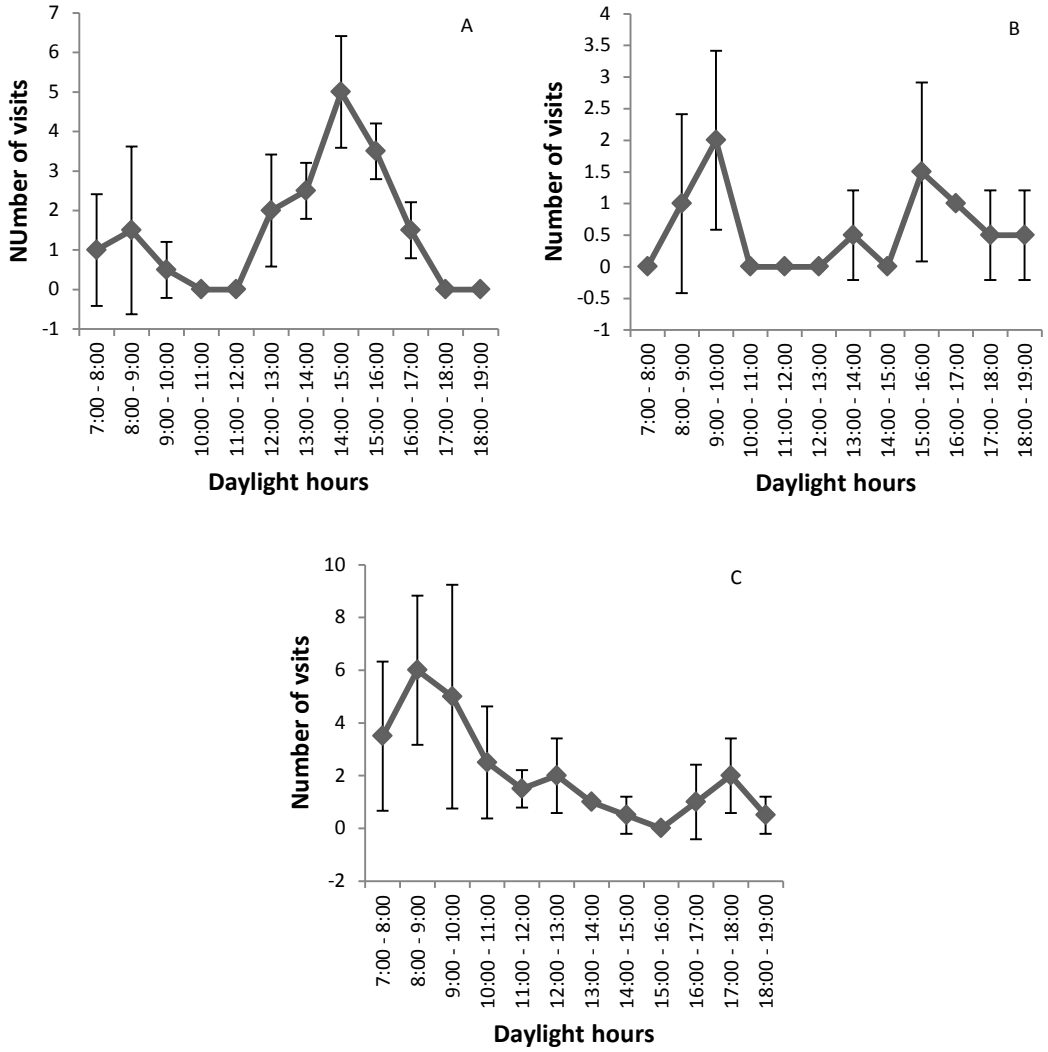

Figure 3. Bee visits in *Zeyheria tuberculosa* flowers throughout the daylight. (A) Mean number of visits per hour of pollen robber bees. (B) Mean number of visits per hour of nectar robber bees. (C) Mean number of visits per hour of medium-sized bees *Bombus* cf *pauloensis*.

The dimensions of the pollinator body are shown in Table 2. The body size and morphology of *Bombus* cf *pauloensis* are compatible with the morphology and dimensions of *Z. tuberculosa* flowers (Fig. 1E, 4A). Considering that the anthers are arranged 10.65 $\pm$ 0.44 mm above the nectariferous chamber basis and that the proboscis of *Bombus* cf *pauloensis* (Fig. 4A) measured 5.9 $\pm$ 0.92 mm, when it reaches the nectar chamber the eye region, the head and the dorsal portion of the thorax contacted anthers and stigma, being this area the site of pollen deposition in the bee (Fig. 4 B-D).

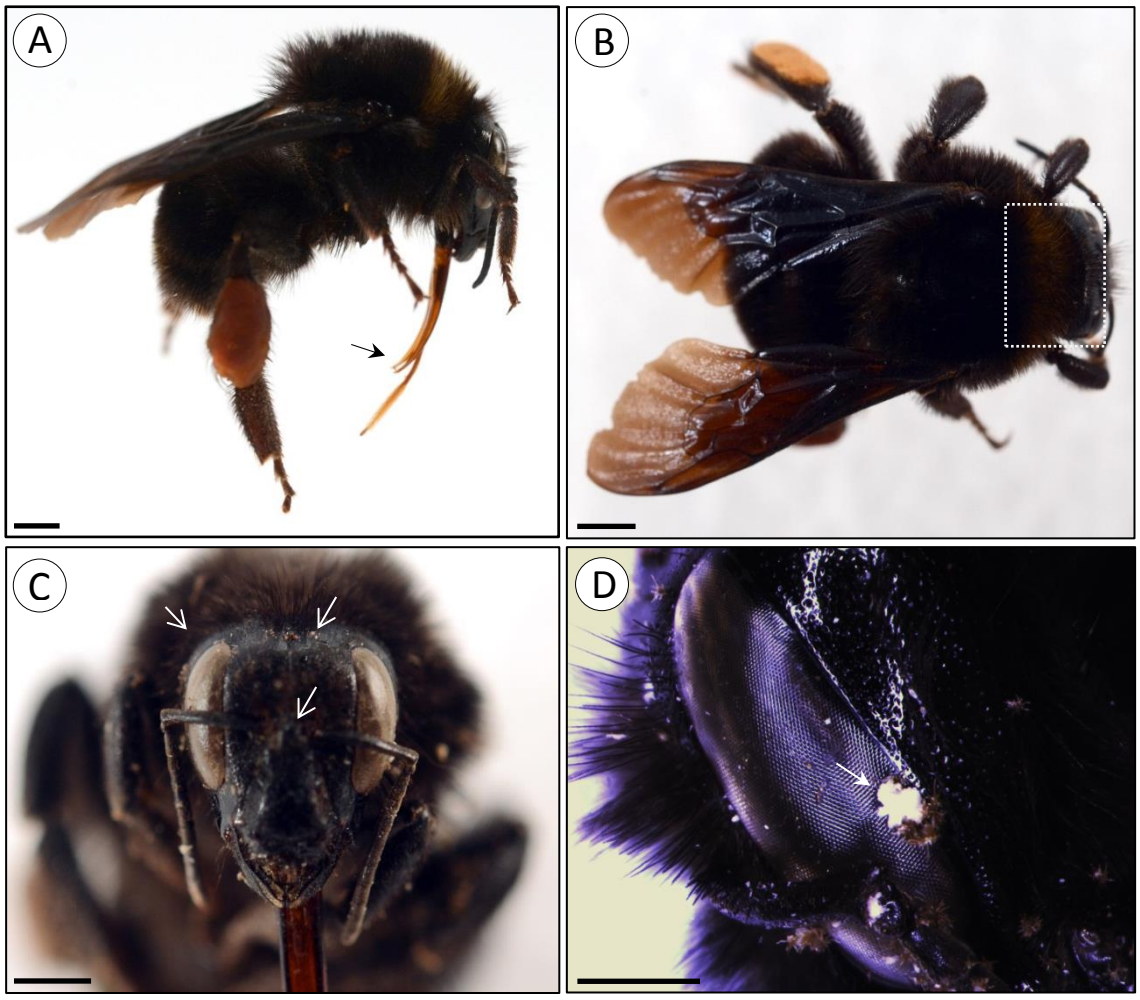

Figure 4. *Bombus* cf *pauloensis*, legitimate visitor of *Zeyheria tuberculosa* flowers. (A) Lateral view of *Bombus* cf *pauloensis* evidencing the proboscis (arrow). Scale bar = 2mm (B). Dorsal view, showing the main site of pollen deposition in the bee thorax and head (square). Scale bar = 2mm. (C) Frontal view of the bee head with pollen grains (arrows). Scale bar = 2mm. (D) Detail showing a pollen mass deposited in the bee eye (arrow). Scale bar = 1mm.

Table 2. Traits dimensions of *Bombus* cf *pauloensis* (Apidae) (n = 10 bees).

Bee traits	**Mean + SD (mm)**
Body length	12.94 + 0.77
Proboscis length	5.90 + 0.91
Thorax dorsiventral thickness	5.90 + 0.48
Thorax latero-lateral thickness	5.52 + 0.27
Head lengh	3.14 + 0.35
Head lateral size	4.34 + 0.57

Mating System

We registered no fruit set in manual self-pollinated flowers. Although *Z. tuberculosa* produces thousands of flowers per plant, the rate of natural fruit set was very low $2.41x10^{-5} \pm 1.41x10^{-5}$.

Pattern of nectar secretion during anthesis and nectar sugar composition

Nectar production in *Z. tuberculosa* started at pre-anthesis stage and was continuous until the second day of anthesis. The parameters volume, concentration and milligrams of total sugar averaged 1.61 ± 0.27 µL, 19.71 ± 2.95 % and 0.35 ± 0.10 mgS, respectively in functional flowers of this species (n = 240 flowers/ 4 plants). Nectar volume and concentration was highly affected by plant, time, and by the interaction between plant and time, however, for milligrams of sugar we did not observe interaction between time and plant (Table 3).

Table 3. Results of factorial ANOVA on nectar volume, concentration and milligrams of total sugars throughout the flower lifetime of *Zeyheria tuberculosa* (Bignoniaceae). (plant = 4 individuals; time = 2-hour flowers, 12-hour flowers and 24-hour flowers).

	Nectar volume			**Concentration**			**Mg of total sugars**		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Plant	3	9.313	*< 0.001*	3	15.07	*< 0.001*	3	21.12	*< 0.001*
Time	2	9.603	*< 0.001*	2	21.05	*< 0.001*	2	12.54	*< 0.001*
Plant*Time	6	2.871	*0.0102*	6	12.12	*< 0.001*	6	0.98	*0.442*

Pos hoc comparisons (Tukey) showed that the nectar volume accumulated at the end of the first (12-hour flowers) and second days of anthesis (36-hour flowers) were significantly different from the volume found when the flowers opened ($p< 0,01$ for all comparisons), with an apparent decrease at the end of the first day and an increase at the end of the second day (Fig. 4A). The same pattern was registered for concentration ($p< 0,002$ for all comparisons),

but in general we registered a gradual increase during the flower lifespan, with exception for Plant1, which showed a remarkable decrease in nectar concentration from the first to the second day (Fig. 4B).

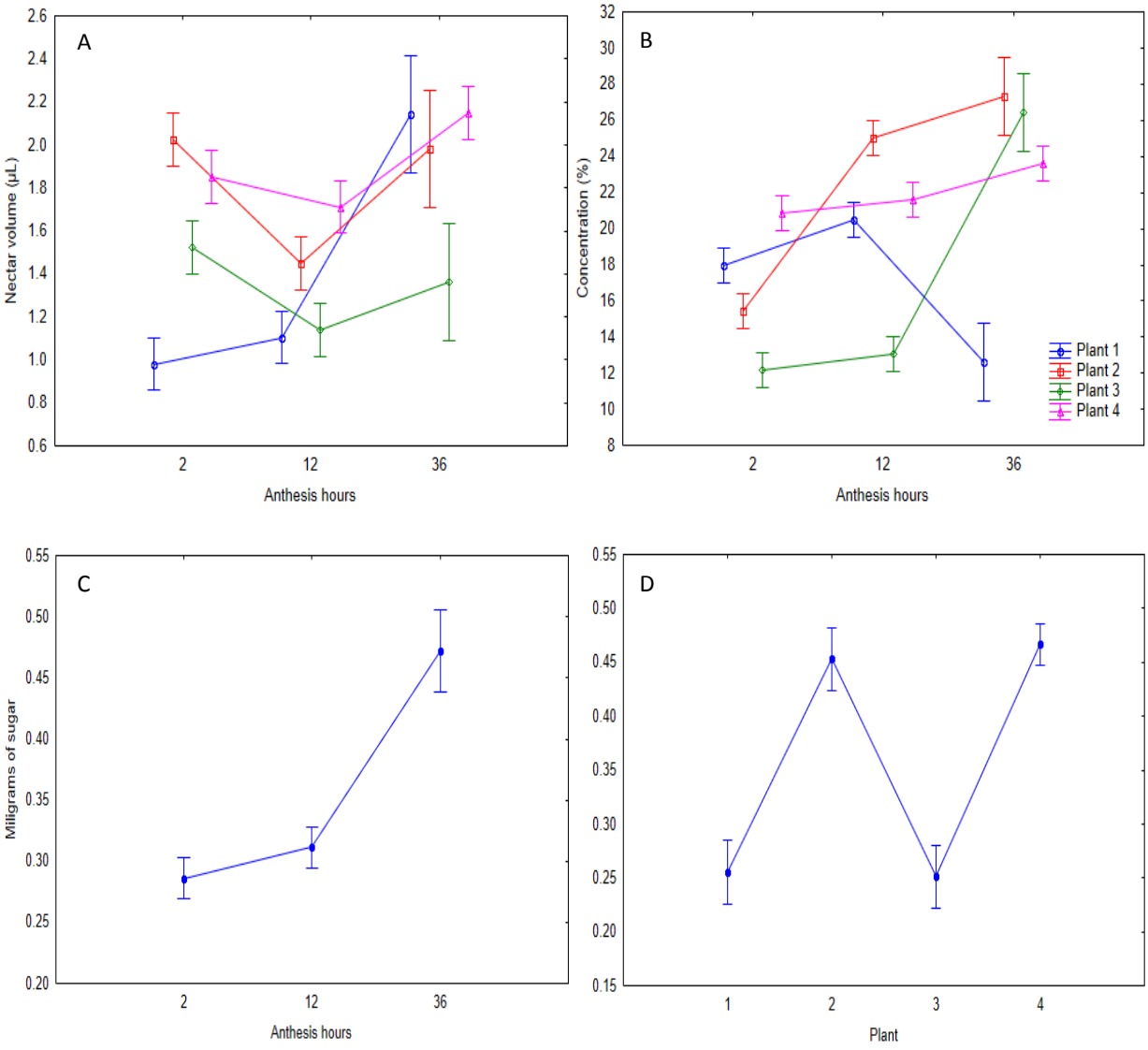

Figure 4. Floral nectar production throughout the flower lifetime of *Zeyheria tuberculosa* (n = 100 flowers/ period; 25 flowers from four trees). (A) Volume. (B) Concentration. (C-D) Total milligrams of sugar. Graphics (A) and (B) indicate interaction between "time" and "plant", graphics (C) and (D) show these two variables independently, absence of interaction according to factorial ANOVA. Vertical bars denote +/- standard errors.

Pos hoc comparison for mgS showed no significant difference along the first day of anthesis when comparing newly opened flowers with 12-hour flowers (p = 0,527), but we registered a significant increase (Fig. 4C) when we compared these two times (2-hour and 12-hour flowers) with 36-hour flowers (p< 0,001). For mgS the individuals could be divided into two groups, with two plants producing larger amount of sugar than the other two (Fig. 4D).

We estimated the total amount of energy available per plant in terms of total milligrams of sugar and we observed that, although each flower produces little nectar, the caloric offer per plant of *Z. tuberculosa* per day is of 1.6 $\pm$ 1.54 g of sugar. Moreover, the caloric offer to pollinator per individual plant during the entire blooming is remarkable, with a mean of 95.73 $\pm$ 93.58 g of sugar per plant.

Floral nectar of *Z. tuberculosa* flowers was dominated by three simple sugars: the disaccharide sucrose and its component monosaccharide sugars, fructose and glucose. The nectar was hexose-rich with overall ratio S/(G + F) = 0.28 $\pm$ 0.13. The carbohydrate composition was 42.24 $\pm$ 27.37 % of sucrose, 69.80 $\pm$ 29.85 % of glucose and 71 $\pm$ 29.90 % of frutose. The G/F ratio was close to 1 (G/F = 0.97 $\pm$ 0.01).

DISCUSSION

Our results on floral biology showed that *Zeyheria tuberculosa* is a melittophylous species, being pollinated by a medium-sized bee of the genus *Bombus*, similar to the pollination vectors described by Gentry (1974) and Gottsberger & Silberbauer-Gottsberger (2006) for several Bignoniaceae species that belongs to *Tabebuia* alliance. The body size and morphology of *Bombus* cf *pauloensis* was totally compatible with the morphology and dimensions of *Z. tuberculosa* flowers. The adjustments between flowers and pollinators in relation to morphology are considered as reflects of the adaptation of these organisms during evolution (Fenster *et al*. 2004; Armbruster *et al*. 2009). In fact, it is remarkable the fine

adjustment between the dimensions of the diameter of zygomorphic plan at corolla mouth and the head dimensions and bee thorax thickness, guaranteeing the narrow contact of superior portion of bee body with the flower reproductive structures. Besides, if we consider the distance of stigma/ anthers to the nectar chamber in relation to the proboscis length, plus head length, we verify that, when *Bombus* cf *pauloensis* reaches the nectar chamber, the eye region, the top of head and the dorsal portion of the thorax contact stigma and anthers.

The self-incompatibility tests indicated that *Z. tuberculosa* is an outcrossing species, what highlights the importance of bee pollinators as a vector for the transfer of pollen among trees, essential to the plant sexual reproduction. The allogamy can be favored by the presence of approach and movement herkogamy (sensu Webb & Lloyd, 1986) that reduces pollen-stigma interference. In *Z. tuberculosa* nototribic pollen deposition occurs following a specific sequence, first the receptive stigma surface (if the lobes are opened) is contacted by bees, and second the anthers area; if they are full of pollen it can adhere to bee body e be transported to the next flower. Additionally, the closure of stigmatic lobes few seconds after touching characterizes the movement herkogamy, which also prevents pollen-stigma interference in several Bignoniaceae species, according to Milet-Pinheiro *et al*. (2009), favoring allogamy.

Nectar is the most important floral resource for the majority of Bignoniaceae, which is a family predominately composed by species having sympetalous nectariferous and zoophilous flowers (Gentry, 1992; Bittencourt & Semir, 2004; Gottsberger & Silberbauer-Gottsberger, 2006; Guimarães *et al*., 2008, Galetto, 2009). In plant species the period of nectar secretion is adjusted to the period of pollinator activity, according to Cruden *et al*. (1983). In fact, we verified that in *Z. tuberculosa* the nectar production occurred at least until the second day of anthesis, floral phases that received bee visits. Additionally, the secretion of floral nectar in *Z. tuberculosa* started before the anthesis ensuring energy supply to pollinators at the moment of flower opening, when we registered a peak of pollinator visits, similar to

observed by Guimarães *et al*. (2015, submitted) for *Anemopaegma album* (Bignoniaceae). Commonly, medium-sized bees, as *Z. tuberculosa* pollinators, have high energy needs and typically do not require water due to the substantial metabolic water production during flight (Nicolson, 2009), consequently they normally interact with flowers that produce little and concentrated nectar (Baker & Baker, 1983).

In general, nectar volume and concentration were low in *Z. tuberculosa* as compared to other melittophylous tree species of Bignoniaceae (Gottsberger & Silberbauer-Gottsberger, 2006) or to the values of several other melittophylous species of Bignoniaceae with different habits (Galetto, 2009). Comparing floral nectar features between Bignoniaceae species belonging to two tribes, Bignonieae and Tecomeae (*sensu* Gentry, 1992), and between Bignoniaceae species associated with distinct pollinator guilds, bees and hummingbirds, Galetto (2009) did not detect any significant differences between tribes, but observed clear differences between pollination syndromes, suggesting that the pollinator type is more important in modulating the nectar features in Bignoniaceae. Thus, concerning nectar concentration, *Z. tuberculosa* does not fit very well in the characteristics of melittophily syndrome described for Bignoniaceae having a diluted nectar, similar to its vicariant pair *Z. montana* (Guimarães *et al*., *in prep*.), suggesting that they may share this feature already present in their common ancestor.

During the light hours of the first day of anthesis occurred a decreasing in nectar volume from the morning until the evening. In the meantime, several authors suggest that the reduction in nectar volume is due to reabsorption that is a mechanism that involves primarily the reabsorption of nectar sugars, allowing the plant to recover the energetically valuable sugars not used by pollinators (Corbet *et al.*, 1979; Nicolson, 1995; Nepi *et al,*, 2001, 2009; Nicolson & Nepi, 2005; Human & Nicolson, 2008; Nepi & Stpiczynska, 2008; Stahl *et al*., 2012). However, in our study, besides volume reduction, we verified an increase in the nectar

concentration in the first day flowers, suggesting the occurrence of evaporation, as suggested by Cruden *et al.* (1983) for other plant species. In general, we registered continuous concentration increasing throughout the flower lifespan in *Z. tuberculosa*. Weather may act as a constraint on pollination systems through modifications in the floral reward (Corbet, 1990), that way, several floral features, such as the corolla shape, can contribute to modulate the evaporation rate (Corbet *et al.*, 1979; Nicolson & Nepi, 2005). In campanulate corollas, such as the *Z. tuberculosa* flowers, the nectar is relatively more exposed than in long tubular corollas of other melittophylous Bignoniaceae species and, therefore, it may suffer major effect of evaporation (Nicolson & Nepi, 2005). Thereby, this is a feature that may contribute to the production of more concentrated nectar in *Z. tuberculosa* flowers than the nectar produced by *Z. montana*, which has a tubular corolla and produces copious nectar more diluted, related to the requirements of hummingbird pollinators (Bittencourt & Semir, 2004). In the beginning of flower lifetime of *Z. tuberculosa* the concentration values are similar to described for *Z. montana* by Gottsberger & Silberbauer-Gottsberger (2006) and Guimarães *et al*. (*in prep*). However throughout the first day of anthesis occurs an increase of nectar concentration with values close to described by Gottsberger & Silberbauer-Gottsberger (2006) to other melittophylous tree of Bignoniaceae from seasonal ecosystems from Brazil.

Beyond the intrafloral nectar variation throughout anthesis, we also observed some variability in the nectar volume and concentration between plants of *Z. tuberculosa*, especially in two individuals, which behave as top nectar producers throughout the entire flower's life span, showing among plants variability similar to that verified by Nicolson & Nepi (2005) for *Aloe castanea*. Floral features related to nectar secretion are expected to be much more variable interplants than structural ones (Herrera, 2009; Galetto, 2009). Corroborating this, Hodges (1993) registered variation among plants of *Mirabilis multiflora* (Nyctaginaceae), although the nectar parameters remained remarkably consistent in individual plants during

three successive flowering periods. Our results showed that the *Z. tuberculosa* top producers presented a relatively constant pattern in terms of nectar volume and concentration during the two first days of anthesis. However, different patterns were observed in the other two individuals. One plant showed a remarkable increase in the nectar production over anthesis and a pronounced decline of concentration by the end of second day of anthesis, producing much diluted nectar. Castellanos *et al*. (2002) suggests that, at least the replenishment of water in nectar could be favored when plants are subjected to low pollinator visitation rates in environments where nectar tends to evaporate. This may be related to the factor that bees may forage dilute nectar when their choices are limited (Human & Nicolson, 2008). Another *Z. tuberculosa* plant showed the same pattern of nectar volume secretion, declining through the first day of anthesis and showing an increase at the end of the second day. However the total nectar volume secreted by this plant was very low as compared to the top producer plants. Besides, nectar concentration was also lower in the first day of anthesis with a remarkable increase at the end of the second day. This suggests that the secretion of sugars exceed that of water and that the evaporation can be the predominant mechanism modulating nectar concentration throughout anthesis in *Z. tuberculosa*.

Nectar volume and concentration in flowers are the combined result of secretion rate, reabsorption, and evaporation rate (Nicolson & Nepi, 2005) so, predictions for these patterns are not simple because they may be related to pollinators behavior, environmental factors, plant resource allocation, and other factors (Galetto & Bernadello, 2004). Anyway, post secretory evaporation increases variability in floral nectar resource (Corbet *et al.*, 1979). The regulation of sugar concentration in nectar may be regarded as an adaptive feature in insect-pollinated flowers much more important than the volume because it guarantees adequate viscosity, permitting the nectar collection by specific mouth apparatus and maintaining the fidelity of pollinators (Baker & Baker, 1975; Nicolson, 1995; Nicolson & Nepi, 2005).

Besides, the adequate viscosity will modulate the energy intake rate during feeding, which may influence foraging efficiency and consequently the plant reproductive success (see Borrel, 2007 and references therein).

Low concentration of nectar may be related to low visitation rate, since pollinators require minimum amounts of energy available in the flowers that compensate the foraging, as proposed by MacArthur & Pianka (1966), concerning the optimal use of a tridimensional environment. However, the high number of flowers produced per plant in this species can ensure enough caloric intakes to compensate foraging investment, despite the low nectar volume per flower and its low concentration. Besides, this species had a mass flowering that lasted just few weeks, which may be an important feature in the advertisement of the pollinator (Wilmer, 2011). *Zeyheria tuberculosa* used to present one cycle of flowering over more than one year (Guimarães, pers. com.), and apparently it can be classified as supra-annual species according to Newstrom *et al*. (1994). The sporadic flowering may difficult the maintenance of the fidelity of pollinators, so supra-annual pattern of flowering may result in pollination by generalist bees, like *Bombus* cf *pauloensis* observed in our study. In general, the eusocial and semi social bees are recognized as generalist (Biesmeijer & Slaa, 2006) because they require a massive succession of flowers along the entire year, resulting in a generalist foraging pattern (Westerkamp, 1991).

Although the nectar volume and concentration parameters do not fit very well on melittophylous syndrome, the nectar sugar composition of *Z. tuberculosa* is hexose-rich matching to the bees preferences, as previously described by Baker & Baker (1983). Nectar sugar composition has been related to pollinator guilds and the constancy of sugar profile may ensure the visitation of specific functional groups (Baker and Baker 1983; Petanidou, 2005; Schmidt-Lebuhn *et al*., 2006; Nicolson, 2007). Some authors have suggested that phylogenetic constraints seems to be also influencing nectar sugar composition, since

taxonomically related plants show similar trend in their nectar sugar composition as they share common ancestors (Van Wyk, 1993; Galetto & Bernadello, 2003, 2004). However, to *Z. tuberculosa* and its ornithophilous vicariant pair, *Z. montana*, which presents sucrose-rich nectar (Guimarães *et al*., *in prep.*), the parameters of nectar composition seems to be modulated by plant-pollinator interactions.

In this study, we verified perfect adjustments between flower and bee pollinator dimensions and we registered nectar secretion pattern, nectar sugar composition and nectar caloric offer very close to the patterns described for bee-pollinated species, although the nectar showed a little diluted as compared to other melittophylous Bignoniaceae. Nevertheless, we observed a very low number of visits and low fruit set in our study plants that were located in a modified landscape. We can list some explanations for these facts: (a) it is possible that the habitat fragmentation and landscape changing may have impacted negatively *Z. tuberculosa* pollinator populations, what may result in pollinator limitation, explaining the low number of visits registered per plant; (b) considering the self-incompatibility system of *Z. tuberculosa*, it is possible that the low fruit set is associated with the lack of compatible mates in the landscape, resulting in a pollen limitation; (c) in a worst scenario, we can speculate that the pollinator species that coevolved with *Z. tuberculosa* was locally extinct and the set of primary and secondary attractives and deterrents of this plant species do not fit very well to the bee species present in this new background, resulting in sporadic bee visits and low plant reproductive success.

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Capítulo 2

Structural and histochemical characterization of floral nectary in *Zeyheria tuberculosa* (Vell.) Bureau ex Verl (Bignoniaceae)

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STRUCTURAL AND HISTOCHEMICAL CHARACTERIZATION OF FLORAL NECTARY IN *ZEYHERIA TUBERCULOSA* (VELL.) BUREAU EX VERL (BIGNONIACEAE)

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Abstract

Bignoniaceae is a family that includes plant species characterized by the presence of a conspicuous nectary disk surrounding the ovary base. However, in some genera the disk is absent or reduced, as in *Zeyheria*, and the nectar production has been attributed to the trichomes of nectar chamber. Nevertheless, detailed studies on the structure and functioning of these reduced disks are absent for most species of Bignoniaceae, except for *Z. montana*. So, the aim of this study was to describe the anatomy, histochemistry and ultrastructure of the reduced disk and of all the tissues that comprise the nectar chamber. We analyzed samples of nectar chamber from first day flowers for a) anatomy and histochemistry by light microscope and b) ultrastructure by scanning and transmission electron microscope. The nectar chamber was composed of a reduced disk placed at the stipe base, the stipe itself, the petals axil and the base of floral tube, which is covered with abundant capitate trichomes and sparse papillae. Histochemical analysis showed that all tissues of nectar chamber are involved in the nectar production. Ultrastructural analysis showed that all the tissues from nectar chamber present evidences of hydrophilic and lipophilic secretion. Furthermore, our analysis enabled us to classify the reduced disk of *Z. tuberculosa* as a functional nectary tissue.

Key words: *Zeyheria tuberculosa*, capitate trichomes, nectary disk, nectar chamber, stipe.

INTRODUCTION

Bignoniaceae is a Neotropical plant family that has showy flowers, with superior ovary, commonly characterized by the presence of a conspicuous floral nectary, as a disk, surrounding the ovary base (Gentry, 1992; Galetto, 1995; Rivera, 2000). However, in some genus the nectary disk is nonexistent or is reduced, as in *Zeyheria* (Gentry, 1992). Nevertheless, detailed studies on the structure, histochemistry and cell characteristics of these reduced disks and of the entire tissues that comprise the nectar chamber are absent for most species of Bignoniaceae.

The genus *Zeyheria* is composed of only two species, *Z. montana*, a typical shrubby species that occurs in Brazilian savanna and *Z. tuberculosa*, a tree species that occurs in rainforest and seasonal semi deciduous forest of Brazil (Gentry, 1992; Rizzini, 1997). These two species differs in their pollination syndromes, *Z. montana* has flowers with characteristics usually attributed to pollination by hummingbirds (Bittencourt and Semir, 2004), while *Z. tuberculosa* is bee pollinated (Vaz et al, Cap.1).

In *Z. montana* the production of the floral nectar was attributed by Bittencourt and Semir (2004) to the glandular trichomes located on the inner wall of the corolla tube, since the authors considered that histological analysis of the rudimentary disk of *Z. montana* did not reveal the presence of secretory tissues. Although *Z. tuberculosa* is a nectariferous species that possess a nectar chamber at the base of corolla (Vaz et al, Cap 1), there is no information about the nectary structure in this species.

The goal of this study was to investigate the structure, histochemistry and ultrastructure of tissues that comprise the nectar chamber of *Z. tuberculosa*, aiming to elucidate the sites of nectar production in this bee-pollinated species.

MATERIAL AND METHODS

Study site and study organism

We studied individuals of *Z. tuberculosa* in Botucatu (22º53'S 48º29'O) and Pratânia (22º48'S 48º44'O) municipalities of São Paulo state, South-eastern Brazil. The climate of the region, according to the classification of Köppen, is Cwa, mesotermal with rains in the summer and drought in the winter (Cunha and Martins 2009).

Zeyheria tuberculosa (Vell.) Bureau ex Verl, occurs in rainforest and seasonal forests from São Paulo (25º S) to Ceará (3º 40' S). *Zeyheria tuberculosa* is a deciduous tree species with thick vertically fissured bark; it can reach 20 m tall; opposite leaves; terminal panicle inflorescence; campanulate flowers without a narrow basal tube; didynamous stamens with the anther thecae divaricate; ovary broadly obovoid, densely stellate-pilose; disk not obviously differentiated from base of ovary; fruit a broadly obovoid to round capsule with the surface tannish stellate tomentose; seeds thin, with a distinctly verrucose surface surrounded by broad hyaline-membranaceous wing (Gentry, 1992; Lohmann e Pirani, 1996).

Vouchers of the studied materials were deposited in the 'Irina Delanova Gemtchujnicov' Herbarium (BOTU) of the Biosciences Institute of the Universidade Estadual Paulista, Botucatu, SP, Brazil, under numbers 30818 and 30819.

Light microscopy

We analyzed the presence, distribution and morphology of secretory structures in dissected flowers at several stages of development, small bud (8 mm), middle bud (11 mm), pre-anthesis flower bud (13 mm), and functional flowers (from first day of anthesis).

For the anatomical studies, we fixed samples of first day flowers in FAA 50 (formaldehyde, acetic acid, and 50% ethanol 1:1:18 v/v) (Johansen, 1940). The samples were subsequently subjected to a gradual dehydration in an ethanol series and embedded in

hydroxyethyl-methacrylate. We cut transverse and longitudinal sections (6-8 μm) with a rotary microtome and stained them with 0.05% toluidine blue (pH 4.3) (O'Brien et al., 1964) for general histology.

Histochemistry

We made fresh hand-sections of functional flowers from first day of anthesis and stained with Sudan Black to detect total lipids (Lison, 1960); Naphthol + dimethyl-paraphenylenediamine (NADI) reagent to detect terpenes (David and Carde, 1964); Dragendorff reagent to detect alkaloids (Svendsen and Verpoorte, 1983); 10% ferric trichloride aqueous solution to detect the presence of phenolic compounds (Johansen, 1940); mercuric bromophenol blue to detect total proteins (Mazia et al., 1953); and Lugol's reagent to detect starch grains (Johansen, 1940). Standard control procedures were carried out simultaneously according to the respective protocols.

Scanning electron microscopy (SEM)

For scanning electron microscopy, we fixed intact and dissected flowers at two developmental stages (buds with 6 mm and first day flowers) in glutaraldehyde (2,5% with 0,1 M phosphate buffer, at pH 7,3, overnight at 4°C), after which they were dehydrated in a graded acetone series, critical point dried, mounted on aluminum stubs, gold-coated (Robards, 1978).

Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM), we fixed the samples of nectar chamber from first day flowers in glutaraldehyde (2.5% with 0.1 M phosphate buffer, pH 7.3) and left them overnight at 4° C. After this we post-fixed the samples with 1% osmium tetroxide

(OsO_4) in the same buffer for 2 h at room temperature, dehydrated them in a graded series of acetone solutions, and embedded them in Araldite resin. We stained ultrathin sections with uranyl acetate and lead citrate (Reynolds, 1963).

RESULTS

Morphological, histochemical and ultrastructural analyses of nectar chamber

The nectar chamber of *Zeyheria tuberculosa* comprises a reduced disk placed at the stipe base, the stipe itself, the petals axil and the base of floral tube. This region, the base of floral tube, is comprised between the petals axil and the thickened base of fillets (Fig. 1A). The space where nectar accumulates, the nectar chamber, is shaped by the upper constriction created between the thickened base of filets and the medium portion of ovary (Fig. 1A). In the middle of chamber is the slender stipe (Fig. 1B). At the base of the stipe occurs a flattened, protuberant region devoid of trichomes, featuring a reduced nectary disk (Fig. 1B-D; 2D). The wall of the nectar chamber is formed by the adaxial surface of the base of floral tube which is lined by abundant capitate trichomes, besides sparse conical papillae (Fig. 1E-G; 2A-C).

The reduced *disk* and *stipe* were composed by a uniseriate epidermis and covered with a thin cuticle, a nectary parenchyma formed by isodiametric and compactly arranged voluminous cells, and a subnectary parenchyma constituted by larger cells loosely arranged and vascularized with phloem and abundant xylem (Fig. 1B-C). The disk epidermis was glabrous (Fig. 1B-D; 2D), while the stipe epidermis was densely covered by glandular and non-glandurar trichomes (Fig. 1A, B; 2D). The histochemical tests revealed the presence of starch grains (Fig. 1H) and oils bodies in the epidermal and parenchyma cells of the disk and stipe (Table 1).

Ultrastructurally, epidermal and parenchyma cells of the disk and stipe presented similar features (Fig. 3A-F; 4A-E). These cells had conspicuous nuclei with evident

nucleolus, abundant cytoplasm and vacuoles of variable sizes (Fig. 3A; 4A). Some vacuoles are filled with finely flocculent material (probably polysaccharides) and others with electron-lucent material (oil) (Fig. 3A, B; 4A, B). These cells also presented abundant ribosomes, mitochondria (Fig. 3B, D; 4C), rough (RER) and smooth (SER) endoplasmic reticulum (Fig. 3C, D; 4C), oil bodies inside vacuoles or scattered in the cytoplasm (Fig. 3C) or juxtaposed to the vacuole membrane (Fig. 4A), multivesicular bodies (Fig. 3D), abundant vesicles that fuse with plasma membrane (Fig. 3D), Golgi bodies (Fig. 3E; 4C) and plastids that are filled with starch grains (Fig. 3A, B; 4A-C, D) or with dense inclusions (Fig. 3C). We observed that the starch grains decreased in size and abundance from nectary parenchyma toward epidermis (Fig. 3A; 4A). Amyloplasts with residual starch grains (Fig. 4D, E) were frequently observed in parenchyma cells subjacent to the epidermis. Images suggesting engulfment of degraded plastids by vacuoles are common in these cells (Fig. 4D, E). The anticlinal walls of epidermal cells showed primary pit fields with abundant plasmodesmata (Figure 3B, white square). Wall invaginations were observed on the entire wall surface of some parenchyma cells (Fig. 4B, C). Phloem elements were registered in the parenchyma of the disk and stipe (Fig. 3A; 4A). Intercellular spaces surrounded by accumulations of pectin (Fig. 3E) were filled with finely flocculated material (Fig. 3F). We observed flocculent secretion at the surface of the disk over the cell wall (Fig. 3B, insert).

The *petals axil* was formed by a uniseriate epidermis (Fig. 1 D, black square) composed by globular cells irregular in shape and size, covered by a smooth and thin cuticle (Fig. 1D; 5A). The subepidermal parenchyma was composed by compactly arranged thick-walled cells of various shapes and sizes (Fig. 1D; 5A). Ultrastructurally, epidermis and parenchyma cells were characterized by presenting conspicuous nuclei, abundant and dense cytoplasm, vacuoles of different sizes (Fig. 5A). Amyloplasts, RER and hyperactive Golgi bodies were the more evident organelles in these cells (Fig 5B-D). Conspicuous oil bodies

occurred scattered in the cytoplasm (Fig. 5D, F). The vacuoles presented flocculent materials and membrane debris (Fig 5A, B). Specifically, in the petal junction with disk (Fig. 1D) small epidermal cells with very thick walls were observed (Fig. 5E, F). The cytoplasm of these cells was abundant, dense and presented large mitochondria, polyrribossomes, Golgi bodies, RER, SER, abundant vesicles, and oil bodies (Fig. 5E, F). Images of vesicle fusion with plasma membrane were frequently observed in the epidermal cells of the petal axil (Fig 5C-F). Flocculent material occurred in the periplasmic space of these cells (Fig. 5D, F). The swell of the middle lamellae and its posterior dissolution along the anticlinal walls of epidermal cells originated channels (Fig. 5E).

The *base of floral tube* at the adaxial surface was composed by a uniseriate epidermis (Fig. 1D-G) covered by a striate cuticle (Fig. 2B, C). The common epidermal cells were irregular in size and shape, presenting reduced cytoplasm and a large central vacuole (Fig. 1E-G). Capitate trichomes and papillae were present in this surface (Fig. 2C). Capitate trichomes were composed by an epidermal basal cell, a stalk with variable number of cells (1-5) (Fig. 1F, G; 2B) and a secretory head with cells disposed in one (Fig. 1F; 6A) or two layers (Fig. 1G; 6E). Trichomes with variable head cell layers occurred side by side (Fig. 1G). The head cells of capitate trichomes reacted positively for lipids, flavonoids, phenolic compounds and proteins tests; papillae reacted positively for lipids and proteins tests (Table 1).

TEM analysis showed that the capitate trichomes with cells disposed in only one layer had machinery involved predominately in hydrophilic secretion (Fig. 6A-D) herein named Type I, while capitate trichomes with head cells disposed in two layers were involved predominately with lipophilic secretion (Fig. 6E-G) herein named Type II. Both trichomes types presented deposits of lipid substances in the lateral walls of stalk cells (Fig. 6A, E). The head cells of capitate trichomes Type I were covered by a thick cuticle that remained attached to the outer cell wall (Fig. 6A, B). The cuticle presented an inner stratum containing a dense

fibrillar network formed by pectin (Fig. 6C). The head cells had conspicuous nuclei, dense and abundant cytoplasm and vacuoles of different sizes (Fig 6A-B). Globular mitochondria (Fig. 6B, D), few profiles of SER (Fig. 6C), RER (Fig. 6B, D), well-developed Golgi bodies (Fig. 6D) are the more abundant organelles in the cytoplasm of head cells of the Type I capitate trichomes. Scattered drops of lipophilic material, which reacted positively to test for flavonoids and phenolic compounds (Table 1), were verified in the cytoplasm of these head cells (Fig. 6A-B, D).

The head cells of capitate trichomes Type II had conspicuous nuclei, dense and abundant cytoplasm and larger vacuoles (Fig 6E). Globular plastids showing dense stroma and tubules filled with dark inclusions (Fig. 6G), extensive and abundant SER (Fig. 6F) and scattered drops of lipophilic material that reacted positively to test for flavonoids and phenolic compounds (Table 1) characterizes the cytoplasm of the head cells (Fig. 6F-G). Fusion of SER dilated cisterns with plasma membrane was frequently observed in these cells (Fig. 6F). As secretion progresses, the exudate accumulates between the cuticle and the outer wall cells in the apical region of the head, originating a subcuticular space (Fig. 6E, F).

DISCUSSION

In this study, we characterized the morphology, histochemistry and ultrastructure of the entire tissues from nectar chamber of *Zeyheria tuberculosa*. Our data allowed us to consider that all these tissues are involved in the nectar secretion and will be referred from here as floral nectary. Additionally, histochemical analysis showed that all tissues of nectar chamber presented evidences of hydrophilic and lipophilic secretion. This study showed that the disk, although with reduced dimensions, have a functional vascularized parenchyma that storage and hydrolyzes starch, besides presenting cellular machinery of secretory cells (Fahn, 1979, Durkee, 1983). Nectar release occurs probably trough the intact cuticle, as reported by

Nepi (2007) for several other plant species, since we didn't find cuticle pores or stomata in nectar chamber tissues.

Our results allowed us to classify the rudimentary disk and stipe as functional nectary tissues in this species, different from the findings of Bittencourt and Semir (2004) for *Z. montana*. Although the disk is reduced, the nectar production is performed together with stipe, which results in a considerable area of tissue with secretory activity inside the nectar chamber. As both, disk and stipe presented very similar ultrastructural and histochemical features, acting as a functional unity; they will be referred as *disk-stipe* from now.

The presence of amyloplasts with voluminous starch grains was remarkable in the parenchyma cells of the disk-stipe of *Z. tuberculosa*. The starch grains were bigger in the stipe suggesting a higher nectar secretion rate in this tissue, what usually requires big amyloplasts differentiating before secretion, according to Nepi et al (1996). These starch grains showed a gradual reduction in size and in abundance from parenchyma cells toward the epidermis of the disk-stipe. In fact, ultrastructural images show clearly the starch grains degradation and the plastids engulfment by vacuoles. Usually, the starch in plastids decreases with the beginning of nectar production, so that several authors suggest that the hydrolysis of starch in the parenchyma contributes directly to nectar carbohydrate content (see Nepi, 2007 and references therein), which reinforces our findings about disk-stipe functionality. The conversion of amyloplasts into vacuoles, observed in this study was similar to the pattern described for other floral nectaries of *Hymenaea stigonocarpa* by (Paiva and Machado, 2008) and *Anemopaegma album* by Guimarães *et al*. (2015, submitted). Additionally, the subcellular features of the nectary parenchyma of disk-stipe was similar to those registered in the floral nectaries of several other species (Fahn and Shimony, 2001; Stpiczynska et al., 2005; Paiva and Machado, 2008; Paiva, 2012), indicating high metabolic activity (Fahn, 1979, 1988; Roshchina and Roshchina, 1993; Nepi, 2007).

The breakdown of the starch grains at the beginning of nectar production indicates that, besides source of carbohydrates for nectar, it may be related to the energy needs during secretion, especially in the presence of abundant mitochondria (Nepi, 1996; Fahn, 2001; Paiva and Machado, 2008), as verified in ultrastructural analysis of disk-stipe of *Z. tuberculosa.* Besides, parenchyma cells of the disk-stipe showed abundant an large mitochondria, rough endoplasmic reticulum (RER), Golgi bodies, abundant vesicles and plasmodemata in the anticlinal walls that are characteristic of nectar-producing tissues (Fahn, 1988, Paiva and Machado, 2008). The parenchyma cells of *Z. tuberculosa* disk showed intercellular spaces where the secretion may flow by apoplast pathway. Additionally, the pectin accumulations located in the intercellular spaces may function as channels for secretion and may facilitate the apoplastic transport of nectar in *Z. tuberculosa* floral disk, as referred by Paiva and Machado (2008). Cell wall labyrinthine projections in parenchyma cells of disk-stipe increase the surface for symplastic and apoplastic transport (Fahn, 1988). In addition, a symplastic via of nectar transport can also take place in the floral disk of *Z. tuberculosa* via plasmodesmata that connect epidermal cells and them with parenchyma cells (Wist and Davis, 2006; Vassilyev, 2010).

The petals axil also showed cellular machinery involved in both, lipophilic and hydrophilic secretion. Additionally, we hypothesize that the protuberant shape of epidermal cells, which creates an expanded surface of changes, and the remarkable thickness of the cell wall can be related to reabsorption of nectar constituents, since a decrease in nectar concentration was verified in some plants by Vaz *et al*. (Cap. 1). Nectar sugar recovering through reabsorption has been reported for several plant species and it can represent an energy-saving strategy (Nicolson, 1995; Stipczynska and Nepi, 2006, Stahl et al., 2012(Stpiczynska et al., 2012), which can be particularly important in this species that produces thousands of flowers per day (Vaz *et al*., Cap. 1).

Although *Z. tuberculosa* and *Z. montana* have different pollinators, both have a nectar chamber covered by capitate trichomes, which were referred by Bittencourt and Semir (2004) as responsible for nectar secretion in *Z. montana* flowers. The head cells of capitate trichomes of *Z. tuberculosa*, both Types I and II, contain subcellular features of cells involved in lipophilic and hydrophilic secretion (Fahn, 1979; Durkee, 1983). From the stalk towards the head cells the secretion probably flows by symplast pathway, because the apoplastic route is impeded by cutinization of the lateral walls of stalk cells (Fahn, 1979; Nepi, 2007).

All the ultrastructural characteristics of the capitate trichomes of the base of floral tube in *Z. tuberculosa* flowers corroborated the results of histochemical tests, being the cellular machinery of trichomes consistent with the substances detected at histological level. These trichomes seems to be directly related to nectar enrichment, since histochemical analysis demonstrated that they are involved in the production of hydrophilic and lipophilic substances as lipids, proteins, terpenes and flavonoids. The same pattern was described by Guimarães *et al*. (2008) for the capitate trichomes of the staminode of *Jacaranda oxyphylla* (Bignoniaceae).

The differences found in the ultrastructural organization among the disk-stipe, petals axil and base of floral tube suggests a differential contribution given from each nectary region to nectar secretion, characterizing a complex and integrate system of nectar production in *Zeyheria tuberculosa* flowers.

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FIGURE LEGENDS

Fig. 1. Characterization and morphology of *Zeyheria tuberculosa* floral nectary. (A-G = Light microscopy, H = Polarized light microscopy). (A) Longitudinal cut of flower evidencing the nectary (square) that comprises a chamber (nc) formed by the reduced nectary disk (nd), the stipe (st), the petals axil and the base of floral tube covered with abundant capitate trichomes. ca = calyx, co = corolla, fb = fillets basis, ov = ovary. (B) Detail of nectar chamber showing the stipe (st), the nectary disc (nd) and the capitate trichomes of the petal basis (arrows). (C) Nectary disc (nd) composed by uniseriate epidermis, nectary parenchyma and subnectary parenchyma supplied by abundant xylem (xy). (D) Detail of the nectary disk evidencing the region of junction of the petal axis and the disk (square). The petal axis is formed by uniseriate epidermis covered by a thin cuticle. (E) Capitate trichomes of the petal basis. Note the epidermal cells with irregular in size and shape. (F) Detail of a short-stalk capitate trichome presenting stalk with one cell. (G) Detail of a long-stalk capitate trichome presenting stalk with three cells. (H) Parenchyma of nectary disk showing starch grains (sg). Scale bars = 200 μm (A), 500 μm (B), 150 μm (C), 100 μm (D, E, G), 50 μm (F, H).

Fig. 2. Nectar chamber of *Zeyheria tuberculosa* (SEM). (A) adaxial surface of the base of floral tube covered with capitate trichomes. (B) Detail of capitate trichomes showing the expanded cuticle at the apical region of the secretory head. (C) Conical papilla (pa) and abundant capitate trichomes. (D) Reduced nectary disk (nd) devoid of trichomes. Scale bars = 150 μm (A), 25 μm (B), 50 μm (C), 100 μm (D).

Fig 3. Reduced nectary disk of *Zeyheria tuberculosa* fuctional flowers (TEM). (A) A general view of the secretory epidermis and nectary parenchyma cell with conspicuous nuclei (nu), vacuoles of variable sizes (va), starch grains (sg), oil (ol) bodies and phloem elements (ph). Note that starch grains (sg) decreased in size and abundance from nectary parenchyma toward epidermis. (B) Epidermal cells showing vacuoles filled with finely flocculent material (va) and others with electron-lucent material (ol), mitochondria (mi), plastids filled with starch grains (sg) and abundant plasmodemata (square). (B

– insert) Detail showing flocculent secretion on the disk surface. (C) Epidermal cells showing oil bodies scattered in the cytoplasm (ol), SER, RER and plastids (pl) filled with dense granulations. (D) Epidermal cells showing thick cell wall, abundant ribosomes and mitochondria (mi), SER. Note the mutivesicular bodies (mb) and abundant vesicles (arrows) fusing with the plasma membrane. (E) Detail of disk parenchyma showing intercellular spaces (is) containing accumulations of pectin (pc). Golgi bodies (Gb) and wall invagination filled with fibrillar material (*) are seen in the cytoplasm adjacent to the corner of the cells. (F) Detail of disk parenchyma showing a wide intercellular space (is) with accumulations of pectin (pc) and filled with finely flocculent material. Scale bars = 5 μm (A), 1 μm (B, E), 400 nm (B insert), 500 nm (C, F), 200 nm (D).

Fig. 4. Stipe of *Zeyheria tuberculosa* fuctional flowers (TEM). (A) A general view of the epidermis and parenchyma cell with conspicuous nuclei (nu), vacuoles of variable sizes (va), oil bodies (ol), and amyloplasts with starch grains (sg) and phloem elements (ph). Note that the starch grains decreased in size and abundance from nectary parenchyma toward epidermis. (B) Part of two parenchyma cells showing conspicuous nuclei (nu), vacuoles filled with finely flocculent material (va), larger starch grains (sg), and wall invaginations (*). (C) Detail of cytoplasm from the epidermal cell showing Golgi body (Gb), abundant ribosomes, mitochondria (mi), RER, SER and wall invaginations filled with fibrillar material (*). Note the plastid with a voluminous starch grain (sg). (D) Detail of cytoplasm from the parenchyma cells containing amyloplast (pl) with residual starch grains (sg). Note the vacuole (va) engulfing the degraded plastid (pl). (E) Parenchyma cells subjacent to the epidermis showing oil bodies scattered (*), plastids filled with starch grains (sg), amyloplasts (pl) with residual starch grains, and engulfment of degraded plastids by vacuoles (va). Scale bars = 5 μm (A), 2 μm (B, E), 1 μm (C), 500 μm (D).

Fig. 5 Petal axil of *Zeyheria tuberculosa* fuctional flowers (TEM). (A) A general view of the epidermis and parenchyma cell with conspicuous nuclei (nu), abundant and dense cytoplasm, and

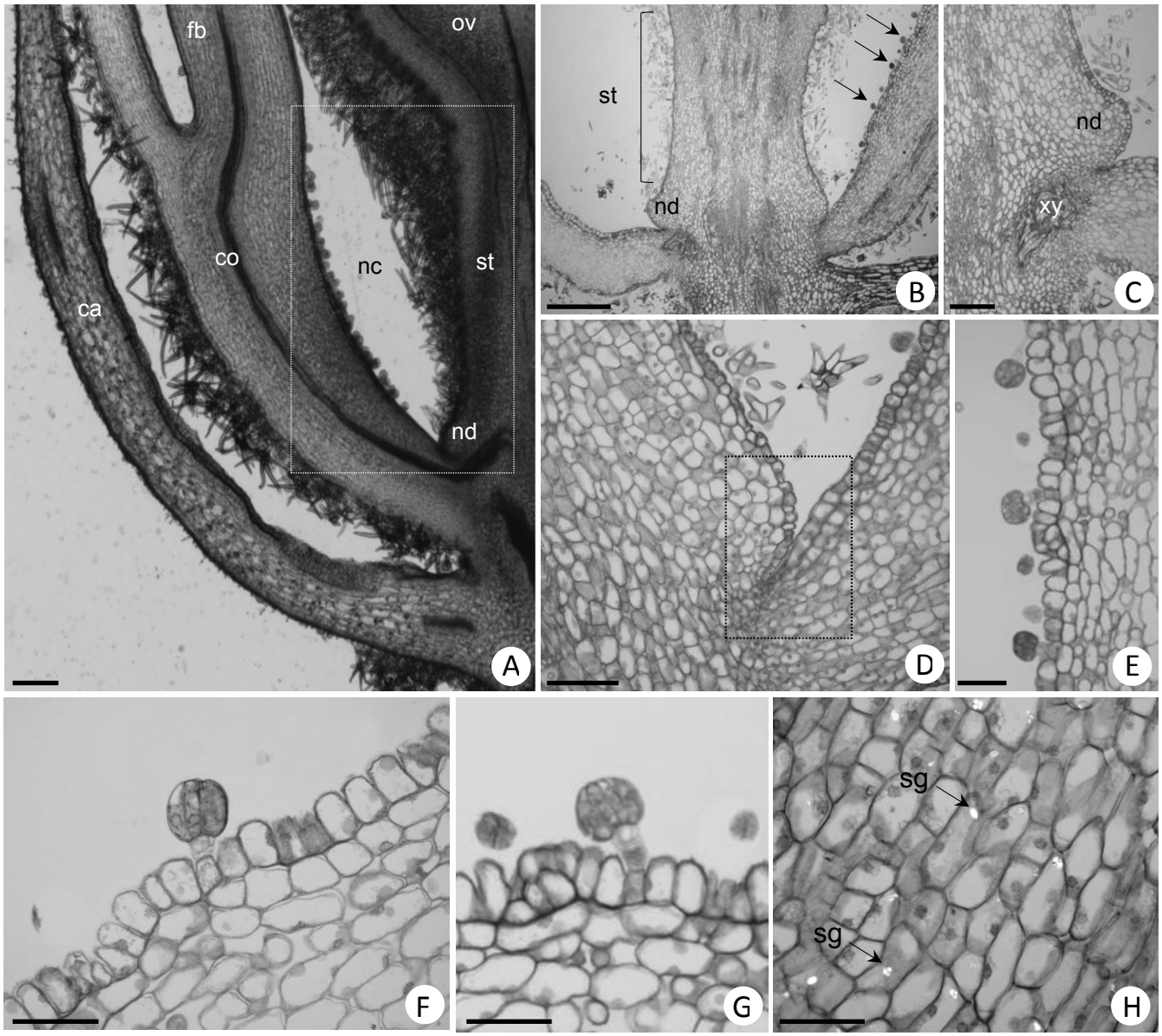
vacuoles (va) of variable sizes. (B) Detail of epidermal cell showing plastids (pl) containing starch grains (sg) of variable sizes, mitochondria (mi) and Gogi body (Gb). Note the vacuoles (va) presented flocculent materials and membrane debris. (C) Detail of cytoplasm of an epidermal cell showing abundant RER, mitochondria (mi), vacuole (va) and hyperactive Gogi bodies (Gb). Note the vesicles fusing with the plasma membrane (arrowhead). (D) Epidermal cell showing oil bodies scattered (ol), RER, hyperactive Golgi body (Gb), vesicles fusing with the plasma membrane (arrowheads), flocculent material in the periplasmic space and thick cell wall (cw). (E) Epidermal cell in the junction of the petal and the disk presenting a very thick cell wall (cw), dense and abundant cytoplasm showing large mitochondria (mi), RER and Golgi bodies (Gb). Note the formation of channels by the dissolution of middle lamellae along the anticlinal cell walls (arrows). (F) Detail of the epidermal cell showing oil body (ol) scattered in the cytoplasm, large mitochondria (mi), polyribosomes, proliferate RER and SER, vesicles fusing with the plasma membrane (arrowheads) and flocculent material in the periplasmic space. Scale bars = 5 μm (A), 1 μm (B, D, E), 500 nm (C), 250 nm (F).

Fig. 6. Capitate trichomes of *Zeyheria tuberculosa* (TEM). (A) A general view of the capitate trichomes (Type I) showing stalk cell covered with a very ticked cuticle, reduced cytoplasm and conspicuous vacuole filled with dark inclusions and; head cells covered with a thick cuticle presenting an inner stratum containing a dense fibrillary network formed by pectin (ct), conspicuous nuclei (nu) and scattered drops of dark inclusion. Th = trichome head, ts = trichome stalk. (B) Part of head cells showing ~~a~~ dense cytoplasm with voluminous mitochondria (mi), RER, plastids (pl) filled with dark inclusions, scattered flavonoids droplets and thick cuticle that remained attached to the outer cell wall. (C) Detail showing the thick cuticle (ct) presenting an inner stratum containing a dense fibrillar network formed by pectin. Note SER in the peripheral cytoplasm. (D) Detail of a head cell evidencing globular mitochondria (mi), flavonoids drop (fl), Golgi body (Gb) and RER. (E) A general view of the capitate trichome (Type II) showing stalk and head cells with conspicuous vacuoles (va). Note the formation of a subcuticular space (arrow) at the central region of the head. (F) Part of head cells evidencing the proliferation of SER cisternae and the fusion of dilated cisterns with the plasma

membrane. Note the presence of flavonoids droplets (fl), globular plastids (pl), large periplasmic space (ps), thick cuticle (ct) and a subcuticular space (*). (G) Detail of a head cell showing globular plastid with dense tubules and dark granules (pl), and flavonoid drop (fl). Scale bars = 5 µm (A, E), 1 µm (B, F), 500 nm (C, D, G).

FIGURES

Figure 1



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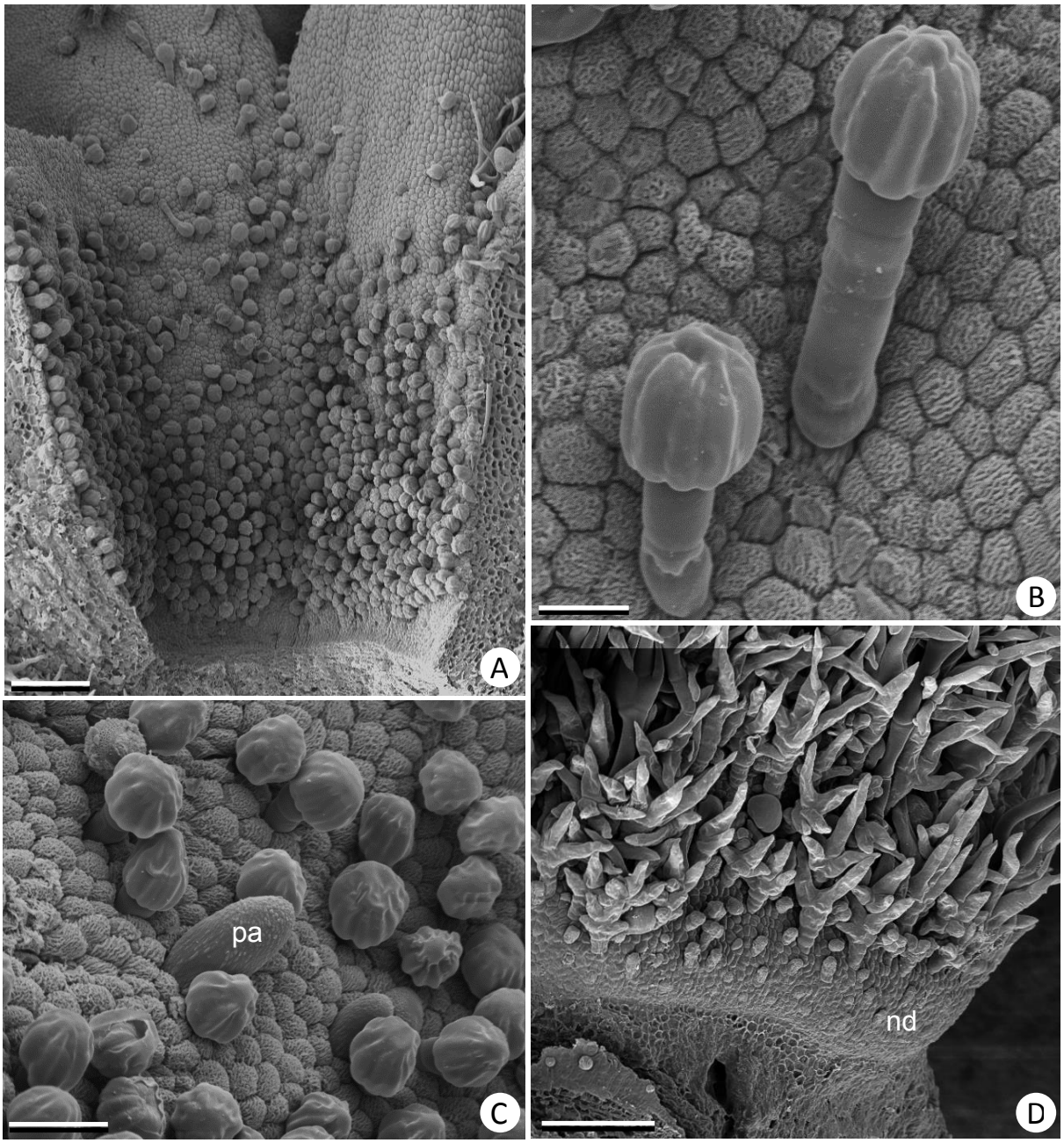

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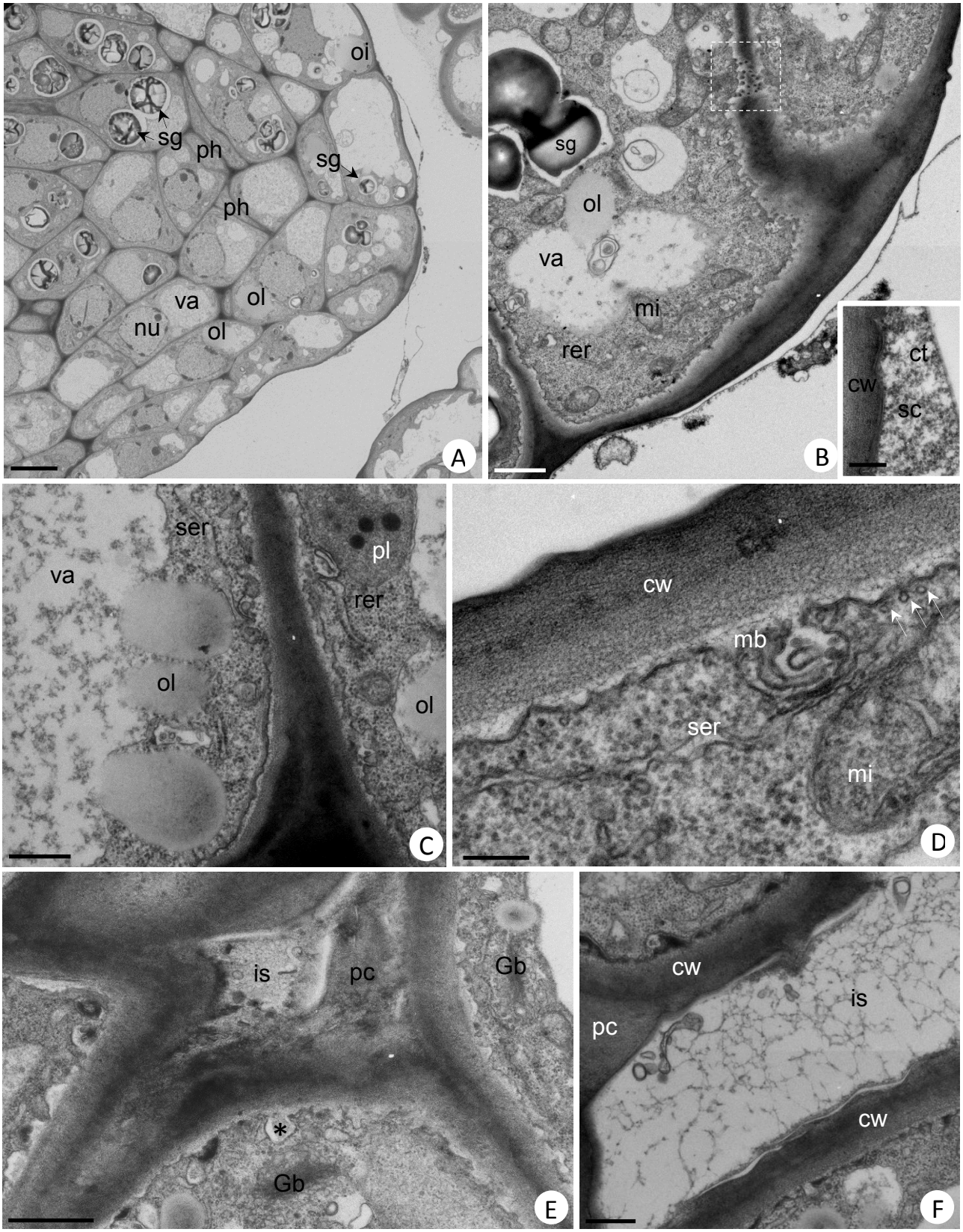
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Figure 4

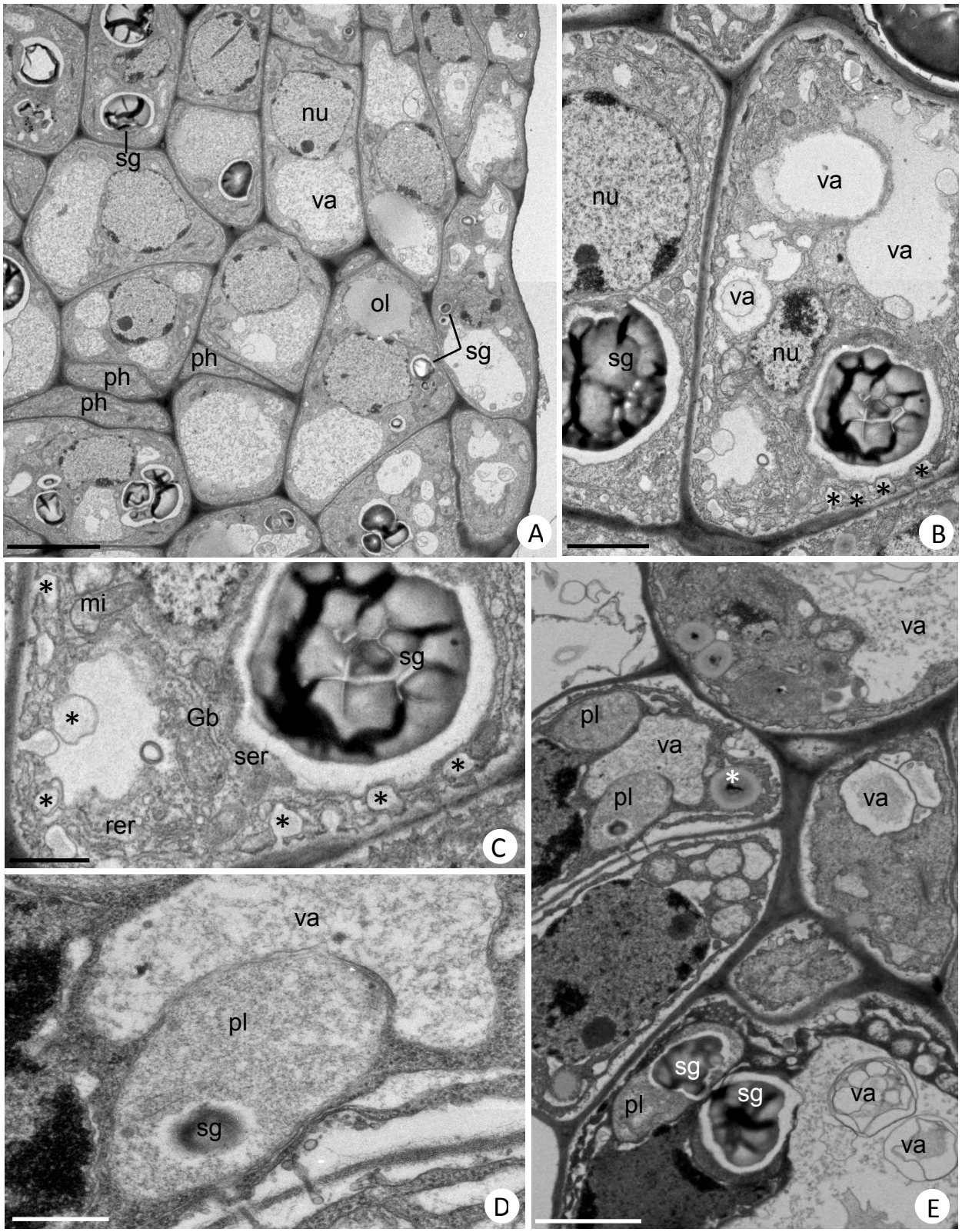
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Figure 5

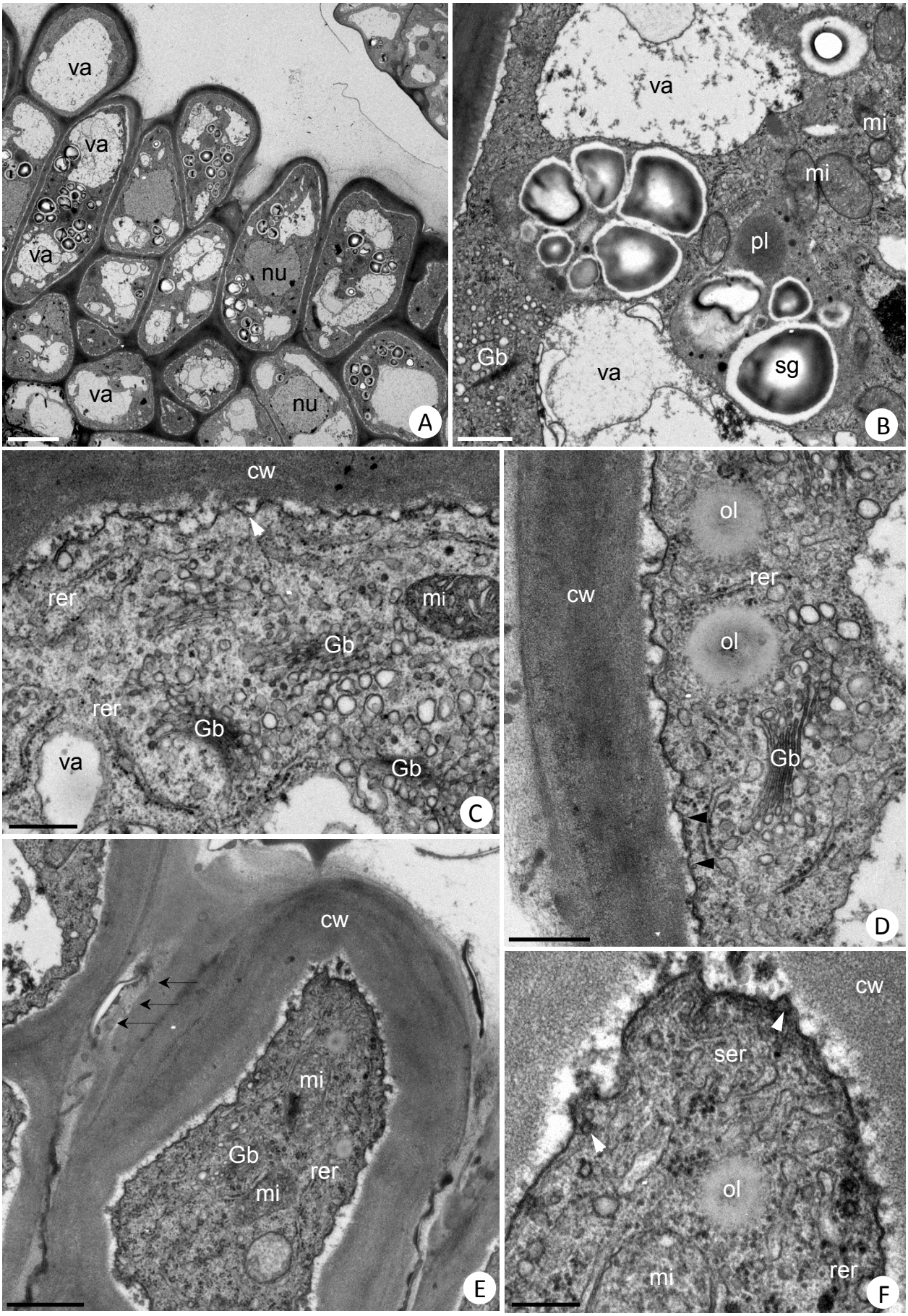
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Figure 6

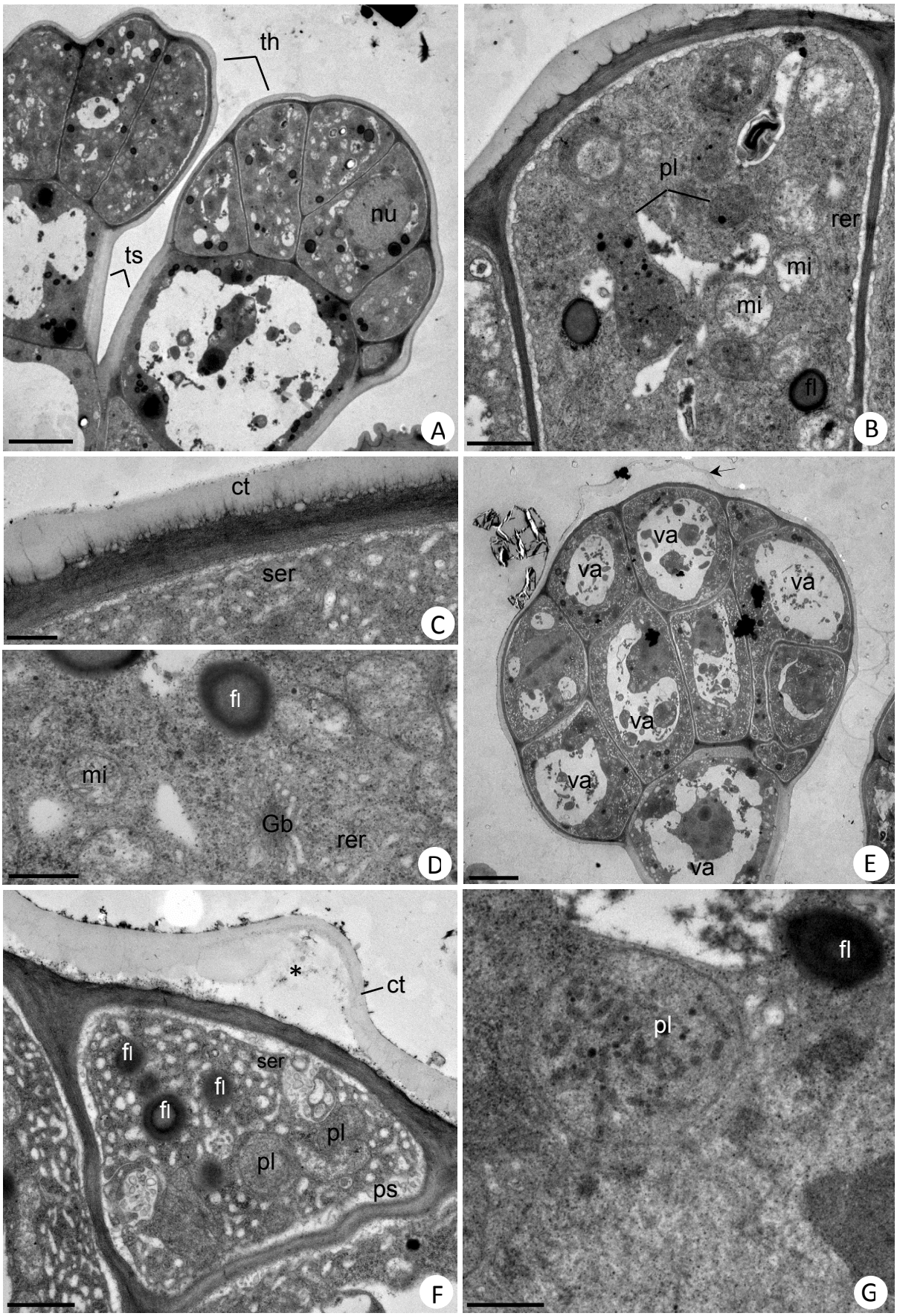
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Table1. Histochemical analyses of nectar chamber tissues from functional flowers of *Zeyheria tuberculosa* (Bignoniaceae).

Staining procedure	Target coumpounds	Capitate trichomes	Papillae	Parenchyma and epidermal cells of disk	Epidermal cells of petals axil
Sudan III	Total lipids	+	+	+	+
NADI	Terpenes	+	-	-	-
Ferric trichloride	Phenolic compounds	+	-	-	-
Mercuric bromophenol blue	Proteins	+	+	+	+
Lugol	Starch grains	+	-	+	+
Neutral lead acetate	Flavonoids	+	-	-	-

CONSIDERAÇÕES FINAIS

Zeyheria tuberculosa apresenta diversos atributos florais que correspondem à síndrome de melitofilia e neste estudo, nós confirmamos isto registrando, em campo, a visita de abelhas de médio porte, pertencentes ao gênero *Bombus*, que apresentaram comportamento de vista e dimensões corporais que as qualificam como polinizadoras efetivas. Além disso, as características do néctar se enquadram bem nas preferências de abelhas, embora o volume seja relativamente pequeno e a concentração mais baixa do que descrito para outras Bignoniaceae melitófilas. Entretanto, verificamos que ao longo do dia ocorre um pequeno aumento na concentração do néctar, o que poderia resultar em um recurso mais adequado às abelhas após algumas horas de antese. Adicionalmente, o nectário floral de *Z. tuberculosa* é complexo, sendo formado pelos diversos tecidos que compõem a câmara nectarífera e sua secreção mista pode estar associada ao enriquecimento do néctar, considerando que além dos açúcares detectados em testes histoquímicos, há traços estruturais e reações histoquímicas que indicam a presença de aminoácidos/ proteínas no néctar, bem como de lipídios, ambos com elevado valor nutricional e energético.

Assim, consideramos que estudos como este que envolvem investigações complementares sobre as necessidades e preferências dos polinizadores, as características dos atrativos florais primários e as estruturas glandulares que secretam o recurso envolvido na interação podem auxiliar na compreensão dos ajustes mútuos presentes neste mutualismo. Além disso, a integração de informações das áreas de ecologia e anatomia vegetal pode ajudar a entender os fatores que levaram as espécies vegetais à especialização na polinização por certos grupos funcionais.

Os resultados obtidos neste estudo indicam certa especialização em relação às dimensões e formato das flores, ao padrão de secreção de néctar e à estrutura e funcionamento

do nectário floral. Entretanto, constatamos em campo baixa frequência de visitas das abelhas às flores e baixo sucesso reprodutivo, o que pode ser decorrente da redução das populações de polinizadores ou da diminuição da variabilidade genética da população local de *Z. montana*, o que poderia levar à limitação de polinizadores e de pólen na área de estudo. Este cenário é preocupante considerando-se que a espécie encontra-se em situação vulnerável à extinção, o que reforça a necessidade de estudos com abordagem integradora, especialmente aqueles que envolvem mutualismos, como a polinização, pois quebras neste tipo de interação podem ter consequências importantes em médio em longo prazo sobre o ecossistema alterado.