POLIANA RAMOS CARDOSO

Desenvolvimento floral em espécies de Pleurothallidinae (Orchidaceae) com ênfase nas estruturas secretoras

Tese apresentada ao Instituto de Botânica da Secretaria do Meio Ambiente, como parte dos requisitos exigidos para a obtenção do título de DOUTOR em BIODIVERSIDADE VEGETAL E MEIO AMBIENTE, na Área de Concentração de Plantas Vasculares em Análises Ambientais.

SÃO PAULO
2014

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À Profa. Dra. Marilia de Moraes Castro, dedico

If you're going to try, go all the way.
otherwise, don't even start.
if you're going to try, go all the way.
this could mean losing girlfriends,
wives, relatives, jobs and maybe your mind.
go all the way.
it could mean not eating for 3 or 4 days.
it could mean freezing on a park bench.
it could mean jail,
it could mean derision,
mockery, isolation.
isolation is the gift,
all others are a test of your endurance,
of how much you really want to do it.
and you'll do it
despite rejection and the worst odds
and it will be better than
anything else you can imagine.
if you're going to try, go all the way.
there is no other feeling like that.
you will be alone with the gods
and the nights will flame with fire.
you will ride life straight to
perfect laughter, its
the only good fight there is"

"Roll the dice", C. Bukowski

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Índice

Resumo

Pleurothallidinae é reconhecida como a maior subtribo de orquídeas polinizadas por Diptera, e têm sido foco de estudos filogenéticos devido à natureza poli e parafilética de alguns gêneros. Estudos recentes acerca da biologia floral e sistemas de reprodução de representantes da subtribo baseiam a proposta de divisão da atual circunscrição de Pleurothallidinae em duas subtribos, uma que recupera sua antiga delimitação e a criação de outra contendo o pequeno clado composto por gêneros polinizados por aves, incorporado às Pleurothallidinae de acordo com a filogenia molecular vigente. O principal objetivo desta tese foi o levantamento de resultados que auxiliem no esclarecimento de homologias e nas interpretações filogenéticas de Pleurothallidinae, e que também possam ser aplicados no entendimento dos eventos de polinização. Aqui descrevemos o desenvolvimento e a vascularização floral, além da morfologia, anatomia e os aspectos ultra-estruturais das glândulas florais em busca de homologias. Para tanto, efetuamos as análises das flores em microscopia de luz, microscopia eletrônica de varredura e transmissão, e microscopia confocal. Realizamos, adicionalmente, a análise química dos voláteis emitidos pelas flores em CG/MS. As espécies contempladas neste estudo pertencem aos gêneros *Octomeria*, *Echinosepala*, *Acianthera*, *Anathallis*, *Zootrophion* e *Phloeophila*, todas ocorrentes no Brasil, escolhidas de modo a representar um clado basal (*Octomeria-Brachionidium*, irmão do restante de Pleurothallidinae) e clados derivados (*Echinosepala-Barbosella*, *Acianthera* e *Zootrophion* - *Trichosalpinx*), de acordo com a filogenia molecular vigente. Os principais resultados obtidos referem-se à natureza anatômica de alguns aspectos morfológicos das flores, a comparação entre o desenvolvimento e a vascularização floral, a identificação e descrição detalhada das glândulas florais, incluindo a descrição de uma nova glândula de secreção heterogênea. Foram propostas uma nova interpretação para o sistema secretor constituinte do estigma, que explica sua dinâmica de secreção, e as tendências evolutivas de coléteres e osmóforos. Finalmente, caracteres relacionados à vascularização e aos compostos voláteis permitiram hipotetizar duas novas sinapomorfias para a subtribo.

Abstract

Pleurothallidinae is recognized as the largest orchid subtribe pollinated by Diptera, and has been a focus of phylogenetic studies due to the poly- and paraphyletic nature of some genera. The floral biology and mating systems of its species have been recently assessed, inspiring a proposal of splitting Pleurothallidinae as currently circumscribed into two subtribes, recovering the old delimitation and creating a subtribe with the small bird-pollinated clade incorporated by the currently molecular phylogeny. Our primary objective was to provide data that aim to clarify homologies and the phylogenetic interpretation of Pleurothallidinae, as well as to provide insights to the knowledge of the events of pollination by the study of floral secretory tissues. We studied floral development and vascularization, besides the morphology, anatomy and ultrastructural aspects of floral glands seeking putative homologies. To achieve our objectives, we performed analysis under light, scanning and transmitting electron microscopies, and confocal microscopy. We also performed chemical analysis of the headspace volatiles emitted by flowers on GC/MS. Species chosen for this study belong to Brazilian *Octomeria*, *Echinosepala*, *Acianthera*, *Anathallis*, *Zootrophion* and *Phloeophila* and represent a late-divergent clade (*Octomeria*-*Brachionidium*, sister of the remaining Pleurothallidinae) and early-divergent ones (*Echinosepala*-*Barbosella*, *Acianthera* and *Zootrophion*-*Trichosalpinx*) according to the currently accepted molecular phylogeny. Our pivotal results regard the anatomical nature of some morphological aspects of the flower, comparison between flower development and vascularization, identification and detailed description of floral glands, including the recognition of a new gland of heterogeneous secretion. We proposed another interpretation to the secretory system constituting the stigma that explains their secretion dynamics, besides evolutionary tendencies of colleters and osmophores. Finally, our vascularization and volatile analysis allowed hypothesizing two new synapomorphies for this subtribe.

Introdução geral

A subtribo Pleurothallidinae (Orchidaceae, Epidendroideae, Epidendreae) compreende cerca de 4000 espécies neotropicais distribuídas em 30 gêneros (Luer 1986, Dressler 1993), o que perfaz aproximadamente 20% das espécies de Orchidaceae (Pridgeon *et al.* 2001), e é considerada o maior grupo de orquídeas polinizadas por insetos da Ordem Diptera (Pridgeon *et al.* 2001, Borba *et al.* 2011).

Esta subtribo foi objeto de uma profunda reformulação taxonômica desde a revisão de Luer (1986), e a filogenia atualmente vigente (Pridgeon *et al*. 2001), totalmente baseada em caracteres moleculares (DNA nuclear e plastidial), divide a subtribo em nove clados que apresentam gêneros poli e parafiléticos. Inclui, ainda, um pequeno clado composto por três gêneros procedentes da América Central (*Dilomilis*, *Neocogniauxia* e *Tomzanonia*), cujas espécies são ornitófilas e auto-compatíveis.

De acordo com Pridgeon *et al.* (2001), a identificação de sinapomorfias morfológicas e anatômicas na subtribo é dificultada pela abundante homoplasia observada em caracteres vegetativos e florais. Assim, as características morfológicas e anatômicas consideradas em estudos filogenéticos prévios (Prigeon 1982, Neyland *et al.* 2005) ocorrem em espécies não relacionadas, ou são observadas em plantas submetidas ao mesmo ambiente (Pridgeon et al. 2001), o que as torna inadequadas para uso como caracteres filogenéticos.

Recentemente, Borba *et al.* (2011) baseados em estudos do sistema reprodutivo de espécies de Pleurothallidinae, propuseram a divisão da subtribo, em sua concepção atualmente vigente, em duas, uma considerando a antiga circunscrição, e outra compreendendo o pequeno clado recém incluído de espécies ornitófilas. Esta divisão, além de resgatar a principal sinapomorfia morfológica que unificava Pleurothallidinae, qual seja, a presença de uma articulação entre o pedicelo e o ovário, ainda inclui duas novas sinapomorfias biológicas, a

miiofilia e a auto-incompatibilidade. Neste cenário, estudos cujo enfoque seja a busca por caracteres que auxiliem na melhor circunscrição e pelo reconhecimento de novas sinapomorfias em Pleurothallidinae, são imprescindíveis.

A ontogênese é, provavelmente, o melhor critério para o reconhecimento de homologias morfológicas primárias e, freqüentemente, o estabelecimento de homologias em dado órgão é inviável sem o conhecimento de seu desenvolvimento (Kocyan & Endress 2001). Esta abordagem disponibiliza um recurso valioso para a avaliação das relações em alguns grupos de orquídeas (Kurzweil *et al.* 2005) e, embora a descrição do desenvolvimento floral tenha sido efetuada em diferentes espécies pertencentes à subfamília Epidendroideae (entre elas, duas inseridas em Pleurothallidinae; Kurzweil 1987, Kurzweil & Kocyan 2002), a observação dos detalhes ocorrentes após os estádios iniciais pode auxiliar na compreensão e na confirmação de homologias propostas.

A vascularização floral sempre constituiu um tópico relevante de investigação em pesquisas taxonômicas e filogenéticas (Saburi *et al.* 2005). É uma ferramenta importante no entendimento de quaisquer alterações nos padrões morfológicos externos e nas seqüências destas alterações (Puri 1951). A vascularização floral é, também, útil nos estudos de homologia e na determinação da origem de estruturas florais (Bachelier & Endress 2008, Pirani *et al.* 2010, Sajo *et al.* 2010). Estes estudos são escassos em Orchidaceae, restritos à ampla descrição dos padrões de vascularização de 40 espécies pertencentes a 24 gêneros por Swamy (1948), e em grupos de orquídeas basais como Apostasioideae (Rao 1969, 1973, 1974) e Cypripedoideae (St-Arnaud & Barabé 1989), uma análise pontual em *Telipogon* (Epidendroideae: Telipogoninae; Pabón-Mora & González 2008), e considerações esparsas em outras espécies (Horta 1935, Rudall & Bateman 2002, Rudall *et al.* 2013).

As estruturas secretoras apresentam grande significado taxonômico, cujos caracteres distintivos abrangem o tipo, a ocorrência, a posição e a ontogênese (Teixeira *et al.* 2004, Judd *et al.* 2009). Em Pleurothallidinae, as estruturas secretoras registradas em órgãos reprodutivos restringem-se aos nectários e osmóforos (Pridgeon & Stern 1983, 1985; Blanco & Barboza 2005; Barbosa *et al.* 2009; Melo *et al.* 2010; Duque-Buitrago *et al.* 2014). Os compostos químicos voláteis emitidos pelos osmofóros, embora sejam resultado direto da convergência com os polinizadores, podem apresentar marcadores químicos (compostos que se repetem nas diferentes espécies) que possuem relevância filogenética e que podem constituir dados aplicáveis na reconstrução taxonômica ou filogenética (Steiner *et al.* 2011).

As espécies escolhidas para a presente investigação pertencem aos gêneros *Octomeria*, *Echinosepala*, *Acianthera*, *Anathallis*, *Zootrophion* e *Phloeophila*, todas elas ocorrentes no Brasil. Os gêneros foram escolhidos de forma a representar um clado basal (clado *Octomeria-Brachionidium*, irmão do restante da subtribo) e aqueles mais derivados (clados *Echinosepala-Barbosella*, *Acianthera* e *Zootrophion-Trichosalpinx*), de acordo com as relações filogenéticas hoje aceitas (Pridgeon *et al.* 2001, 2010).

Esta tese teve como principal objetivo utilizar técnicas clássicas em anatomia vegetal (microscopia de luz e câmara clara) aliadas àquelas contemporâneas (microscopia confocal e eletrônica de varredura e transmissão) e análises químicas (CG/MS), no estudo dos aspectos referentes ao desenvolvimento floral e estruturas secretoras presentes nas flores de espécies de Pleurothallidinae, com o intuito de responder às seguintes questões:

(1) As características do padrão de desenvolvimento floral de espécies de Pleurothallidinae apresentam apomorfias em relação ao modelo descrito em Epidendroideae (Kurzweil 1987,

Kurzweil & Kocyan 2002)? O padrão de vascularização dos representantes desta subtribo obedece ao modelo descrito por Swamy (1948) para orquídeas monândras[1]?

(2) Quais são as estruturas secretoras presentes nos órgãos florais das espécies estudadas? É possível observar padrões evolutivos nestas glândulas?

(3) É possível hipotetizar sinapomorfias a partir do estudo morfológico e anatômico das flores?

A partir destas questões, pretendeu-se levantar características aplicáveis à taxonomia e filogenia desta subtribo, e contribuir para o entendimento da biologia floral das espécies estudadas.

O texto desta tese está organizado em quatro capítulos e dois apêndices. O primeiro capítulo contempla o desenvolvimento floral e a vascularização das espécies-alvo; o segundo corresponde ao artigo proveniente do estudo dos coléteres presentes em órgãos florais. O terceiro capítulo é dedicado ao estudo das glândulas relacionadas à atração e recompensa de polinizadores, enquanto o quarto detalha o papel das células secretoras que constituem o rostelo e o tecido transmissor presentes no ginostêmio destas espécies. Durante o desenvolvimento desta tese, tornou-se necessária a elaboração e a validação de uma técnica viável para o estudo da reabsorção do néctar, resultando em dois artigos apresentados nos apêndices I e II.

Parte dos resultados do Capítulo 1 foi apresentada sob a forma de pôster na 18ª Reunião Anual do Instituto de Botânica (2011), recebendo o Prêmio "Frederico Carlos Hoehne", 1º lugar na categoria "Doutorado"; a parte restante foi apresentada, também sob a forma de pôster, no Monocots V – 5th International Conference on Comparative Biology of Monocotyledons (2013), recebendo o Prêmio "The Trevor Clifford Best Student Poster Award" de melhor pôster.

[1] Orquídeas que apresentam apenas uma antera fértil (Swamy 1948).

O desenvolvimento desta tese contou, além da orientação do Dr. Fábio de Barros, com a supervisão e/ou colaboração dos seguintes pesquisadores: Dra. Solange Cristina Mazzoni-Viveiros, Núcleo de Pesquisa em Anatomia (Instituto de Botânica), onde a maior parte da tese foi desenvolvida; Dra. Lisa M. Campbell, em período sanduíche realizado no The New York Botanical Garden (EUA); Profa. Dra. Cleusa Bona, em período sanduíche desenvolvido na Universidade Federal do Paraná (UFPR); Prof. Dr. Arthur R. Davis, em período sanduíche realizado na Univesity of Saskatoon (Saskatchewan, Canadá). A técnica de visualização da reabsorção de néctar foi desenvolvida com a colaboração do Prof. Dr. Emerson Ricardo Pansarin (Universidade de São Paulo, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto).

Capítulo 1

Comparative floral vascularization in Pleurothallidinae (Epidendroideae, Orchidaceae)

Cardoso-Gustavson P, Campbell LM, Mazzoni-Viveiros SC, Barros F

(a ser submetido para Botanical Journal of the Linnean Society)

ABSTRACT

Pleurothallidinae has been a focus of phylogenetic studies due to the poly- and paraphyletic nature of some genera. In light of a recent proposal of split Pleurothallidinae in two subtribes, in which two putative floral synapomorphies for the subtribe were recognized, we describe the floral vascularization with the aim that these data might clarify homologies and phylogenetic interpretation. We studied floral vascularization and described pivotal phases of the development of seven Pleurothallidinae species in seeking putative homologies. Species were selected to represent both early- and late-divergent clades of this subtribe and also two varieties (one teratological) of Laeliinae, a sister group of Pleurothallidinae, for comparison. We provide an anatomical definition of the articulation, a morphological synapomorphy of the subtribe, and also describe the nature of the "lateral windows" formed by the sepals of some Pleurothallidinae. The number of floral vascular traces is suppressed in both sterile whorls and more pronouncedly in the gynostemium. Divergences in gynostemium vascularization are not reflected in external morphological distinctions. Our results comparing Pleurothallidinae to the literature and to Laeliinae taxa indicate that the gynostemium vascularization is reduced and accompanied by the loss of stigma lobe traces. We hypothesize that this reduction is another morphological synapomorphy for this subtribe.

ADDITIONAL KEYWORDS: articulation – *Encyclia* – floral development – Laeliinae – placenta – postgenital coherence

INTRODUCTION

According to Rudall and Bateman (2002: 418), "floral vasculature in monocots is a complex subject urgently requiring a fresh overview". Especially considering the recent advances in understanding floral vascularization in some families, such as Velloziaceae (Sajo, Mello-Silva & Rudall, 2010), Liliaceae (Novikoff & Kazemirska, 2012), and Xyridaceae (Remizowa *et al.*, 2012), this aspect of the flower has provided essentialdata to clarify homologies and phylogenetic interpretations. Despite the large size of Orchidaceae, data on floral vascularization are scarce, restricted to the extensive description of 40 species belonging to 24 genera by Swamy (1948), in addition to detailed studies of *Apostasia* (Apostasioideae; Rao, 1969, 1973, 1974), *Cypripedium* (Cypripedoideae; St-Arnaud & Barabé, 1989), species of *Telipogon* (Epidendroideae, Telipogoninae; Pabón-Mora & González, 2008), and some discussion in the literature (Horta, 1935; Rudall & Bateman, 2002; Rudall, Perl & Bateman, 2013). Indeed, the support that floral vascularization may offer to clarify the relationships among orchid groups has been understudied and underestimated.

The subtribe Pleurothallidinae (Epidendroideae, Epidendreae), with 37 genera and ca. 4100 species (Pridgeon *et al.*, 2010; Borba *et al.*, 2011), is one of the largest of Orchidaceae and has been a focus of phylogenetic studies and taxonomic revision as a result of the polyphyletic and paraphyletic nature of some genera (Pridgeon & Chase, 2003; Pridgeon, Solano & Chase, 2001; Pridgeon *et al.*, 2010). This subtribe is also recognized as the largest orchid group pollinated by Diptera (Borba *et al.*, 2011), likely involving a deceit-syndrome, and some unique floral morphology features that were probably phenotypic responses to selection pressures imposed by pollinators (Pridgeon *et al.*, 2001). The currently accepted phylogeny was based only on the sequence data as both vegetative and floral morphology characters are considered highly homoplastic (Pridgeon *et al.*, 2001 and references therein). In addition, a small clade consisting of members formerly included in Laeliinae was incorporated to Pleurothallidinae (Pridgeon *et al.*, 2001). However, Borba *et al.* (2011) recently proposed

the splitting of Pleurothallidinae as presently recognized in two subtribes, one composed of the former circumscription of Pleurothallidinae (*sensu* Luer, 1986; Dressler, 1993) which would enable recognizing an already wide-known morphological synapomorphy of Pleurothallidinae (an articulation between the pedicel and ovary) and the two newly biological ones identified (self-incompatibility and myophily), and the other subtribe with the self-compatible, ornitophilous small clade presently incorporated. This study highlighted the relevance and the need for floral studies to improve the understanding of internal relationships in this subtribe.

In the course of an investigation of the gynostemia[1] in Pleurothallidinae, we observed some differences in the vascularization of the species examined that could be interpreted in a phylogenetic context, suggesting that structural details of floral vascularization may be important to systematic studies. In this study, we focused on a description of flower vascularization and some aspects of flower development in seven Pleurothallidinae species. Species chosen for this study represent both early- and late-divergent clades (Pridgeon *et al*. 2001, 2010).To evaluate Pleurothallidinae floral vasculature, particularly that of the gynostemium, we also described the vascular pattern in a sister group (van den Berg *et al.* 2005), Laeliinae (50 gen./1500 spp.; van den Berg *et al.* 2009).

MATERIAL AND METHODS

Plant material

Flowers of Pleurothallidinae (Fig. 1) and Laeliinae species were collected from plants cultivated in the living orchid collection of the "Núcleo de Pesquisa Orquidário do Estado", Instituto de Botânica (São Paulo, Brazil). All were collected in Brazil and are listed on Table

[1] In orchids, the gynostemium is commonly termed a 'column' (the latter being defined as a structure formed by union of stamens, style and stigma), though increasing number of authors reject the use of the term 'column' in favor of 'gynostemium'. The two structures are not strictly fully homologous, as the gynostemium does not include the stigma in all orchids. The term 'column' is also ambiguous in comparative terms: in the other angiosperm families it is used to describe a connate staminal structure in which the stamens are fused to each other to form a tube, but not adnate to gynoecium as in many Iridaceae (Rudall and Bateman 2002).

1, following their phylogenetic position, with provenance and accession numbers. *Acianthera fenestrata* ("number pending") was recently incorporated into the collection.

Table 1. Specimens of Pleurothallidinae and Laeliinae examined, their provenance and accession numbers.

Taxon	Origin and accession number
Pleurothallidinae	
Brachionidium-Octomeria clade	
Octomeria crassifolia Lindl.	Serra da Piedade, 5666; São Paulo, Campos do Jordão, 12096; Minas Gerais, Carangola, 14866.
Echinosepala-Barbosella clade	
Echinosepala aspasicensis (Rchb.f.) Pridgeon & M.W.Chase	Roraima, Serra da Neblina, 12306,16306.
Acianthera clade	
Acianthera aphthosa (Lindl.) Pridgeon & M.W.Chase	São Paulo, Reserva Ecológica do Morro do Diabo, 14854, 14855; Minas Gerais, Camanducaia, P1082.
Acianthera fenestrata (Barb.Rodr.) Pridgeon & M.W.Chase	number pending
Zootrophion-Trichosalpinx clade	
Anathallis obovata (Lindl.) Pridgeon & M.W.Chase	Paraná, Guaira, Região de Sete Quedas, 12599, 12611, 13299; São Paulo, Reserva Ecológica doMorro do Diabo, 14263, 14294; São Paulo, Boracéia, 14359; Minas Gerais, Carangola, 14961.
Zootrophion atropurpureum (Lindl.) Luer	São Paulo, Serra do Mar, 678; Minas Gerais, Carangola, 14998; São Paulo, Boracéia, 16936.
Remaining Pleurothallidinae clade	
Phloeophila nummularia (Rchb.f.) Garay	São Paulo, Bananal, 18235.
Laeliinae	
Encyclia patens Hook. var. *patens*	São Paulo, Cananéia, Ilha do Cardoso, 18119; São Paulo, Analândia, Cacheira da Bocaina, P3654.
Encyclia patens var. *serroniana* (Barb.Rodr.) Romanini & F.Barros	São Paulo, Cananéia, Ilha do Cardoso, 18097; São Paulo, Cananéia, Ilha do Cardoso, 18144.

The material was fixed in Karnovsky solution (Karnovsky, 1965, modified by Kraus & Arduin, 1997) for 24 h for morphological and anatomical purposes, washed, dehydrated, and stored in 70% ethanol.

Scanning electron microscopy (SEM)

To describe floral development buds at several stages of development were dissected under a Leica MZ 7.5 stereoscope, further dehydrated to 100% ethanol and rinsed in a

hexamethyldisilazane (HMDS) series (33.3%, 50.0%, and 66.6% v/v in 100% ethanol) and then three times in 100% HMDS for 1min each (Jeger *et al.*, 2009) to dry the material. Samples were mounted on stubs, coated with gold palladium in a Hummer 6.2 sputtering system (Anatech, Union City, California, USA) and viewed with a JSM-541OLV SEM (JEOL, Tokyo, Japan) at 10 kV. Digital images were edited using Adobe Photoshop version 7.0.

Light microscopy

To describe the floral vascularization pattern entire floral buds and mature flowers of Pleurothallidinae and excised Laeliinae gynostemia from flowers at anthesis were processed using standard methods for Paraplast® X-tra™ (Leica Biosystems, Richmond, Inc.), embedding and transverse and longitudinal sectioned serially (7–10 µm thickness) using a rotary microtome. Prepared sections were stained with safranin and astra blue (Gerlach, 1969) and mounted in Permount® (Fisher Scientific, Pittsburgh, PA, USA). Relevant features were observed and drawn under a Zeiss light microscope with an attached camera lucida. Drawings were scanned and then edited using Corel DRAW X6.

Comparisons of ontogenetic sequences in Pleurothallidinae and Laeliinae are based on previous reports by Kurzweil (1987) and Kurzweil & Kocyan (2002). The phylogenetic framework used for optimization and comparison used are those by Pridgeon & Chase (2003) and Pridgeon *et al.* (2001). Terminology and graphic representation of vascular traces (e.g., Fig. 12) are modified from Swamy (1948):

Outer whorl of perianth:	dorsal sepal – DS
	lateral sepals – LS
Inner whorl of perianth:	median petal (labellum) – L
	lateral petals –LP
Outer whorl of stamens:	median stamens – A1
	lateral stamens – A2 and A3
Inner whorl of stamens:	lateral staminodes* – a1 and a2
Whorl of stigma lobes:	median stigma lobe: G1
	lateral stigma lobes: G2 and G3

*Staminodes in all Pleurothallidinae species examined here and in *Encyclia patens* var. *patens*; fertile stamens in the teratological *E. patens* var. *serroniana.*

Encyclia patens Hook. has two taxa with different gynostemia structures. *Encyclia patens* var. *patens* possesses one fertile stamen and two large lateral wings, while *Encyclia patens* var. *serroniana*, a teratological variety, presents three fertile stamens, a regular median and two lateral ones (Cardoso-Gustavson, Mazzoni-Viveiros & Barros, 2012).

For better understanding, the traces in the region of the ovary were numbered, instead of named, with their reciprocal whorl because the traces of two or more whorls can be derived from a common origin.

RESULTS

FLOWER ORGANOGRAPHY

Flowers are resupinate, and the labellum is free from the lateral petals and articulated to the base of the gynostemium in all Pleurothallidinae described below.

Octomeria crassifolia (Fig. 1A) – The inflorescence is congested with flowers produced simultaneously in a fascicle. Flowers are tiny, yellowish, and membranaceous. The perianth consists of free alternated sepals, lateral petals and labellum.

Echinosepala aspasicensis (Fig. 1B, C) – The inflorescence consists of a single flower at the apex of the cauloma. Flowers are fleshy, purple, and are pubescent throughout. The lateral sepals are coalescent and free from the dorsal sepal. The lateral petals and labellum are diminutive.

Acianthera aphthosa (Fig. 1D) – The inflorescence is a very short congested raceme of two to five flowers that open one at a time. The flowers are fleshy, purple and pubescent throughout. The perianth is constituted by free alternate sepals, diminutive lateral petals and a labellum.

Acianthera fenestrata (Fig. 1E) – The inflorescence is a very short raceme with several small purple flowers. The sepals converge at their apices and bases, but are free in the middle, presenting two lateral "windows". The lateral petals and labellum are diminute.

Anathallis obovata (Fig. 1F) – The inflorescence is fasciculate with flowers at different stages of development. Flowers are yellowish and membranaceous as in those of *O. crassifolia*. The perianth consists of free, alternate sepals, lateral petals and a labellum.

Zootrophion atropurpureum (Fig. 1G) – The inflorescence consist in one or two flowers at the apex of the cauloma. The sepals have "windows" as described in *A. fenestrata* flowers, formed from the apices and bases converging, but free in the middle. The lateral petals and labellum are diminute.

Phloeophila nummularia (Fig. 1H) – The inflorescence is composed of a single flower arising from the apex of the cauloma. The sepals are externally pubescent, free and alternate with the lateral petals and labellum.

FLOWER VASCULARIZATION AND ANATOMICAL DETAILS

General aspects

Regardless of the stage of the flower (from very young buds to flowers at anthesis), the pattern of vascularization from the pedicel to the apex of the gynostemium and median portion of the perianth remained constant, with no secondary branches. All bundles and traces showed the same collateral orientation.

An articulation, a zone of constriction was observed between the pedicel and the ovary (Fig. 2A). In this region, the vascular bundles, clearly demarcated by sclerenchyma cells (Fig. 2B), became traces that then entered the ovary (Fig. 2C).

Octomeria crassifolia, *Acianthera fenestrata*, *Anathallis obovata* and *Phloeophila nummularia*

The flowers of these four species presented the same pattern of vascularization. Here we illustrate the vascularization pattern using *Anathallis obovata*. Six traces were present at the base of the ovary (Fig. 3A). The ramifications that supply both perianth and gynostemium initiated above the ovary, in the region of the insertion of the whorls. All the traces anastomosed at the same level. Trace 1 bifurcated just once, originating the central trace of the dorsal sepal and anther (A1). Traces 2 and 3 bifurcated twice, initially giving rise to one of the lateral sepals and, afterwards, to one of the lateral petals and lateral stigma lobes (G2 and G3). Traces 1', 2' and 3' bifurcated twice, each one originating one trace to the labellum, and, afterwards, to two traces of each lateral sepal (Fig. 3B–H).

Echinosepala aspasicensis

The major feature that differentiated this species from the others was the occurrence of supernumerary traces in the ovary (Fig. 4A, B). Six traces penetrated the pedicel (Fig. 5A). Each one of the ovary traces bifurcated at the base of this organ, totaling twelve traces (Fig. 5B). Each of two newly formed traces aligned in front of the placenta (see the asterisk in Fig 4B) such that every other trace possessed two additional small traces in front of it (Fig. 4, 5C). The other three traces directly supplied, without bifurcation, each each stigma lobe (G1–G3; Fig. 5D–I). Few differences were observed in the size of the collateral traces between the whorls, highlighting that traces to the placenta and stigma lobes that were smaller than all the remaining traces.

The major ramifications began above the ovary. Trace 1 was the first to bifurcate, originating the anther trace (A1) and the central trace of the dorsal sepal. Immediately following, traces 2 and 3 bifurcated four times, each one originating two traces that supplied the dorsal sepal, one in the lateral petal and one to each lateral staminode (a1 and a2). The bifurcations of trace 1' originated all the traces of the labellum, while trace 2' and 3' both gave rise to three traces of the lateral sepals and the two remaining petal traces (Fig. 5D–J).

Acianthera aphthosa

Six traces entered the axis of the flower (Fig. 6A). The ramifications that supply both the perianth and gynostemium started above the ovary, in the region of the insertion of the whorls. The first trace to bifurcate was 1, supplying the central trace of the dorsal sepal and the anther. In contrast to the other species examined here, *A. aphthosa* possessed a strong reduction in the number of traces that supplied the gynostemium, and only the trace to the anther (A1) was observed (Fig. 6G–I). Traces 2 and 3 bifurcated once, giving rise to one trace to the lateral sepal and another to the lateral petal. Traces 1', 2' and 3' split once into three traces, each originating a trace to the labellum and 2' and 3' each contributing two to the respective lateral sepals (Fig. 6B–I).

Zootrophion atropurpureum

Six traces were observed at the base of the ovary (Fig. 7A). The ramifications that supply both the perianth and the gynostemium initiated above the ovary, in the region of the insertion of the whorls. The median trace bifurcated just once, originating the central trace of the dorsal sepal and the anther (A1). Lateral traces 2 and 3 went through two consecutive bifurcations such that each of the first three traces originated another two, resulting in two traces that supplied the dorsal sepal: two in the lateral petals and one in each stigma (G2 and G3). Meanwhile, 1' bifurcated twice, giving rise to three traces to the labellum and two to the periphery of each lateral sepal. Traces 2' and 3' also underwent two bifurcations, each one originating four traces to the lateral sepals and the one remaining trace of the lateral petals (Fig. 7B–I).

Encyclia patens var. *patens* and *E. patens* var. *serroniana*

Encyclia patens var. *patens* was characterized by the presence of the anther (A1) and two conspicuous lateral gynostemium wings (a1 and a2; Fig. 8A–D). The teratological variety *E. patens* var. *serroniana* possessed three fertile anthers, the median (A1) and two lateral ones (a1 and a2; Fig. 8E–I). In both species each stigma lobe was vascularized by one trace (G1–G3; Fig. 8A–I). The floral ground plans of both species presented the same pattern, because the traces that correspond to the lateral wings and fertile lateral anthers occupied the same position (Fig. 8J, K).

Anatomical details of sepals and gynostemium

Observations of the transverse sections in the regions of the juxtaposition of sepals in *Zootrophion atropurpureum*, *A. fenestrata*, and also between the lateral sepals of *E. aspasicensis* (free at the apex) did not reveal fusion of the epidermis; the sepals were coherent and shared a cuticle (Fig. 9A–C). In the region where the lateral windows started to become visible a progressive detachment of the sepals was seen (Fig. 9B).

An overview of the vasculature throughout the gynostemium is shown in Fig. 10. Differences in the number of traces in the species were observed to start from the region of the style. In *E. aspasicensis*, six traces were observed in the region of the style, corresponding to the anther (A1), lateral staminodes (a1 and a2), and stigma lobes (G1–G3). Because the stigma traces ended up in the region of the stigmatic surface, only A1, a1 and a2 were observed at the base of the anther (Fig. 10B, C). In strong contrast to this species, *Acianthera aphthosa* exhibited an extreme reduction, presenting only a single trace corresponding to the anther (Fig. 10D, E). In the remaining species, three traces were observed in the style region (A1, G1 and G2), whereas from the stigmatic cavity region to the anther, only A1 was present (Fig. 10A, F–L).

FLOWER DEVELOPMENT

The differentiation of the floral primordium, the sequence of sepal and petal initiation and the early development of the anther were completely uniform among the species examined, essentially following the sequences described in Kurzweil (1987). Fusion between sepals or petals was not observed in the early stages. In all species, the staminodes a1 and a2 emerge as massive bulges at the base of the lateral petals in early ontogeny immediately after the initiation of the anther, but before the initiation of the median stigma lobe. The development is identical amongst the species until the point at which the anther, staminodes, and stigma lobes can be identified. The primordium of the median stigma lobe grows out just below the anther followed, by the lateral stigma lobes. Their apices protruded as separate bulges only in *Acianthera aphthosa* (Fig. 11A), while in the other species examined, they emerged completely fused, even reaching a "U"-shape as in *Echinosepala aspasicensis* (Fig. 11C). In both separated or connated stigmas, the apices of the lateral stigma lobes were laterally connected to the pillar-like staminodes (a1 and a2, Fig. 11A–C). Later in development, the apices of staminodes became situated just beside the anther, while the clinandrium wall, a structure that fully or partly surrounds the anther, continued development,

even covering the anther (Fig. 11D, E, G–I, K, L), except in *Octomeria crassifolia* and *Anthallis obovata* (Fig. 11F, J).

As a consequence of the flower's allometric growth, the anther, staminodes, and stigma lobes were elevated. The basal part of the median stigma lobe was also part of the receptive stigma of the adult gynostemium, wherein the rostellum was formed by the upper part of the median stigma lobe, becoming very prominent in *Echinosepala aspasicensis* (Fig. 11G). Together with the anther, the rostellum bent down to almost a horizontal position. At anthesis, the clinandrium wall around the anther was very prominent in *Acianthera fenestrata*, *A. aphthosa* and *Zootrophion atropurpureum*, making it difficult to delimit the staminodes and the clinandrium wall (Fig. 11G–I, K, L), because of their location (beside the anther). Moreover, below the anther of *E. aspasicensis*, *Z. atropurpureum* and *Phloeophila nummularia*, broad wing-like structures were developed below the anther during the expansion of the organs (Fig. 11D–E), as a unique structure from the base of the gynostemium to the top of the clinandrium (Fig. 11G, K, L). Otherwise, the staminodes remained as little protrusions beside the anther in *Octomeria crassifolia* and *Anathallis obovata*, in which neither the clinandrium wall nor a broad wing-like structure were seen (Fig. 11F, J).

Fig. 1. Flowers of Pleurothallidinae at anthesis. (A) *Octomeria crassifolia*, the perianth of similar membranaceous segments. (B) Fleshy, solitary flower of *Echinosepala aspasicensis*. (C) A few-flowered raceme of *Acianthera aphthosa*, note the fleshy perianth. (D) A congested inflorescence of *Acianthera fenestrata* near the substrate. (E) *Anathallis obovata* with a membranaceous perianth. (F) A solitary flower of *Zootrophion atropurpureum*. (G) Solitary flowers of *Phloeophila nummularia* close to the substrate. Arrows (D, F) indicate the opening of the lateral sepals. Scale bars: 1cm.

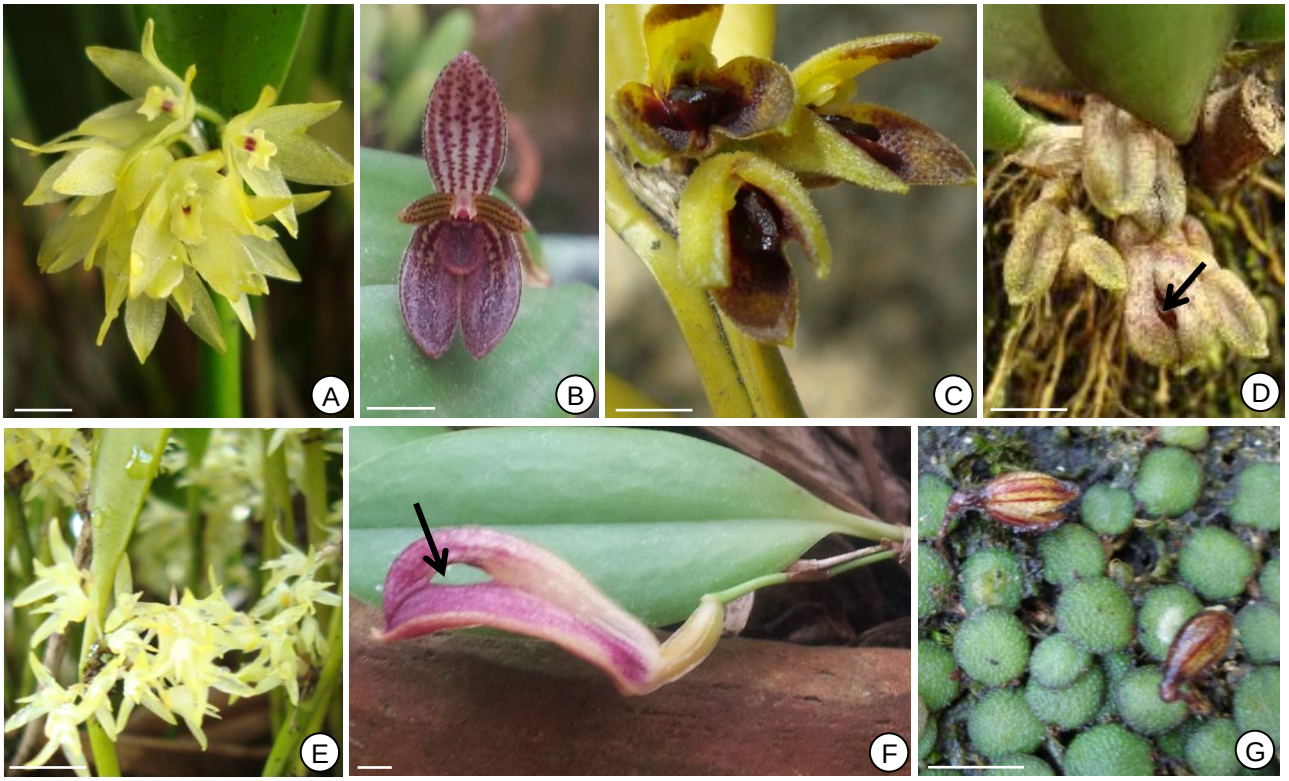
A
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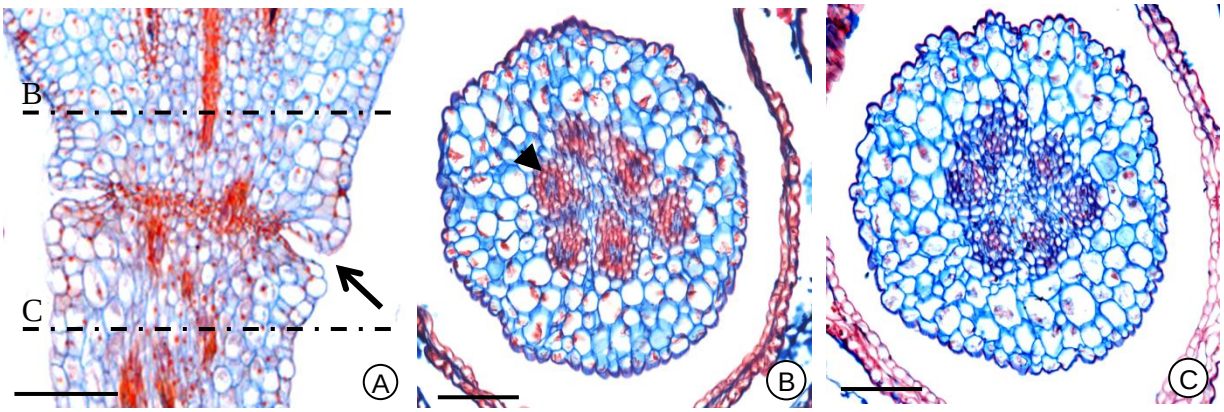
Fig. 2. Vascularization in the pedicel and base of the ovary in *Octomeria crassifolia*. (A) Longisection of the pedicel and ovary, showing the articulation (arrow) between these structures. Dashed lines correspond to the regions where transverse sections (B and C) were made. Vascular bundles below (B) and traces above (C) the articulation, both distinguished by the presence (arrowhead) and absence of sclerenchymatic sheath, respectively. Scale bars: 50µm.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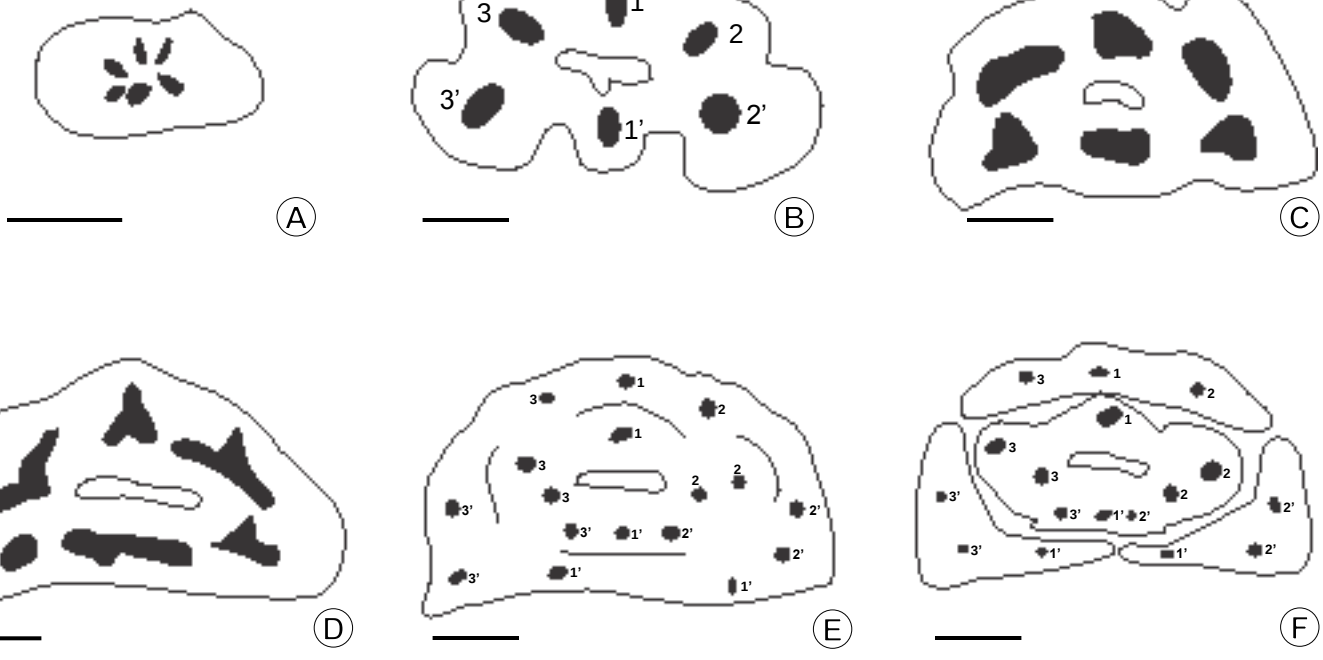
C

Fig. 3. Floral vascularization in *Anathallis obovata*. (A) The base of the ovary has six traces. (B–H) Acropetal series from the middle of the ovary to the anther. The traces broaden then bifurcate simultaneously. Trace 1 supplies both the dorsal sepal and the anther; 2 and 3 give rise to the remaining traces of the dorsal sepal, lateral petals and lateral stigma lobes. Traces 1', 2' and 3' supply both the labellum and lateral sepals. A1, anther trace; G2 and G3, lateral stigma lobe traces. Sepals in yellow, lateral petals and labellum in orange, and gynostemium in red. Scale bar: 100µm.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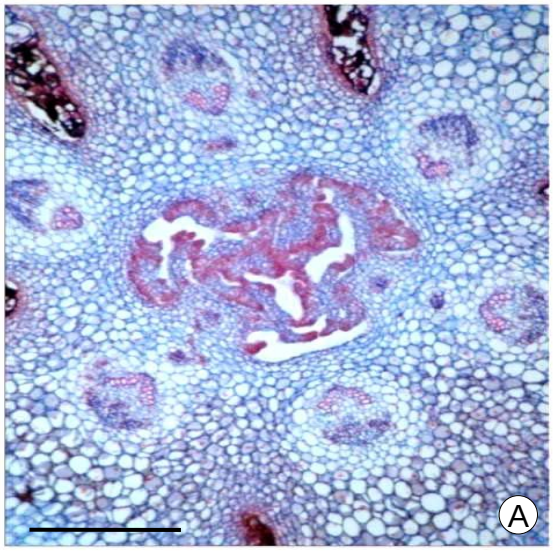
A1
H

Fig. 4. Transverse section of *Echinosepala aspasicensis* ovary. Light micrograph (A) and drawing (B) of vascular traces. Note the three innermost traces (*) that directly supply the placenta. Scale bars: 150µm (A), 100µm (B).


A

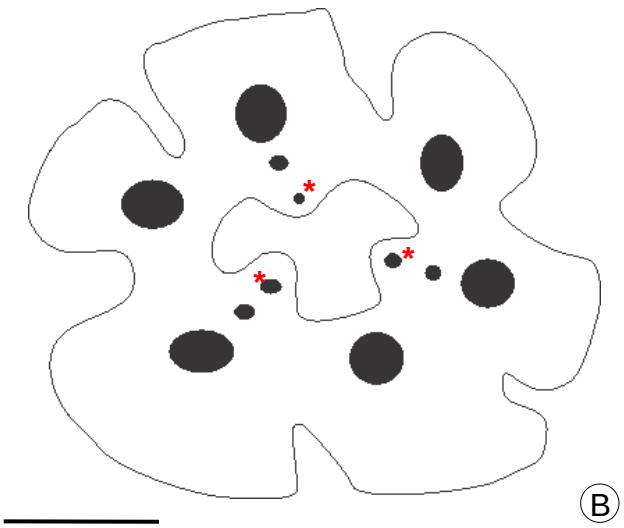
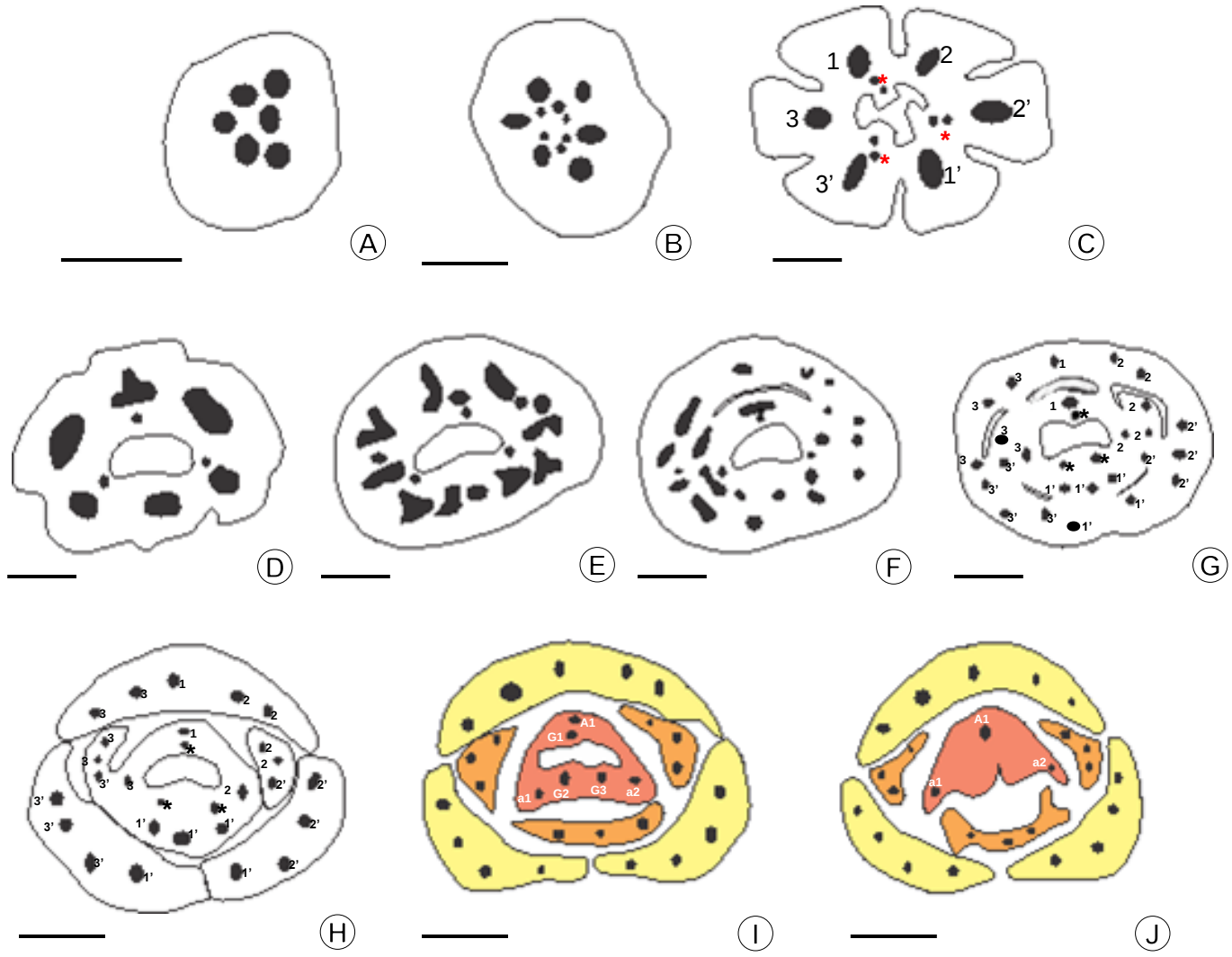

B

Fig. 5. Floral vascularization in *Echinosepala aspasicensis.* (A) The pedicel has six traces. (B) Two minor traces diverge from each main, resulting in twelve traces at the base of the ovary. (C–J) Acropetal series from the middle region of the ovary to the anther. Trace 1 is the first to bifurcate, supplying both the dorsal sepal and anther. Both traces 2 and 3 supply the remaining dorsal sepal traces, and each to one of the lateral petals and lateral staminodes. Bifurcations of 1' supply the labellum, while 2' and 3' give rise to the lateral sepals and the petals. Note that the traces of each lobe of the stigma come from the base of the ovary (*) with no further bifurcations. A1, anther trace; a1 and a2, lateral staminode traces; G1–G3, stigma lobe traces. Sepals in yellow, lateral petals and labellum in orange, and gynostemium in red. Scale bar: 100µm.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a2
I
J

Fig. 6. Floral vascularization in *Acianthera aphthosa*. (A) Six traces are observed at the base of the ovary. (B–I) Acropetal series from the middle region of the ovary to the anther. Trace 1 is the first to bifurcate, supplying both the dorsal sepal and anther, while 2 and 3 constitute the traces serve on the dorsal sepal and lateral petals. 1', 2' and 3' originate the traces serving on the labellum and lateral sepals. A1, anther trace. Sepals in yellow, lateral petals and labellum in orange, and gynostemium in red. Scale bar: 100µm.

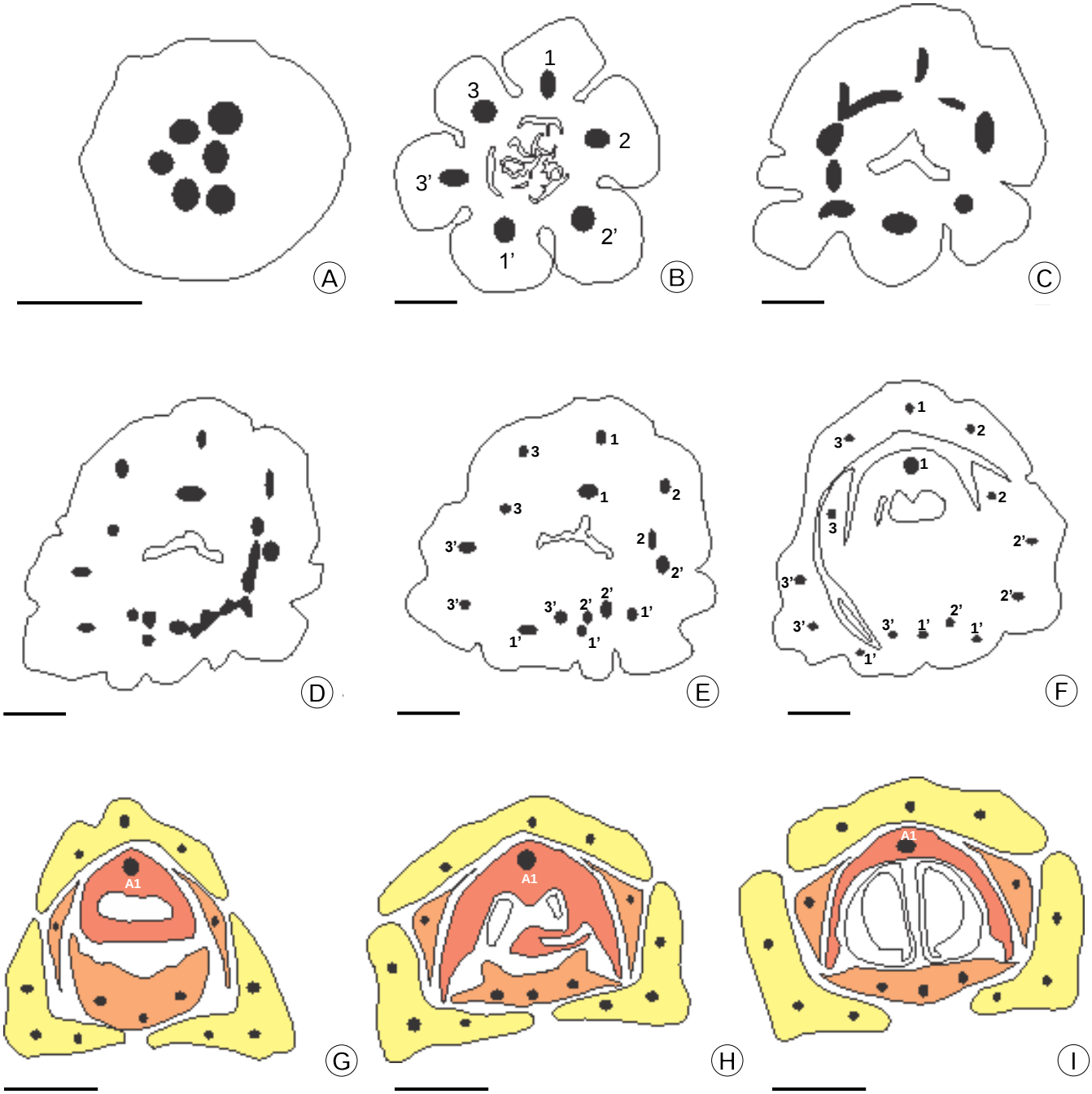
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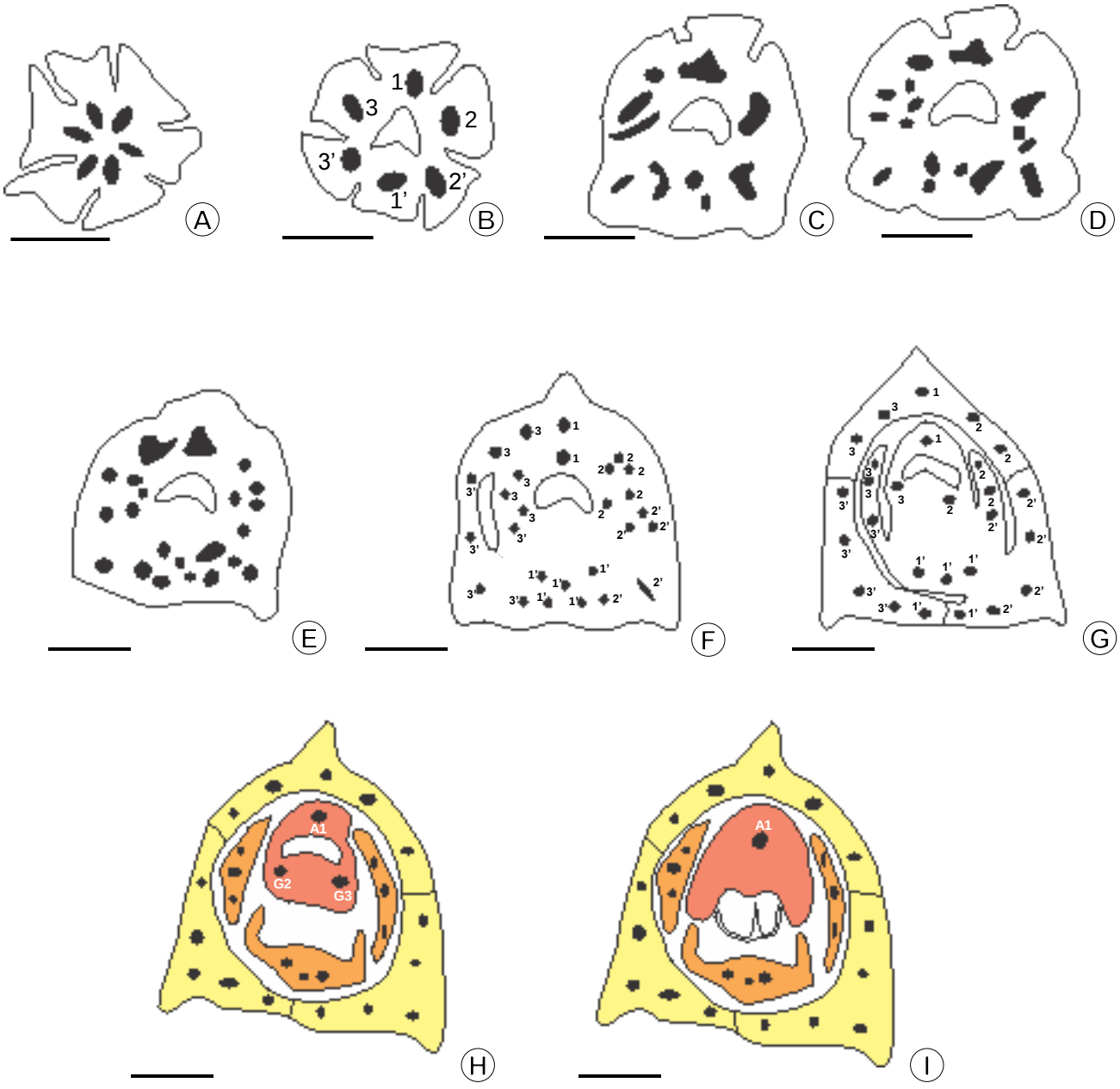
Fig. 7. Floral vascularization in *Zootrophion atropurpureum.* (A) Six traces are observed at the base of the ovary. (B–I) An acropetal series from the middle region of the ovary to the anther. Trace 1 is the last one to bifurcate, supplying both the dorsal sepal and anther, while 2 and 3 serve on the dorsal sepal, part of the lateral petals and the lateral stigma lobes. Trace 1’ supplies the labellum and part of the lateral sepals, while 2’ and 3’ supply the remaining traces of the lateral sepals and petals. A1, anther trace; G2 and G3, lateral stigma lobe traces. Sepals in yellow, lateral petals and labellum in orange, and gynostemium in red. Scale bars: 100µm.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A1
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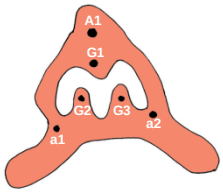
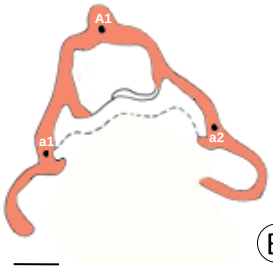
Fig. 8. Vascularization (A–I) and ground plans (J, K) of the gynostemia in two resupinate Laeliinae flowers. (A–D, J) *Encyclia patens* var. *patens.* (E–I, K) *Encyclia patens* var. *serroniana.* Dashed lines in G, H represent the lateral fertile stamens. A1, anther trace; a1 and a2, lateral stamens or staminode traces; G1–G3, stigma lobe traces. Scale bars: 100μm.



A



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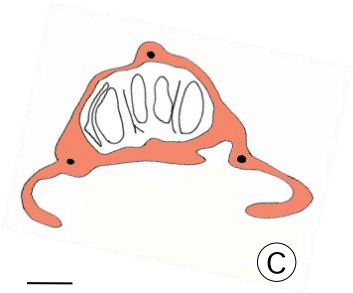

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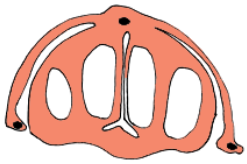

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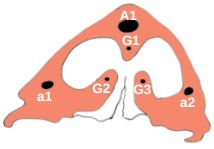

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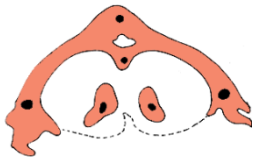

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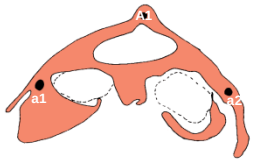

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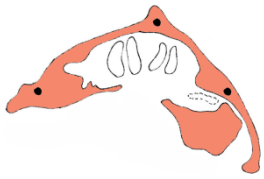
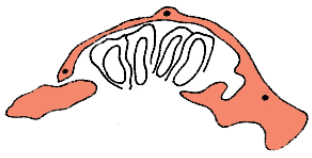


H



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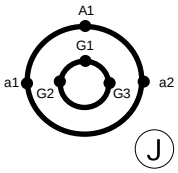

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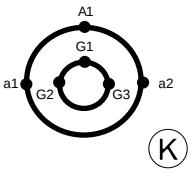
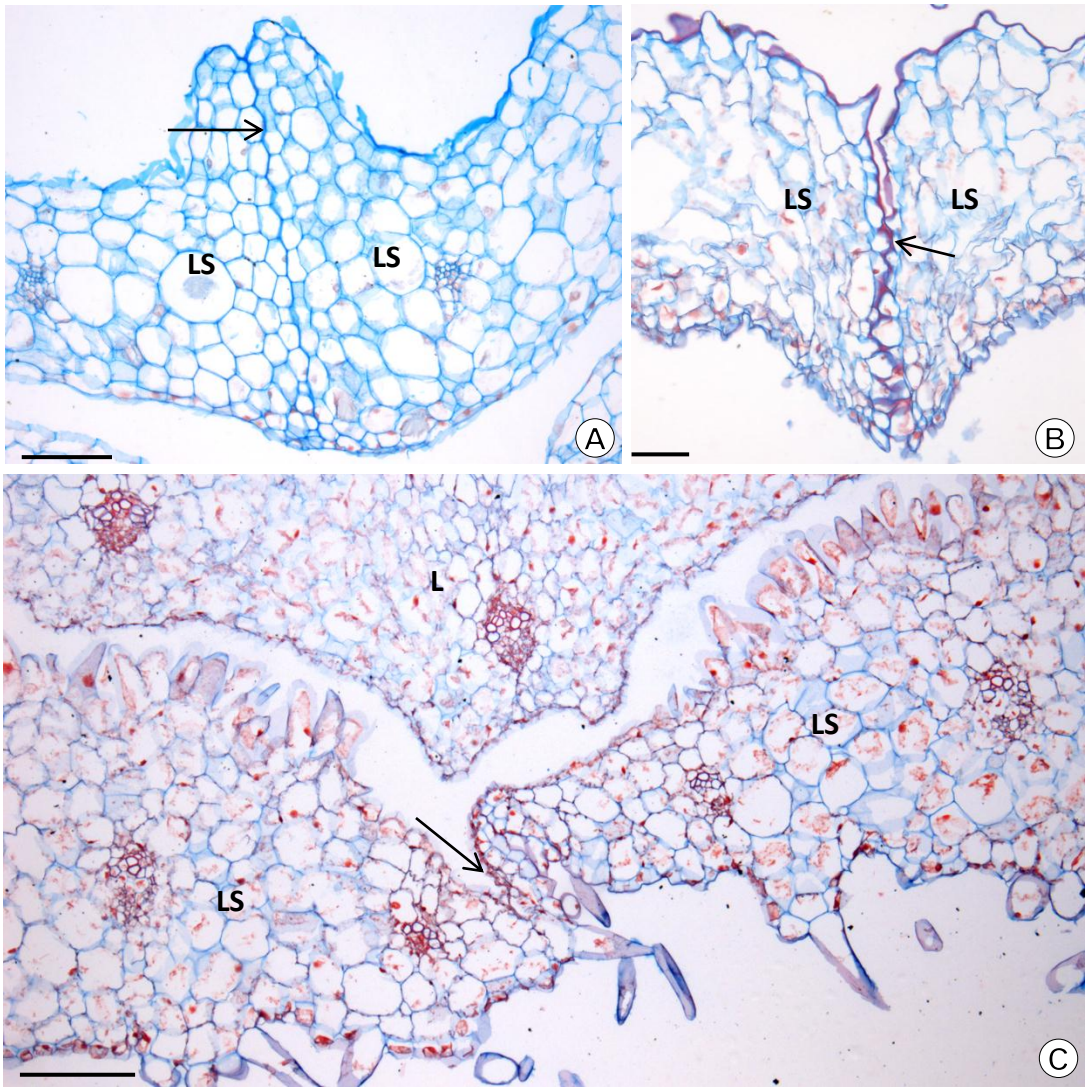


K

Fig. 9. Details of the coherence between sepals. (A) *Zootrophion atropurpureum*: both sepals are coherent, sharing a common cuticle (arrow). (B–C) In both *Acianthera fenestrata* (B) and *Echinosepala aspasicensis*, the coherence occurs between the papillae; note that they appear to fit together (arrows). LS, lateral sepals; L, labellum. Scale bars: 150µm.



C

Fig. 10. Overview of gynostemium vascularization in Pleurothallidinae. (A) *Octomeria crassifolia*. (B, C) *Echinosepala aspasicensis*. (D, E) *Acianthera aphthosa*. (F, G) *Acianthera fenestrata*. (H) *Anathallis obovata*. (I–J) *Zootrophion atropurpureum*. (K, L) *Phloeophila nummularia*. (A, B, D, I) Style region. (F, K) Stigma region. (C, E, G, H, J, L) Anther region. Flowers shown resupinate. A1, anther trace; a1 and a2, lateral staminode traces; G1–G3, stigma lobe traces. Scale bars: 100µm (B–G, I, J), 75µm (A, H, K, L).

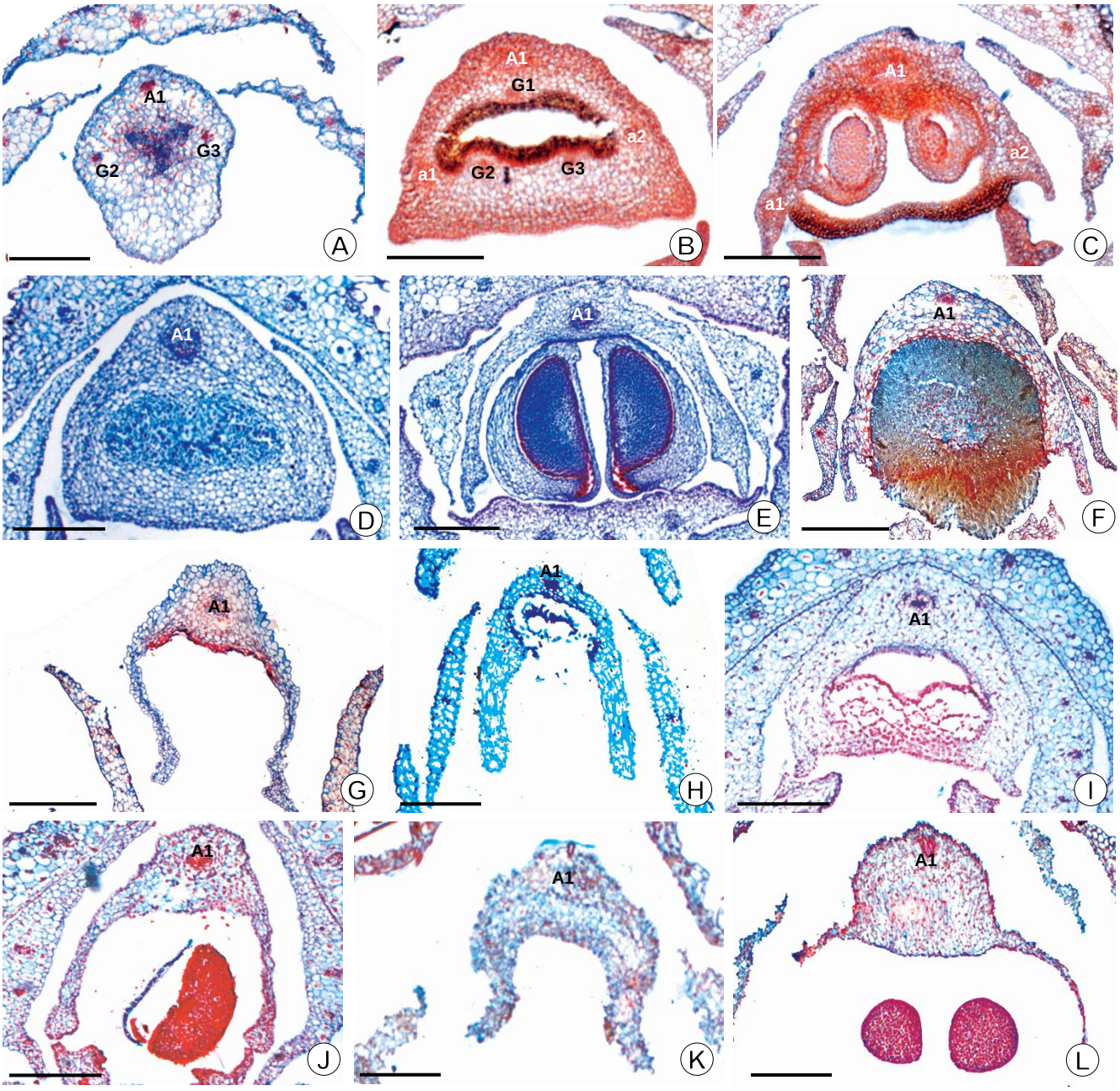

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Fig. 11. Summary of the main stages of floral development of Pleurothallidinae species as viewed with SEM. (A, B) *Octomeria crassifolia*. (C–E) *Echinosepala aspasicensis.* (F, G) *Acianthera aphthosa.* (H–J) *Acianthera* fenestrata. Note the absence of the pollinia (star). (K, L) *Anathallis obovata*. (M, N) *Zootrophion atropurpureum*. (O) *Phloeophila nummularia.* A1, anther; a1 and a2, lateral staminodes; ls, lateral stigma lobes; ms, median stigma lobe; r, rostellum; ss, stigmatic surface; arrows, lateral wings of the gynostemium; arrowheads, clinandrium wall. Scale bars: 100µm.

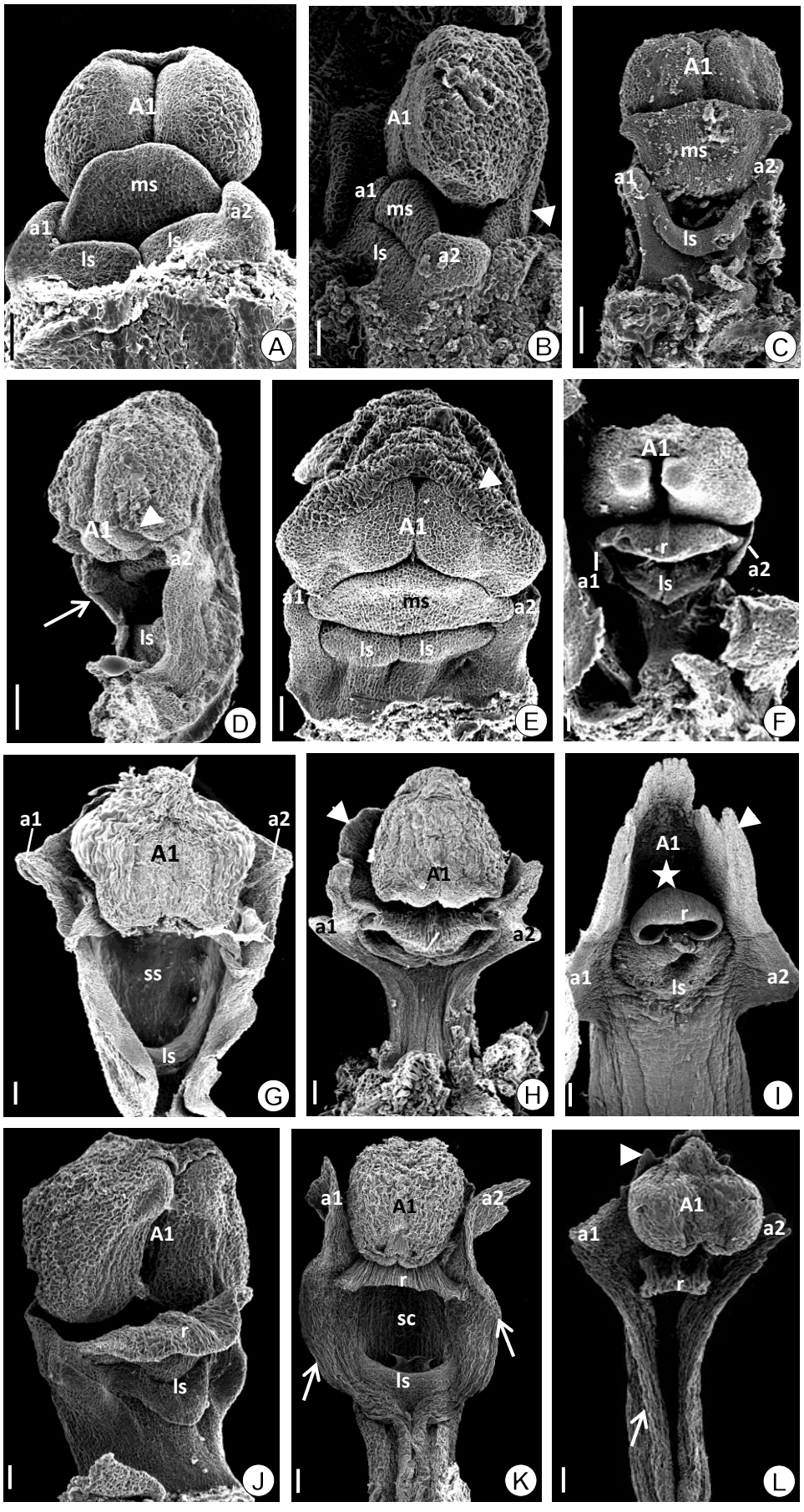
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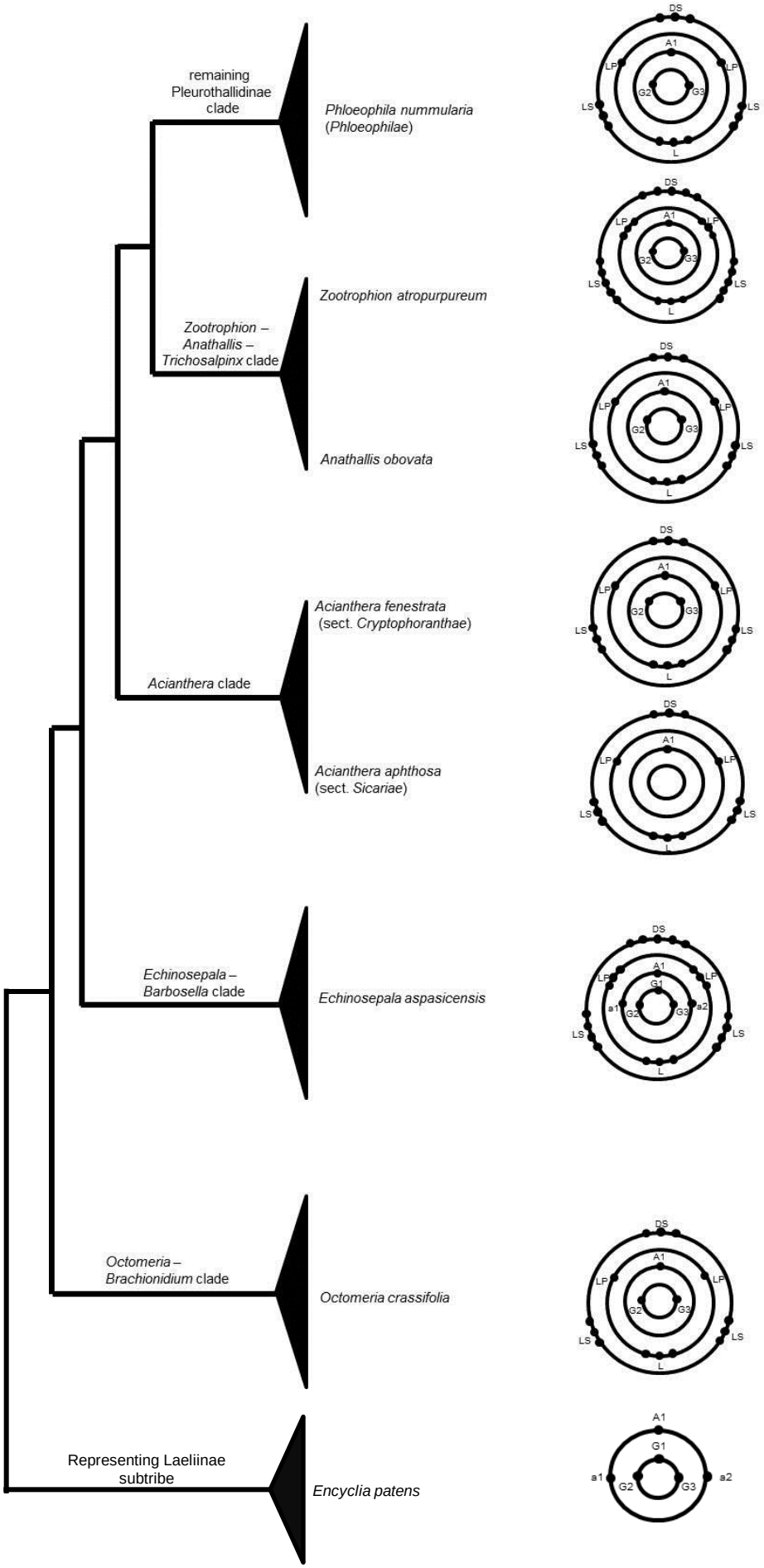
Fig. 12. Simplified phylogeny of Pleurothallidinae based on Pridgeon & Chase (2003) and Pridgeon *et al.* (2001), showing currently recognized clades and representative floral ground plans of the vascular traces. DS, dorsal sepal; LS, lateral sepals; L, labellum (median petal); LP, lateral petals; A1, anther; a1 and a2, lateral staminodes; G1–G3, stigma lobes. Note that only the vascularization of the gynostemium is represented in Laeliinae species.


remaining Pleurothallidinae clade
Phloeophila nummularia (Phloeophilae)
Zootrophion – Anathallis – Trichosalpinx clade
Zootrophion atropurpureum
Anathallis obovata
Acianthera clade
Acianthera fenestrata (sect. Cryptophoranthae)
Acianthera aphthosa (sect. Sicariae)
Echinosepala – Barbosella clade
Echinosepala aspasicensis
Octomeria – Brachionidium clade
Octomeria crassifolia
Representing Laeliinae subtribe
Encyclia patens
DS
A1
LP
G2
G3
LS
L
G1
a1
a2

DISCUSSION

Articulation and ovary

A morphological synapomorphy regarded as uniting Pleurothallidinae in older classifications (*sensu* Luer, 1986; Dressler, 1993) was an articulation between the ovary and pedicel. This structure has not been anatomically characterized. Because our observations indicate that this narrow region is where each pedicel vascular bundle loses its sclerenchyma sheath and then enters the base of the ovary, we designated the articulation as the region in which the vascular bundles become traces that will supply the flower.

The articulation is ubiquitous in Pleurothallidinae, apart from the small clade containing the genera *Dilomilis*, *Neocogniauxia*, and *Tomzanonia*, currently included in this subtribe based on molecular evidence (Pridgeon *et al.*, 2001, 2010). The recent suggestion of Borba et al. (2011) to split Pleurothallidinae *s.l.* (as presently recognized) into two subtribes (one with the previous delimitation of Luer 1986 and another with the small clade recently incorporated) recuperates the articulation as a morphological synapomorphy, and include two putative biological synapomorphies, self-incompatibility and myophily. In this scenario, it should be highlighted that both biological and morphological synapomorphies were recognized in flowers, in spite of the critical taxonomic discussion of the use of floral features in phylogenetic analysis of this subtribe (Neyland, Urbatsch & Pridgeon, 1995; Pridgeon *et al.*, 2001, 2010; Pridgeon & Chase, 2003).

The pattern of the number of bundles that serve the pedicel and ovary is the same in all of the species examined here. The major difference occurs in the ovary vascularization in *Echinosepala aspasicensis*, in which three traces directly supply the placenta. Although Swamy (1948) earlier reported an absence of a direct supply to the placenta in orchids, a variable number of these traces (up to ten) are described in *Apostasia* (Rao, 1973) and *Cypripedium* species (St-Arnaud & Barabé, 1989). Considering the available data, it is possible to hypothesize that the direct supply of the placenta is a homoplasic character in

Orchidaceae, restricted to basal groups and to species that retains ancestral character such as *E. aspasicensis.*

Perianth

Most of the species examined here possess free sepals, petals, and labellum. The exceptions are the sepals of *Acianthera fenestrata* and *Zootrophion atropurpureum* that never open, except from an aperture between the median and lateral sepals during anthesis, and the lateral sepals of *E. aspasicensis,* free just at the apex. In these species descriptions (see Pridgeon *et al.*, 2010, and references therein), the juxtaposition is termed as a connation, or fusion, within a whorl. Our studies, however, demonstrate that, in fact, a postgenital coherence (see details of this terminology in El Ottra, Pirani & Endress, 2013) occurs in sepal whorl, in which the sepals only share their cuticle, i.e., the absence of fusion of epidermal cells or adjacent tissues. In the region of the lateral windows, only a progressive detachment of the cuticle over the epidermis of the sepal is observed.

Fig. 12 plots the floral ground plans of the Pleurothallidinae species examined here in the optimized phylogeny of this subtribe (based in both Pridgeon & Chase (2003) and Pridgeon *et al.* (2001)), in which it is possible to comparatively visualize the divergences in the number of traces from sepals, petals and labellum. Three major features can be seen in the perianth: (*i*) in all species the labellum possessed three traces; (*ii*) in most species there was a reduction in the number of lateral petal traces concomitant with three traces to the sepals; (*iii*) the occurrence of supernumerary traces in the sepals of *E. aspasicensis* and *Z. atropurpureum.* These differences, however, are not reflected in the development of the perianth that is homogeneous and essentially follows the detailed description of Kurzweil (1987). The supernumerary traces observed can be related to the relative size of these flowers compared to the other species examined here (but see St-Arnaud & Barabé (1989) in which no correlation between vascular complexity, number of traces and size of flowers was found). The reduction

in the number of the perianth traces may be an evolutionary apomorphy of Pleurothallidinae such as observed in the prominent reduction in the traces of the gynostemium (see discussion below).

Gynostemium

The gynostemium exhibited the greatest differences in the number of traces. All the Pleurothallidinae species examined here possess the trace corresponding to the anther (A1) which also has the same origin as the median region of the dorsal sepal trace (1). Except in *Echinosepala aspasicensis*, the traces that correspond to both the inner whorl of stamens (or staminodes, a1 and a2) and the median stigma lobe (G1) were suppressed. In *Octomeria crassifolia*, *Acianthera fenestrata*, *Anathallis obovata*, *Zootrophion atropurpureum*, and *Phloeophila nummularia*, the lateral stigma lobes (G2 and G3) originate from traces 2 and 3, respectively. In *Acianthera aphthosa*, an extreme suppression of the traces was observed, in which only the anther trace is observed towards the gynostemium. Unlike this suppression pattern, the gynostemium of *Echinosepala aspasicensis* exhibits six traces: one belonging to the anther, two corresponding to the staminodes and the three from the stigma lobes (G1, G2 and G3). Both a1 and a2 are supplied by traces 2 and 3, which, interestingly, are the same traces that originate G2 and G3 in other species, while the stigma traces are derived from bifurcations at the base of the ovary, continuing through the ovary and penetrating the gynostemium until reaching the stigma lobes with no further bifurcations, a unique pattern observed in the present study.

Three patterns of vascularization of the gynostemium were observed in the Pleurothallidinae examined; three wide divergent points stand: (*i*) the occurrence of six traces in *E. aspasicensis*, (*ii*) the suppression of trace G1 in all the remaining species and (*iii*) a total suppression of the stigma traces in *A. aphthosa*. Based on these observations, we carried out

developmental studies to ascertain if differences in the vascular pattern are reflected in the floral development.

Unlike vascularization, all the species examined here possess the same pattern of development, in which large bulges interpreted as staminode primordia in Epidendroideae species (Kurzweil, 1987; Kurzweil & Kocyan, 2002) are observed. Auricles, lateral projections of the gynostemium nonhomologous to the lateral wings of Pleurothallidinae, are also initiated earlier and remain unvascularized in the musk-orchid *Herminium monorchis* (Rudall *et al.*, 2013) such that the staminodes do not necessarily exhibit vascularization. In *Octomeria crassifolia* and *Anathallis obovata*, the staminodes are completely incorporated into the structure of the gynostemium, and also the clinandrium wall is not well developed, resulting in a very simple and similar gynostemia. Interestingly, *E. aspasicensis* and *Z. atropurpureum* present extremely elaborate lateral wings also fused with a well-developed clinandrium wall, although both exhibit strong differences in relation to the number of traces in the gynostemium. Thus, our evidence indicates that divergences in gynostemium vascularization do not reflect external morphological distinctions. This fact highlights the conflict between flower ontogeny and anatomy with respect to organ homologies, apparently common in orchids (Rudall *et al.*, 2013), making it difficult to interpret evolutionary events.

Considering the homoplasy of the floral traits resulting from the high convergence with pollinators (Pridgeon *et al.*, 2001, 2010 and references therein), it can be argued that the vascular differences detected here could be related to pollinator pressure. It was suggested that the deceit-pollination of some Pleurothallidinae could "literally toss the pollinator against the column for deposition and extraction of pollinia (though this has never been observed in nature)" (Pridgeon *et al.*, 2001: 2303), indicating an "aggressive" behavior of pollinator. Thus, the existence of a broader gynostemium, i.e., one with wide lateral wings, might be associated with the behavior of the pollinator. In this context, the occurrence of several traces

containing xylem would confer a high rigidity against such pollinator behavior due to the lignified constituents of the cells that compose this tissue.

Floral biology studies in large subtribe Pleurothallidinae are still incipient. Studies that coupling observations of both pollinators' behavior in the flower with morphology and vascularization of *Echinosepala aspasicensis* will broaden our understanding of pollination in the subtribe. In *Acianthera aphthosa*, the pollinators are predacious flies of *Idana* sp. (Diptera: Otitidae) that visit the flower to find their prey (Ribeiro, Kohler & Boelter, 2006), and in nectarless *Acianthera* species, small female flies of Chloropidae and Phoridae (both Diptera) lay their eggs in the flowers (Borba & Semir, 2001). However, *Octomeria crassifolia* flies (Sciaridae, Diptera) looking for nectar do not present aggressive behavior (Barbosa, Melo & Borba, 2009). Speculating from these few data, it is possible to relate species that present a simple gynostemium wing to a "non-aggressive" behavior of the pollinator.

Alternatively, as in *Dichorisandra thyrsiflora* C.K.Mikan (Commelinaceae), the presence or absence of traces may be determined by phylogenetic, rather than functional, constraints (Hardy, Stevenson & Kiss, 2000). Another point corroborates this phylogenetic alternative. Specifically, despite the strong convergence found between myophilous Pleurothallidinae and *Bulbophyllum* species (Dressler, 1993; Azevedo *et al*., 2007), the pattern of gynostemium vascularization is completely incongruent, with the latter presenting the common pattern of vascularization described in monandrous orchids (Swamy, 1948). Although a loss or suppression of adaxial stamens from both outer and inner androecial whorls defines Orchidaceae (Rudall & Bateman, 2002), the degree of suppression, including of traces to the stigma, has been never mentioned before in this family. A clear tendency toward reduction in the number of the traces that supply the gynostemium is observed in Fig. 12. In *O. crassifolia* – here representing the sister group of the remaining members of the older delimitation of Pleurothallidinae (*sensu* Luer, 1986; Dressler, 1993) – a suppression in the traces of the stigma and staminodes is observed, while flower vascularization in *E.*

aspasicensis is probably a plesiomorphic condition, because it retains the more complex vascularization observed in *Telipogon* species (Telipogoninae, Maxillarieae, Epidendroideae; Pabón-Mora & González, 2008). Similarly, for additional evidence of the phylogenetic alternative hypothesis, we also studied the vascularization in two taxa of Laeliinae for two reasons: (*i*) this subtribe is sister to the whole larger Pleurothallidinae (Pridgeon *et al.*, 2001), which is early-divergent according to the phylogeny of Epidendroideae (van den Berg *et al.*, 2005), and (*ii*) *Encyclia patens* var. *patens* possesses a broad lateral wing that resembles the structure found in Pleurothallidinae species, while *E. patens* var. *serroniana* is teratological, exhibiting three fertile anthers (Cardoso-Gustavson *et al.*, 2012). In this scenario, we hypothesized that some reduction pattern would be observed. Surprisingly, both taxa present the same pattern of vascularization, indicating that the lateral wings are indeed staminodes. Moreover, they possess six traces supplying the gynostemium (i.e., no suppression), similar to *E. aspasicensis*, *Telipogon* sp. (Pabón-Mora & González, 2008) and other monandrous orchids (Swamy, 1948), indicating that this pattern of reduction might emerge only in Pleurothallidinae. Our comparison with Laeliinae and other Epidendroideae (as literature data from *Telipogon* species) can be performed since presumably they share a common genetic background and genetic mechanisms controlling gynostemium arrangement (Pabón-Mora & González, 2008).

When considering a pattern that reflects a tendency toward reduction observed inside Pleurothallidinae, culminating in a maximum of reduction in *Acianthera aphthosa*, we hypothesize that this reduction is a derived character exclusive to Pleurothallidinae and may perhaps be another morphological synapomorphy of the group, when considering the splitting of Pleurothallidinae as proposed by Borba *et al.* (2011), besides the occurrence of the articulation between pedicel and ovary. Therefore, we strongly encourage vascularization studies not only in more species of Pleurothallidinae but even in other Epidendroideae subtribes to corroborate our hypothesis.

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Capítulo 2

Floral colleters in Pleurothallidinae (Epidendroideae: Orchidaceae)

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Floral colleters in Pleurothallidinae (Epidendroideae: Orchidaceae)[1]

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- *Premise of the study:* The term colleter is applied to trichomes or emergences positioned close to developing vegetative and floral meristems that secrete a sticky, mucilaginous, and/or lipophilic exudate. Several ecological functions are attributed to these glands, but none are exclusive to colleters. Patterns of morphology and distribution of colleters may be valuable for systematics and phylogeny, especially concerning problematic and large groups such as the subtribe Pleurothallidinae, and are also essential to understand the evolution of these glands in Orchidaceae as a whole.
- *Methods:* We used scanning electron and light microscopy to examine the structure and occurrence of trichomes on bracts and sepals and in the invaginations of the external ovary wall (IEOW) in flowers in several developmental stages from species in seven genera.
- *Key results:* The exudate was composed of polysaccharides, lipophilic, and phenolic compounds. Colleters were secretory only during the development of floral organs, except for the glands in the IEOW that were also active in flowers at anthesis. After the secretory phase, fungal hyphae were found penetrating senescent trichomes.
- *Conclusions:* Trichome-like colleters seem to be a widespread character in Epidendroideae, and digitiform colleters are possibly the common type in this subfamily. Mucilage from IEOW colleters may aid in the establishment of symbiotic fungi necessary for seed germination. The presence of colleters in the IEOW may be a case of homeoheterotopy, in which extrafloral nectaries that produce simple sugar-based secretions (as in other orchid species) have changed to glands that produce secretions with complex polysaccharides, as in Pleurothallidinae.

Key words: bracts; colleter; Epidendroideae; mucilage glands; Oncidiinae; Orchidaceae; orchids; Pleurothallidinae; ovary wall; sepals; trichomes.

Traditionally, the term colleter has been applied to glands present in vegetative and/or floral buds that secrete a sticky substance primarily containing mucilage, lipophilic compounds, or both (Fahn, 1979; Mangalan et al., 1990; Thomas, 1991). Terpenes, phenolic compounds, proteins, and alkaloids have also been reported as minor constituents of colleter exudate (Fahn, 1979; Thomas, 1991; Klein et al., 2004; Miguel et al., 2006; Castro and Demarco, 2008; Gonzalez and Tarragó, 2009; Coelho et al., 2013; Mayer et al., 2013). Morphologically these glands appear as multicellular trichomes or emergences that originate from both epidermal and subepidermal tissues (Fahn, 1979).

The main functions attributed to colleters are to lubricate and protect the developing meristem against desiccation (Thomas, 1991; Paiva, 2009; Paiva and Martins, 2011; Mayer et al., 2011), to promote symbiotic associations with bacteria (Lersten, 1975), and to act as a chemical barrier against insects and microorganisms (Miguel et al., 2006; Muravnik and Kostina, 2011). A recent study showed that flowers from mutagenically decaffeinated coffee plants (Decaffito) exhibit comparatively minimal production and secretion of exudate by colleters and, consequently, their flowers open precociously because there was no adhesive compound to keep the sterile organs closed, indicating that the molecular mechanisms controlling production of exudate and flower development/anthesis are related (Mayer et al., 2013). The report extended the known taxonomic distribution of colleters and highlights the interdependence of structures in the processes that comprise flowering.

A functional concept was proposed (Mayer et al., 2011, 2013) in which only the chemical composition and function of the gland are required to characterize it as a colleter. However, the precise function to characterize a gland as a colleter was not defined by the authors. Characters are assumed as homologous among different taxa if they are present in the common ancestor, allowing a common ontogeny to be inferred; thus, homology does not require the structures in question to be identical in form or function (Baum, 2013). Assuming that function is not the best criterion to access homology, the use of a "functional concept" would be difficult to apply in taxonomic and phylogenetic studies. It is important to point out that the role of colleters in plants (secretion of mucilage and/or lipophilic compounds) should not be confused with ecological functions attributed to these glands. Indeed, the features

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that have been used to define a gland as a colleter are the chemical composition of the exudate (mucilage and/or lipophilic compounds), the morphology of the glands, their close proximity to developing meristems, and secretory activity during the development of vegetative or floral organs. These developmental and structural traits govern the functions of the colleters and are under selective pressure.

Colleters are sometimes misinterpreted as "extrafloral nectaries" or "resin glands" (Thomas, 1991). This misinterpretation is likely due to the morphological and chemical similarities between glands, because the main metabolic difference between colleters and nectaries is the production of high-molecular-weight polysaccharides and simple sugars, respectively. On the other hand, lipophilic compounds constituting part or all of colleter exudate has led to their characterization as resin glands. Further, a morphological transition between the extrafloral nectaries and colleters on leaves has been documented in some species of *Piriqueta* (Turneraceae) (González, 1998), highlighting the problematic characterization of some glands and the need for precise circumscriptions. Glands that share the same ontogeny, morphology, and position can produce different exudates, serving completely different functions. According to Vogel (1997), pre-existing secretory units of a more basic function, such as hydathodes, may be transformed to nectaries through reorganization of ultrastructure and metabolism. These "substitutive nectaries" (Vogel, 1997) probably have a homeoheterotopic origin (Baum and Donoghue, 2002) similar to that of the aforementioned colleters derived from extrafloral nectaries.

In the eudicot families Apocynaceae and Rubiaceae, colleters have great taxonomic importance: their morphology, distribution, and number have been employed by many authors (e.g., Lersten, 1975; Simões and Kinoshita, 2002; Klein et al., 2004; Simões et al., 2006; Moraes et al., 2011). However, there are few reports on the taxonomic relevance of the presence and morphological characteristics of colleters in Orchidaceae and in Asparagales as a whole. Colleters and their potential systematic significance may be overlooked due to the application of several terms for these structures, including "intravaginal squamules" (Dahlgren and Clifford, 1982; Paiva, 2009; Mayer et al., 2011). The anatomy of these glands has rarely been studied in monocots, making the interpretation of their ecological role and evolutionary history difficult. In Orchidaceae, description of colleters is limited to subtribe Oncidiinae (Epidendroideae, Cymbidieae), where they occur on the bracts of *Rodriguezia venusta* (Leitão and Cortelazzo, 2008) and on reproductive and vegetative organs of *Oncidium flexuosum* (Mayer et al., 2011).

The subtribe Pleurothallidinae (Epidendroideae, Epidendreae), with 37 genera and ca. 4100 species (Pridgeon et al., 2010), is one of the largest of Orchidaceae and has been a focus of phylogenetic studies and taxonomic revision due to the polyphyletic and paraphyletic nature of some genera (Pridgeon and Chase, 2001; Pridgeon et al., 2001, 2010). During an investigation of flowers of this subtribe, we realized that detailed morphological studies on the glands present on ovaries and sterile organs were needed to evaluate their systematic usefulness. New evidence about the functional relevance of glandular exudates during floral development (Mayer et al., 2013) prompted a detailed study of these glands to ascertain possible secretory functions beyond that of lubricating the developing buds in Pleurothallidinae flowers.

The primary goal of this study was to document the occurrence of colleters on floral organs in Pleurothallidinae and to characterize the morphology, distribution, and histochemistry of the main secretory compounds in these glands.

MATERIALS AND METHODS

***Taxon sampling*—**The species selected for this study (Table 1) are all native to Brazil and were chosen to represent both early- and late-diverging clades of Pleurothallidinae (Pridgeon et al., 2001, 2010). For anatomical and morphological studies, inflorescences and flowers were collected from live plants cultivated at the Instituto de Botânica, São Paulo, Brazil. Vouchers were deposited in the herbarium of the Instituto de Botânica (SP) and are also housed in the spirit collection of The New York Botanical Garden.

***Anatomical studies*—**Material was fixed in Karnovsky's glutaraldehyde solution (modified by Kraus and Arduin, 1997) for 24 h or in formalin-ferrous sulphate, a fixative for detecting total phenolic compounds (Johansen, 1940), for 48 h. The samples were washed in the same buffer in which they were prepared, dehydrated in an ethanol series, and stored in 70% ethanol.

For scanning electron microscopy (SEM), flowers were further dehydrated to 100% ethanol and rinsed in a hexamethyldisilazane (HMDS) series (33.3, 50.0, and 66.6% v/v in 100% ethanol) and then three times in 100% HMDS for 1 min each (Jeger et al., 2009) to dry the material. Samples were mounted on stubs, coated with gold palladium in a Hummer 6.2 sputtering system (Anatech,

TABLE 1. Living collection data for Pleurothallidinae specimens investigated in this study. All plants in cultivation at the Núcleo de Pesquisa Orquidário do Estado, Instituto de Botânica (São Paulo, Brazil).

Species	Collection site (Brazil)	Accession number
Acianthera aphthosa (Lindl.) Pridgeon & M.W. Chase	Reserva Ecológica do Morro do Diabo, SP; Camanducaia, MG	14854, 14855; P1082
Acianthera fenestrata (Barb.Rodr.) Barb. Rodr.		without number
Anathallis obovata (Lindl.) Pridgeon & M.W. Chase	Guaira, Região de Sete Quedas, PR; Reserva Ecológica do Morro do Diabo, SP; Boracéia, SP; Carangola, MG	12599 (01426009[a]), 12611 (01427243[a]), 13299 (01426010[a]); 14263 (01426010[a]), 14294 (01426015[a]); 14359 (01426018[a])
Echinosepala aspasicensis (Rchb.f.) Pridgeon & M.W. Chase	Serra da Neblina, Parima, RR	12306, 16306
Octomeria crassifolia Lindl.	Serra da Piedade, Caeté, MG; Campos do Jordão, SP; Carangola, MG	5666; 12096; 14866
Phloeophila nummularia (Rchb.f.) Garay	Bananal, SP	18235
Zootrophion atropurpureum (Lindl.) Luer	Serra do Mar, SP; Carangola, MG; Boracéia, SP	678; 14998; 16936

[a] Barcode number of liquid-preserved specimen housed in the Plant Research Laboratory, New York Botanical Garden.

Union City, California, USA) and viewed with a JSM-541OLV SEM (JEOL, Tokyo, Japan) at 10 kV. Digital images were edited using Adobe (San Jose, California, USA) Photoshop version 7.0.

For light microscopy observations, material was embedded using standard methods for Paraplast (Fisher Healthcare, Houston, Texas, USA) or Leica historesin (Heraeus Kulzer, Hanau, Germany) and serially sectioned at 5–7 μm thickness. Sections prepared with Paraplast were stained with safranin and astra blue (Gerlach, 1969) and mounted in Permount (Fisher Scientific, Pittsburgh, Pennsylvania, USA); sections prepared with resin were stained with toluidine blue O (Sakai, 1973) and mounted in water. Observations and digital images were acquired with an Olympus BX53 compound microscope equipped with an Olympus Q-Color 5 digital camera and Image Pro Express 6.3 software.

Paraplast-embedded material was used for histochemical analyses as follows: periodic acid-Schiff's reaction (PAS) to detect 1,2-glycol groups present in total polysaccharides (McManus, 1948), tannic acid-ferric chloride for mucilage (Pizzolato and Lillie, 1973), ruthenium red for acid mucilage (Gregory and Baas, 1989), Sudan black B to identify the lipophilic compounds (Pearse, 1985) and copper acetate/rubeanic acid for fatty acids (Ganter and Jollès, 1969). Simultaneous standard control procedures (McManus, 1948; Pizzolato and Lillie, 1973; High, 1984) were performed.

RESULTS

Bracts (involucral and floral) and sepals—In early development, colleters were present on the adaxial surface of involucral bracts and on the abaxial surface of floral bracts as digitiform (or finger-like) uniseriate trichomes (Figs. 1A–C; 2A, C, D, F; 3A, B), except for *Zootrophion atropurpureum,* which possessed capitate trichomes, that were also found on the abaxial surface of the sepals (Fig. 3C, D). These glands produced a relatively abundant exudate in most species (Figs. 2, 3) that sometimes impeded observation of the glands (Fig. 2B, E). Before anthesis, secretion stopped and the colleters abscised (Fig. 3E).

Invaginations of the external ovary wall (IEOW)—In all species examined, there were six grooves along the external ovary wall in which varying amounts of colleters were located (Fig. 4A–O). Nonglandular trichomes occurred outside the invagination (Fig. 4C, E, I), except in *Octomeria crassifolia* and *Anathallis obovata*, which were glabrous (Fig. 4A, K) and possessed stomata (Fig. 4A, M).

Colleters in the IEOW of *Acianthera apthosa*, *Anathallis obovata*, *Echinosepala aspasicensis*, *Octomeria crassifolia*, and *Phloeophila nummularia* were digitiform trichomes (Figs. 1D, E; 4B, D, F, J, L), whereas in *Zootrophion atropurpureum* the glands were capitate (Figs. 1G, H; 4O) and appeared as conical trichomes in *Acianthera fenestrata* (Figs. 1F; 4H). Depending on the species, a more or less abundant, slightly viscous secretory substance was visible by SEM (Fig. 1D, H). Fungal hyphae were observed in *A. fenestrata* (Fig. 1F).

Digitiform and capitate colleters were bicellular and uniseriate with a clear distinction between head and stalk cells. Digitiform colleters possessed an apical cell that was longer and sometimes slightly larger in diameter than the stalk cell due to the accumulation of exudate (Fig. 4J). Capitate colleters possessed a globular apical cell (Fig. 4O). The conical colleters on *Acianthera fenestrata* were unicellular and distally tapered (Fig. 4H).

In all organs examined, colleter exudate that accumulated in the extracellular space had an emulsion-like or dense appearance (Figs. 2; 3C, D; 4B, D, F, H, J, L, O). Sometimes profuse exudate obscured the glands. The apical secretory cell developed a subcuticular space, a gap between the cell wall and the detached cuticle, that characterized all the glands in the secretory phase (Fig. 4J), except for the conical trichomes of *Acianthera fenestrata.* In the latter species, neither a subcuticular space nor a detached cuticle were observed, only an abundant exudate in extracellular spaces (Fig. 4H).

Unlike the colleters on the bracts and sepals, those on the IEOW were only active during the development of the flower and stopped secreting after anthesis and then abscised. The IEOW become obscured during fruit formation and abscised colleters were not easily observed (Fig. 5A); however, fungal hyphae were found growing into these senescent glands (Fig. 5B).

Histochemical analyses—The histochemical tests performed on glands situated on the bracts, sepals, and IEOW yielded the same results, except for the conical trichomes present in the IEOW of *Acianthera fenestrata* in which the exudate consisted only of mucilage (Fig. 6C). The exudate found in extracellular spaces and also inside the secretory cells of capitate and digitiform trichomes consisted of mucilage, lipophilic substances, and phenolic compounds. The hydrophilic fraction was detected by the PAS reaction (Fig. 6A–C), ruthenium red (Fig. 6D), and tannic acid/ferric chloride (Fig. 6E–G), whereas the lipophilic fraction was evidenced by the Sudan black B (Fig. 6H–K) and copper acetate/rubeanic acid (Fig. 6L) reactions. Total phenolic compounds were visualized using the formalin-ferrous sulphate fixative (Fig. 6M–O).

DISCUSSION

All of the glands described here are considered to be colleters due to their location, the primarily mucilaginous nature of their exudate, and their secretory phase coinciding with floral development. We speculate on colleter function based on their position; however, it is important to highlight that none of these ecological roles are unique to colleters. Thus, we do not apply the functional concept of colleters (Mayer et al., 2011, 2013) here.

The major component of mucilage is pectin. Pectins are largely acidic polysaccharides that form gels in the extracellular matrix and are present in all cell walls as well as in mucilage (Western et al., 2000). The complex structure of the pectic polysaccharides and the large number of genes required for plants to synthesize pectin suggest that pectins have multiple functions in plant growth and development (Ridley et al., 2001). Although molecular and biochemical studies have focused on mucilage produced in the seed coat (Haughn and Western, 2012), no molecular studies have addressed colleter mucilage. Colleter secretions in Rubiaceae and Aquifoliaceae (Klein et al., 2004; Miguel et al., 2006; Gonzalez and Tarragó, 2009; Coelho et al., 2013) are chemically composed of polysaccharides and proteins that are involved in protection against microorganisms (Miguel et al., 2006) and are distinct enough to mark taxonomic groups (Gonzalez and Tarragó, 2009). In addition to the presence and location of colleters, chemical characterization of the exudate may prove valuable to our understanding of Pleurothallidinae relationships.

Bracts (involucral and floral) and sepals—Colleters occur on the adaxial surface of involucral bracts of other Epidendroideae: in *Rodriguezia venusta* (Oncidiinae) (Leitão and Cortelazzo, 2008), *Oncidium flexuosum* (Oncidiinae) (Mayer et al., 2011), and *Epidendrum* spp. (P. Cardoso-Gustavson, unpublished data). Their exudate lubricates the growing floral bud so that it can slide over the bract as it expands (Leitão and Cortelazzo,

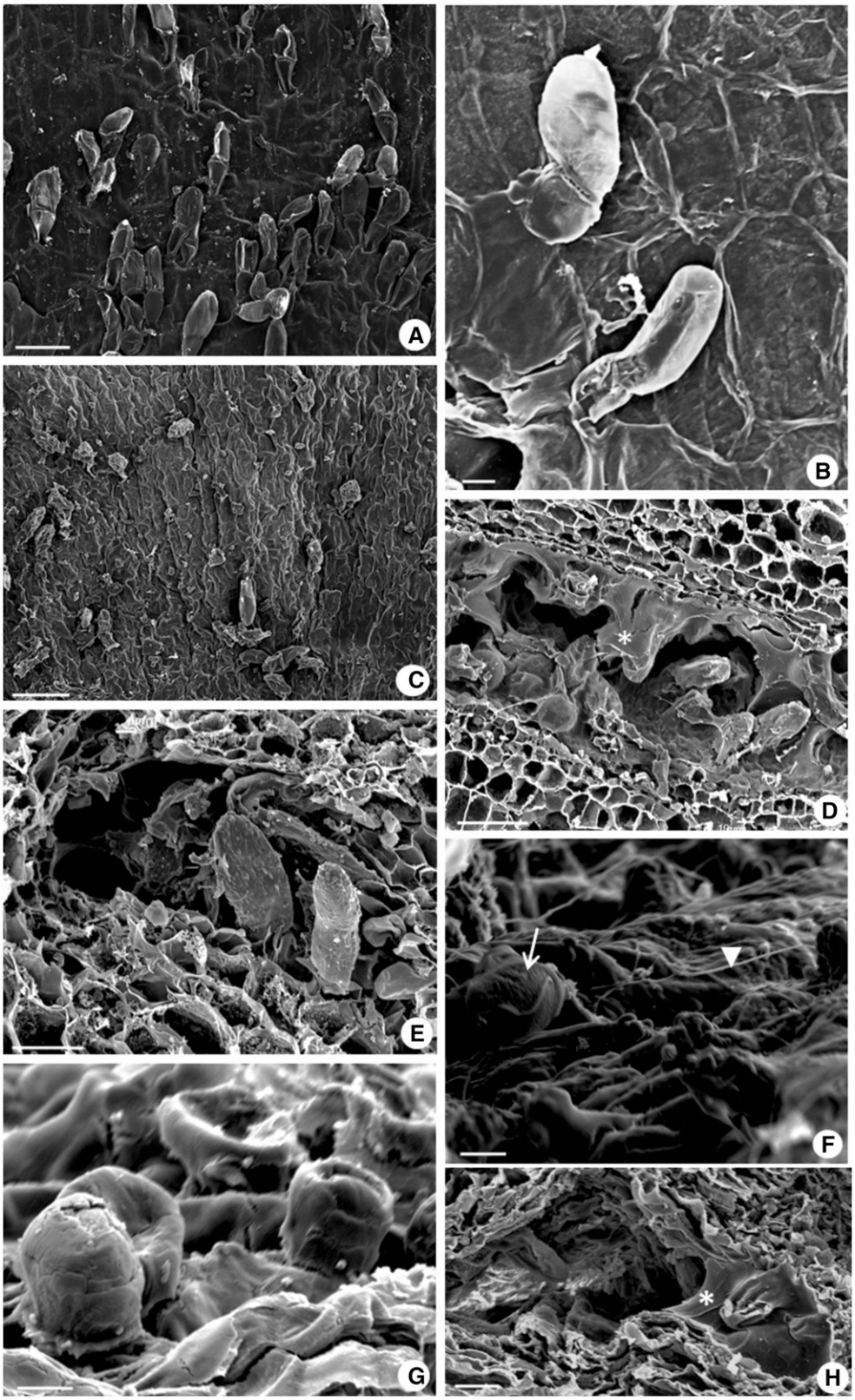

Fig. 1. Scanning electron micrographs of colleters on the (A, B) involucral and (C) floral bracts and in the (D–H) invagination of the external ovary wall of Pleurothallidinae species. (A–E) Digitiform trichomes in (A) *Octomeria crassifolia*, (B) *Anathallis obovata*, (C, D) *Echinosepala aspasicensis* and (E) *Acianthera aphthosa*. (D, H) Note the profuse exudate (*). (F) Conical trichomes (arrow) in *Acianthera fenestrata*. Note the fungal hyphae (arrowhead). (G, H) Capitate trichomes, *Zootrophion atropurpureum.* Scale bars = 100 μm (A, B), 10 μm (C–H).

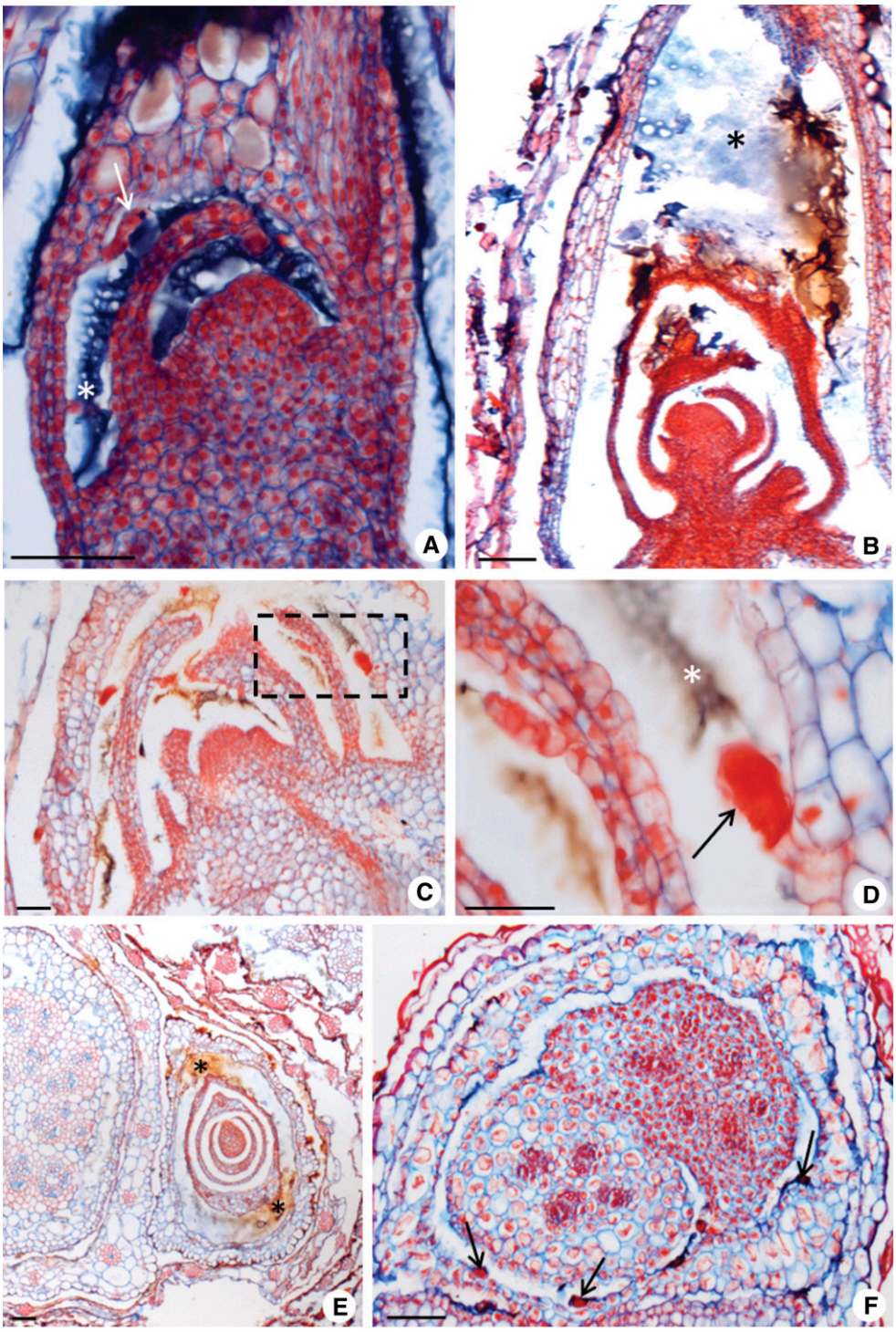

Fig. 2. (A–D) Longitudinal and (E, F) transverse sections of developing meristems in Pleurothallidinae. (A) *Octomeria crassifolia.* (B) *Echinosepala aspasicensis.* (C, D) *Zootrophion atropurpureum.* (E, F) *Acianthera aphthosa.* Colleters (arrows in A, D, F) in the bracts secrete a profuse exudate (* in A, D) that impede the observation of the glands (E). (D) Detail of the colleters from the region indicated in (C): the apical cell (arrow) is intensely colored with safranin, an indication of a secretory phase. Scale bars = 25 μm.

2008). Colleters commonly occur exclusively on the adaxial surface of plant organs (Paiva, 2009) because it is the zone of contact between developing structures. As far as we know, this is the first report of colleters present on the abaxial surface of floral bracts and sepals.

There does not seem to be a direct relationship between secretion volumes and the types of tissues that constitute the colleters or their size (Paiva, 2009). However, the presence of colleters on both bracts and sepals could increase the number of secretory glands and thus the volume of mucilage. Secondary

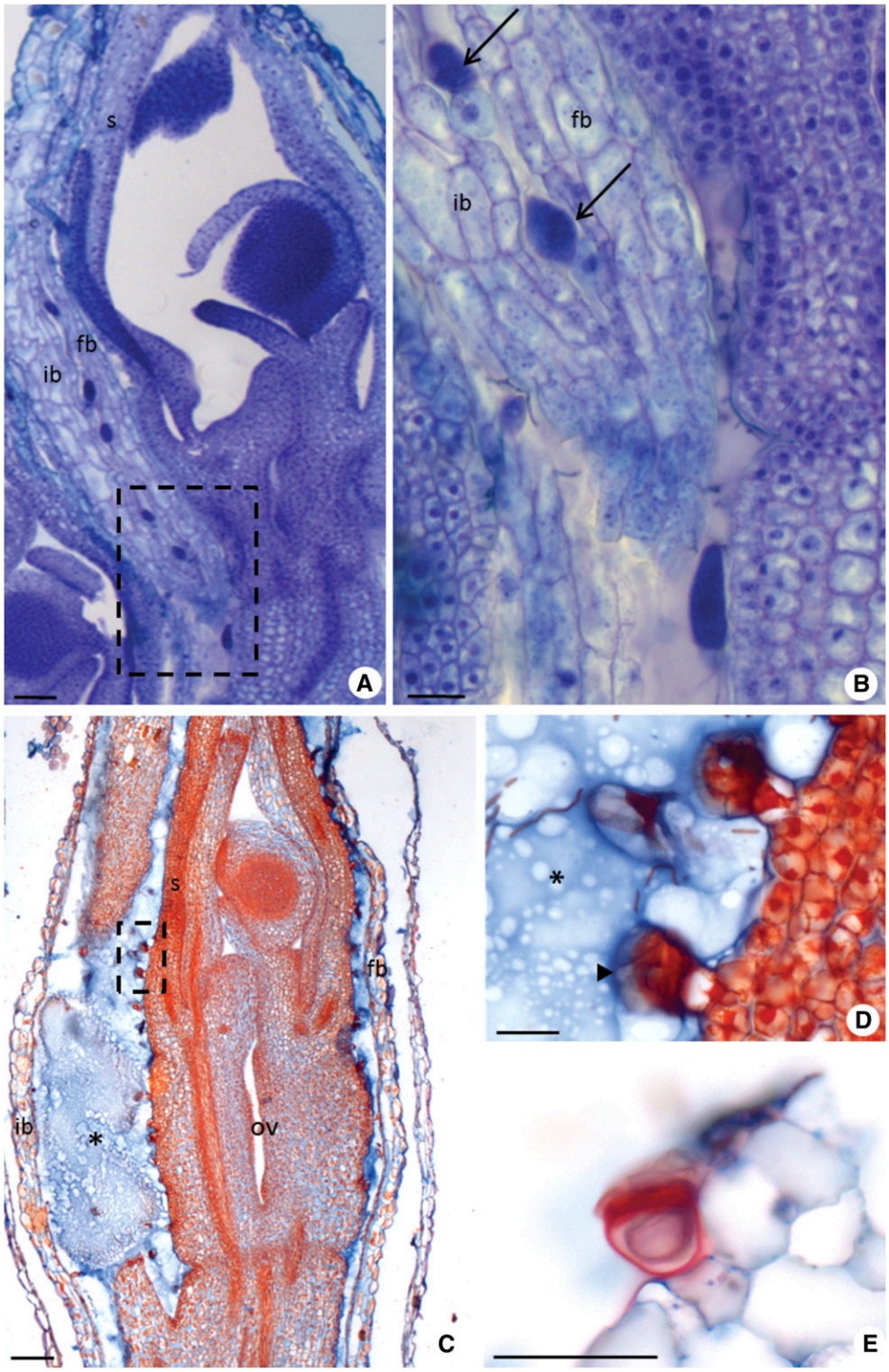

Fig. 3. Longitudinal sections of young flowers of (A, B) *Anathallis obovata* and (C–E) *Zootrophion atropurpureum*. (A) Apex of the flower with the colleters outlined. (B) Detail of the region indicated in (A): colleters (arrows) on the adaxial surface of involucral bract (ib) and on the abaxial surface of floral bract (fb). (C) Profuse exudate (*) released by glands on adaxial surface of involucral bract (ib) and on abaxial surface of sepals (s). (D) Detail of region indicated in (C): cuticle detachment (arrowhead) indicative of secretory phase of the gland. (E) Postsecretory phase of the colleter on abaxial surface of sepal of opened flower: note the abscised apical secretory cell. (ov) ovary. Scale bars = 25 μm.

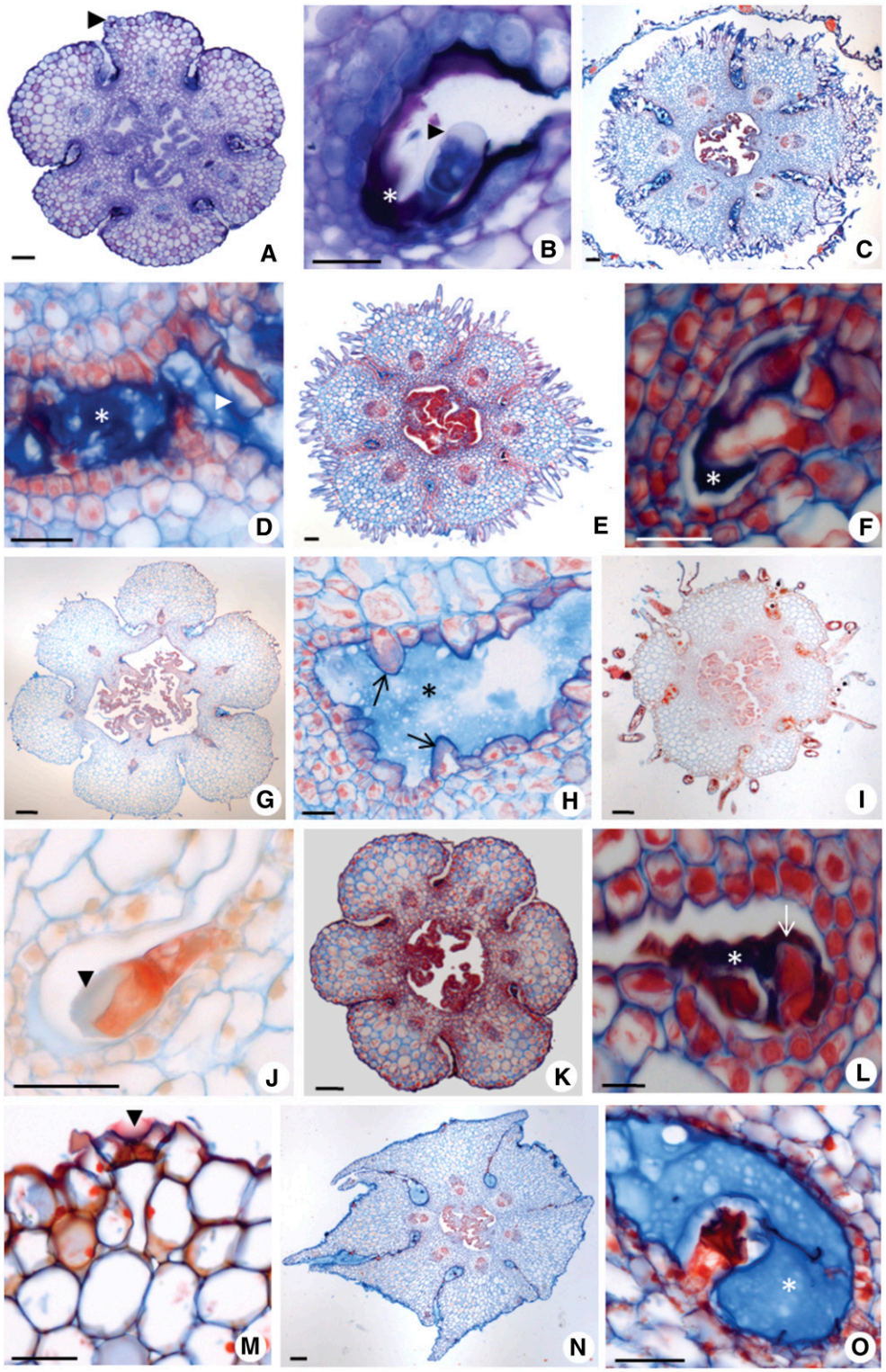

Fig. 4. Transverse sections of the ovary of Pleurothallidinae species. (A, B) *Octomeria crassifolia*. (C, D) *Echinosepala aspasicensis*. (E, F) *Acianthera aphthosa*. (G, H) *Acianthera fenestrata*. (I, J) *Phloeophila nummularia*. (K, M) *Anathallis obovata*. (N, O) *Zootrophion atropurpureum*. (A, M) Presence of stomata (arrowheads) in the epidermis. (A–L, N, O) Secretory trichomes (arrows) in the invaginations of the external wall of ovary secrete a profuse exudate (*). (B, D, J) Cuticle detachment (arrowhead) caused by the accumulation of the exudate in the subcuticular space. Scale bars = 25 μm.

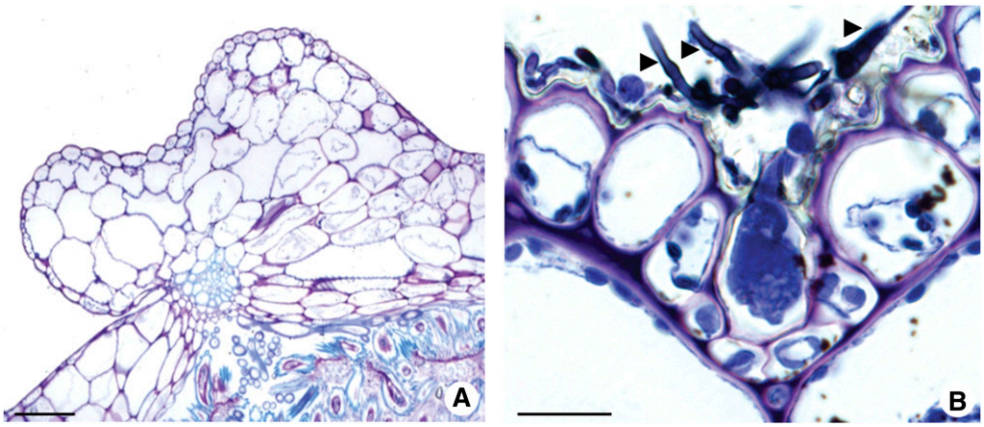

Fig. 5. Longitudinal sections of the fruit of *Anathallis obovata.* (A) Region of invagination of the external ovary wall: note the absence of glands. (B) Detail of an abscised colleter: note fungal hyphae (arrowheads). Scale bars = 25 μm.

metabolites (as lipophilic and phenolic compounds) present in the mucilaginous secretion produced by *Zootrophion atropurpureum* sepal glands may act as a deterrent to herbivory, as occurs in the buds of *Spathodea campanulata* (Bignoniaceae; Trigo and Santos, 2000), or to microorganisms as evidenced by the presence of fungi on *Acianthera fenestrata,* which did not produce secondary metabolites.

Invaginations of the external ovary wall (IEOW)—The ovary of the Pleurothallidinae studied is generally described as triquetrous in *Acianthera*, trivalvate in *Anathallis* and *Phloeophila*, often winged or carinate in *Zootrophion* (Luer, 2004; Pridgeon et al., 2010), and 6-sulcate in *Octomeria* (Luer, 1991; Forster, 2007). Neither an IEOW nor the presence of glands in this region have been mentioned, possibly due to artifacts of preservation and rehydration and the difficulty to observe these structures at low magnification.

The number, distribution, morphology, and internal structure of colleters proved to be taxonomically significant in Apocynaceae (Simões et al., 2006). In all of the species examined here, colleters were found on bracts, sepals, and in the IEOW. The presence of colleters in three subtribes suggests that colleters may be common in Epidendroideae. Future investigations of their occurrence in other subtribes of Epidendroideae, and even in other subfamilies of Orchidaceae, are needed to ascertain if colleters are common in the family as a whole.

We observed three trichome morphologies: conical trichomes in *Acianthera fenestrata*, capitate trichomes in *Zootrophion atropurpureum*, and digitiform trichomes in the other species examined. Despite these morphological differences, no functional distinction was observed in the exudate produced, suggesting that structural differences may have a phylogenetic basis rather than a physiological role. The digitiform colleter type occurs in *Octomeria crassifolia*, from an early-divergent clade (Pridgeon et al., 2001, 2010), as well as in *Oncidium flexuosum* and *Rodriguezia venusta*, and may be more wide spread and represent the common type of colleter in Epidendroideae.

The presence of phenolic compounds inside the colleters and in extracellular spaces has not yet been reported in Orchidaceae but has been described in Rubiaceae and Apocynaceae (Barreiro and Machado, 2007; Martins, 2012). Their main function is hypothesized to be protection against microorganisms and herbivory. This is supported by our finding fungal hyphae on colleters of *Acianthera fenestrata,* the only species in which phenolic compounds were not detected.

Lipids impregnated on the anticlinal walls of stalk cells may prevent apoplastic transport, thus facilitating secretion and blocking the reabsorption of the exudate (Fahn, 1988; Ascensão et al., 1999; Paiva and Martins, 2011). Although we found detached cuticle in capitate trichomes, cuticle rupture was not observed. Exudate may be released via micropores, as suggested by Mayer et al. (2011) for the colleters of *Oncidium flexuosum.* In *Acianthera fenestrata* the unicellular, conical trichomes lack impregnated lipids, which may have facilitated penetration by the fungal hyphae observed during the secretory phase.

Glands active in the IEOW, especially in flowers at anthesis, might be related to orchid seed germination, in which seeds must be infected by fungal hyphae that can penetrate the ovary wall through stomata. We observed stomata on the external walls of the ovaries of *Octomeria crassifolia* and *Anathallis obovata*; furthermore, fungal hyphae were found penetrating senescent colleters in the latter. An analogous situation occurs in some species of Rubiaceae and Myrsinaceae, where the mucilaginous exudates contain bacteria and may act as a space for leaf nodule symbionts to enter substomatal cavities (Herman et al., 1986). The possible role of mucilage as a medium for fungi is supported by our observations of fungal hyphae in the IEOW of *Acianthera fenestrata* and over abscised colleters on the fruit of *Anathallis obovata.* There is still no published evidence for a specific mechanism that attracts fungi to orchid seeds. The hyphae seem to encounter the seeds by chance, and if orchid and fungus are compatible, germination can take place (Arditti, 1992); thus, the presence of fungal hyphae over the fruit epidermis should increase the chance of contact between seeds and fungi. Pleurothallidinae is the largest orchid group pollinated by dipterans (Borba et al., 2011). The need for specific pollinators may limit their reproductive success and some taxa, such as *Octomeria crassifolia* and *Anathallis* spp. are largely self-incompatible (Barbosa et al., 2009; Gontijo et al., 2010). Thus, mucilaginous secretions that facilitate the entrance of hyphae, and hence germination, may be a strategy to increase fitness.

Secretory trichomes around the external ovary wall that are unrelated to pollination have been described in few angiosperm families. Peltate trichomes along the ovary and/or on the inner corolla wall of *Zeyheria montana* (Bignoniaceae), produce mono- and triterpenes and also appear to be a site of synthesis

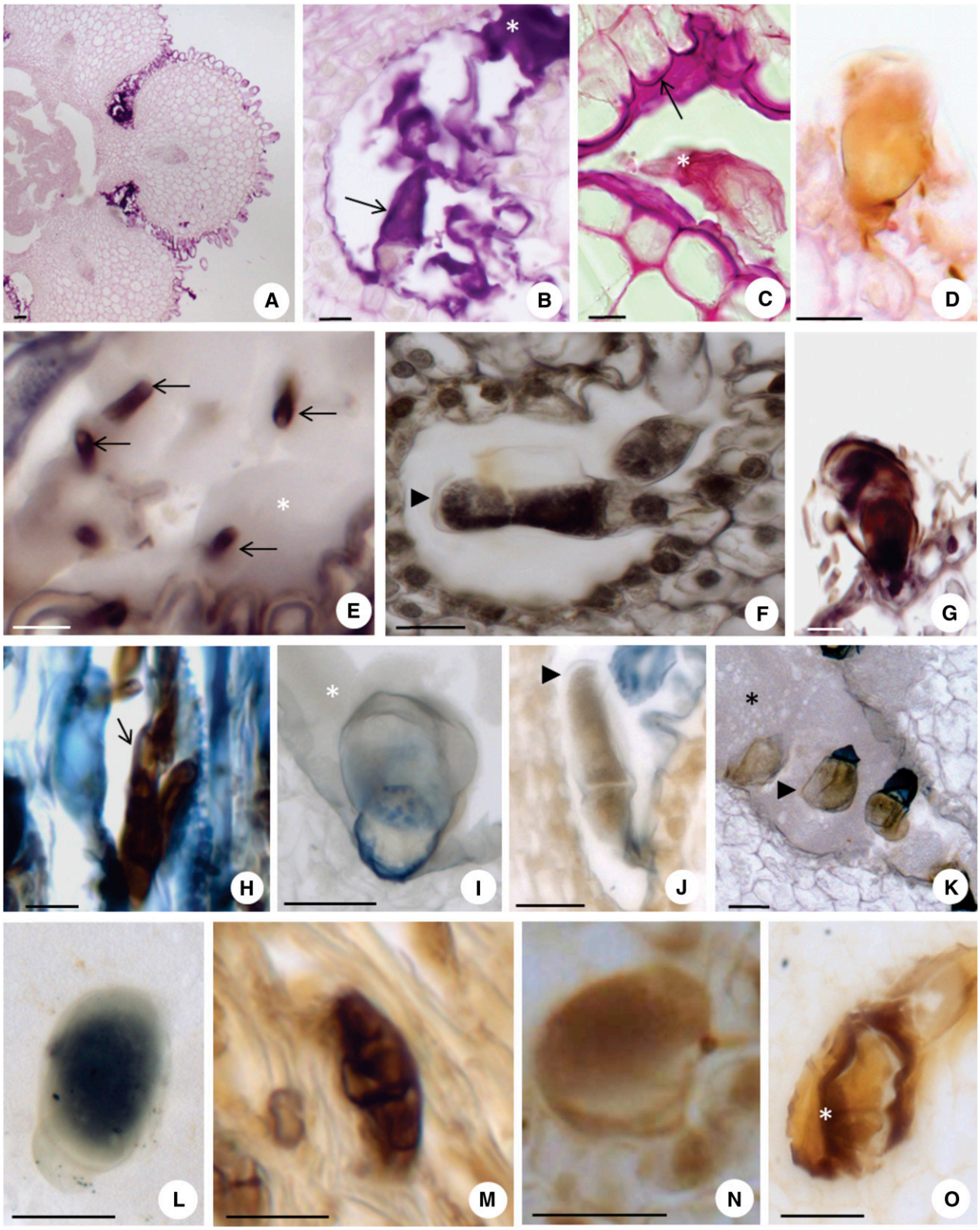

Fig. 6. Histochemical characterization of the exudate from colleters of Pleurothallidinae species. (A–C) PAS reaction. (D) Ruthenium red. (E–G) Tannic acid/ferric chloride. (H–K) Sudan black B. (L) Copper acetate/rubeanic acid. (M–O) Formaline-ferrous sulphate. (A, B, F, J) *Acianthera aphthosa,* digitiform trichomes (arrow) in the invaginations of the external ovary wall (IEOW). (C) *Acianthera fenestrata*, conical trichomes (arrow) in the IEOW. (D, G, I, K, L, N, O) *Zootrophion atropurpureum,* capitate trichomes on the sepals (D, I, N, G) and in the IEOW. (E, H, M) *Octomeria crassifolia,* digitiform trichomes (arrows) on the meristems. Note the cuticle detachment (arrowheads in F, J, K) and the extracellular exudate (*). Longitudinal (E, H, M) sections. Scale bars = 25 μm.

and/or storage of alkaloids, suggesting that these glands may be involved in chemical defense and not related to pollination (Machado et al., 2006). In Orchidaceae, extrafloral nectaries around the external ovary wall are occur in some unrelated genera, such as *Cymbidium*, *Grammatophyllum*, *Spathoglottis*, *Vanda* (Elias, 1983), *Epidendrum* (Almeida and Figueiredo, 2003), *Brassavola* (Stpiczynska et al., 2010), and *Coleogyne* (Subedi et al., 2011). In these species, the nectaries are located in the same regions where colleters are found in Pleurothallidinae (on the outer surface, at the base of the sepals and floral bracts) and often functional in the bud stage (Elias, 1983). Colleters and extrafloral nectaries differ metabolically, the former producing polysaccharides of high molecular weight and the latter simple sugars. A complete transference of function from a nectary to colleter may be a special case of homeoheterotopy defined as homeosis (Baum and Donoghue, 2002).

We have documented the first record of colleters in the invaginations of the external ovary wall in Pleurothallidinae, as well as their typical locations on the bracts and sepals. We hypothesize that the occurrence of colleters in the IEOW may be a case of homeosis, in which the metabolism of the glands in this position changed from producing simple sugars (as in nectaries of other orchid species) to complex polysaccharides (as from other colleters in Pleurothallidinae). We also hypothesize that the digitiform colleters are the common form in Epidendroideae. Finally, we hypothesize that both glands and exudate can be sites for fungal penetration associated with developing seeds because they were observed over senescent glands. Our findings can be applied to studies of plant reproductive biology, anatomy, and development.

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Capítulo 3

Floral volatiles, osmophores, nectaries, and sticky-exudate glands in Pleurothallidinae (Orchidaceae: Epidendroideae)

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Abstract

Premise of research. The Pleurothallidinae are recognized as the largest orchid group pollinated by Diptera, and has been focus of ongoing phylogenetic studies due to the occurrence of para and polyphyletic genera. The floral biology and mating systems of their representatives have been recently assessed and some synapomorphies pointed out, all of them regarding features or processes occurring in flowers. However, these studies are still incipient and data about the structure of glands directly involved in pollination as well as the volatiles emitted by the flowers are scarce, although may offer valuable information about the floral biology and phylogeny of this subtribe.

Methodology. Sepals and labellum were examined using light, scanning and transmitting electron microscopies. Nectar reabsorption was assessed by confocal analysis. The profile of volatiles emitted from flowers of most species was determined by GC-MS analysis of the headspace.

Pivotal results. Osmophores occurred in the sepals of all examined species, and also in the labellum of the ones that exhibit sapromyophilous features, except in *Echinosepala aspasicensis.* Floral nectaries were present only in the labellum of the myophilous species *Octomeria crassifolia* and *Anathallis obovata,* in which reabsorption was demonstrated in the latter. Sticky-exudate glands of heterogeneous secretion (lipids and polysaccharides) were unprecedentedly described in the lateral sepals and labellum of *E. aspasicensis*. The outstanding feature regarding the floral scent profile of these species is the ubiquitous occurrence of alkanes.

Conclusions. The presence of scent glands in the labellum of early-divergent species may be a case of homeoheterotopy, in which the floral nectaries have changed to osmophores. We propose that the alkanes are a chemical marker in volatiles of Pleurothallidinae flowers. Comparing our results to the available data in Laeliinae it is possible to hypothesize the occurrence of alkanes as

another synapomorphy (considering the older delimitation) of Pleurothallidinae.

Keywords: alkanes, myophily, Lucifer Yellow CH, sapromyophily, smooth endoplasmic reticulum.

Introduction

The subtribe Pleurothallidinae comprises about 4100 Neotropical species in 37 genera (around 20% of the total species of the family), and is the largest orchid group pollinated by Diptera (Pridgeon et al. 2001, 2010; Borba et al. 2011). Due to the poly and paraphyletic nature of some genera (Pridgeon et al. 2001, 2010), this subtribe has been a focus of phylogenetic studies and taxonomic revision. Recently, Borba et al. (2011) proposed the split of Pleurothallidinae as presently recognized (Pridgeon et al. 2001, 2010) in two subtribes: one comprising the former representatives (*sensu* Luer 1986 and Dressler 1993) with self-incompatibility and myophily as biological synapomorphies, and another one comprising the small clade consisting of ornitophilous, self-compatible genera (*Dilomilis*, *Neocogniauxia,* and *Tomzanonia*). This new proposal based on floral features highlights the need of multidisciplinary studies to evaluate morphological, chemical and biological synapomorphies that may aid in a cohesive circumscription of Pleurothallidinae.

Orchid flowers display visual, chemical and structural advertisements to guide their pollinators, and may offer rewards as nectar, pollen, fragrance[1] or oil, or simply not offer anything (Dressler 1993, Pemberton 2010). In fact, a high occurrence of non-rewarding flowers is noticed in orchids compared to other plant families (Jersáková et al. 2006, Humeau et al. 2011). Pleurothallidinae species present both rewarding species that offer nectar (Barbosa et al. 2009, Borba et al. 2011), and non-rewarding flowers that are food, brood place or sexual-deceived (Borba and Semir 2001, Blanco and Barboza 2005, Pemberton 2010, CaraDonna and Ackerman 2012, Duque-Buitrago et al. 2014).

Myophily is a syndrome corresponding to elements of anatomical, behavioral and physiological adaptations of flower-visited Diptera (Borba and Semir 1998, Woodcock et al.

[1] The volatile chemicals from flowers, fruits, feces, decaying wood or tree wounds collected by Euglossine bees and that are not ingest but accumulate outside their body in voluminous leg pockets (Dressler 1993)

2014). Nectar is the main attractant and reward to some pollinators while visual, chemical and structural cues provide information to potential pollinators about location and access to this reward (Jersáková et al. 2006, Woodcock et al. 2014). Sapromyophyly is a special case of deception in which plants deceive their pollinators by producing odors mimicking the decaying flesh where these flies normally lay their eggs (Jersáková et al. 2006, Melo et al. 2010, Humeau et al. 2011, Woodcock et al. 2014).

In Pleurothallidinae, detailed descriptions of nectaries refer to glands located in the labellum that produces small quantities of nectar (Borba and Semir 2001, Barbosa et al. 2009, Melo et al. 2010). Although nectar is not commonly found among sapromyophilic plants, it seems to be an important element in the mechanism of deceit-pollination in some partially deceitful species of *Acianthera*. Borba and Semir (2001) studied the pollination biology of a group of Brazilian *Acianthera* species and found that species pollinated by Phoridae flies have nectar on the labellum, while those pollinated by Chloropidae flies are nectarless and deceit-pollinated. When present, the nectary cells are unicellular trichomes (Melo et al. 2010). Data concerning the reabsorption of nectar in Pleurothallidinae species is still unavailable, although it was registered by microautoradiography in other non-related orchids (Stpiczyńska 2003; Nepi and Stpiczyńska 2007, 2008).

The outstanding glands in Pleurothallidinae are the osmophores (scent glands), apparently ubiquitous in this subtribe (Pridgeon et al. 2010), mediating the different strategies of pollination that Pleurothallidinae species exhibit. Although its widespread distribution, studies concerning morphology and structure of this osmophores are really scarce, restricted to studies in *Restrepia* spp. that present papillae osmophores[2] in the apices of dorsal sepal and petals (Pridgeon and Stern 1983), *Scaphosepalum* spp. in which glandular pads or papillae occur on adaxial surfaces of

[2] Here we apply the terms "papillae osmophores" following the term "trichome elaiophores" designated by Buchmann (1987).

sepals (Pridgeon and Stern 1985), and species of *Acianthera* that exhibit papillae osmophores and regions with stomata pore in the adaxial face of sepals (Melo et al. 2010). The presence of osmophores was also indicated by the use of Neutral red in *Pleurothallis marthae* (Duque-Buitrago et al. 2014), or by comparison with the probable morphology (papillae) usually described in the literature in *Lepanthes glicensteinii* (Blanco and Barboza 2005).

Floral scents stand out for their chemical complexity and variation, both among and within taxa (Raguso 2004). Floral scent constituents often correlate strongly with specific phylogenetic related groups of pollinators that are not necessarily tied to only one or a few specific compounds in a scent bouquet (Steiner et al. 2011). Studies on chemical profiles of the volatiles emitted by Pleurothallidinae flowers are incipient, restricted to *Masdevallia*, *Dracula* and *Dryadella* species (Kaiser 1993). Besides the use of chemical markers in a phylogenetic frame, studies that characterize the volatile floral compounds are essential for a better understanding on Pleurothallidinae species and their (mostly still unknown) pollinators.

We investigated *Octomeria crassifolia*, representing the sister group of remaining Pleurothallidinae (Pridgeon et al. 2011), a species that offer nectar and is pollinated by Sciaridae flies (Barbosa et al. 2009, Borba et al. 2011), and six other myophilous and sapromyophilous species representing early-divergent clades of this subtribe whose floral biology is still unknown. Our aims here were to identify and characterize the structure of glands present in sepals and labellum, besides describe the profile of the volatiles emitted by their flowers. We compared the profiles with the ones provided by literature to some Laeliinae species seeking for phylogenetic insights.

Specifically, we addressed the following questions: sapromyophilous species examined here produce nectar? If so, since the flowers are opened, the nectaries are able to reabsorb nectar?

Is it possible to propose some evolutionary trend concerning the floral glands identified? Is it possible to identify a chemical marker from the analysis of the volatile emitted by these flowers?

Material and Methods

Plant material

Flowers at several days of anthesis were collected from plants cultivated in the living orchid collection of the "Núcleo de Pesquisa Orquidário do Estado", "Instituto de Botânica" (São Paulo, Brazil) (Fig. 1). All were collected in Brazil and are listed below with provenance and accession numbers. *Acianthera fenestrata* ("number pending") was recently incorporated to the collection and lacks a registration number.

Taxon	Origin and accession number
Brachionidium-Octomeria clade	
Octomeria crassifolia Lindl.	Serra da Piedade, 5666; São Paulo, Campos do Jordão, 12096; Minas Gerais, Carangola, 14866.
Echinosepala-Barbosella clade	
Echinosepala aspasicensis (Rchb.f.) Pridgeon & M.W.Chase	Roraima, Serra da Neblina, 12306,16306.
Acianthera clade	
Acianthera aphthosa (Lindl.) Pridgeon & M.W.Chase	São Paulo, Reserva Ecológica do Morro do Diabo, 14854, 14855; Minas Gerais, Camanducaia, P1082.
Acianthera fenestrata (Barb.Rodr.) Pridgeon & M.W.Chase, number pending.	
Zootrophion-Trichosalpinx clade	
Anathallis obovata (Lindl.) Pridgeon & M.W.Chase	Paraná, Guaira, Região de Sete Quedas, 12599, 12611, 13299; São Paulo, Reserva Ecológica do Morro do Diabo, 14263, 14294;

	São Paulo, Boracéia, 14359; Minas Gerais, Carangola, 14961.
Zootrophion atropurpureum (Lindl.) Luer	São Paulo, Serra do Mar, 678; Minas Gerais, Carangola, 14998; São Paulo, Boracéia, 16936.
Remaining Pleurothallidinae clade	
Phloeophila nummularia (Rchb.f.) Garay	São Paulo, Bananal, 18235.

Glicofita Plus® (Bayer, S.A.)[3] was used to test for the occurrence of glucose (Aguiar-Dias et al. 2011) in living plants whenever the exudate from sepal or labellar glands was profuse.

Scanning electron microscopy (SEM)

The material was fixed in Karnovsky solution (Karnovsky 1965, modified by Kraus and Arduin 1997) for 24 h for morphological and anatomical purposes, since the use of formalin-based fixatives resulted in plasmolysis of the flowers' cells. The samples were washed, dehydrated and stored in 70% ethanol.

Sepals and labellum were isolated from flowers, further dehydrated to 100% ethanol and rinsed in a hexamethyldisilazane (HMDS) series (33.3, 50.0, and 66.6% v/v in 100% ethanol) and then three times in 100% HMDS for 1 min each (Jeger et al. 2009) to dry the material. Samples were mounted on stubs, coated with gold palladium in a Hummer 6.2 sputtering system (Anatech, Union City, CA, USA) and viewed with a JSM-541OLV SEM (JEOL, Tokyo, Japan) at 10 kV. Digital images were edited using Adobe Photoshop version 7.0.

Light microscopy

[3] Glicofita Plus® is based on Benedict's reagent to detect the presence of reducing sugars. This includes all monosaccharides and many disaccharides. Even more generally, Benedict's test will detect the presence of aldehydes, and alpha-hydroxy-ketones, including those that occur in certain ketoses. Thus, although the ketones fructose is not strictly a reducing sugar, it is an alpha-hydroxi-ketone, and gives a positive test because it is converted to the aldose glucose by the base in the reagent (Benedict 1909).

Sepals and labellum were processed using standard methods for Leica Historesin® (Heraeus Kulzer, Hanau, Germany) embedding media, serially sectioned at 5 µm thickness, stained with toluidine blue O (TBO) (Sakai 1973) and mounted in Neomount resin. Specific regions where the glands were observed were processed using LR White (Ted Pella Inc., USA) embedding media, and sectioned at 1.5–2 µm thickness. Histochemical tests were then performed: TBO and p-phenylenediamine were used for metachromasy and lipid localization (Kivimäempää et al. 2004), Sudan black B for total lipids, phenolic compounds and starch (Bronner 1975), PAS reaction for total polysaccharides (McManus 1948) and followed by p-phenylenediamine to co-localize polysaccharides and lipids. Tests were performed with appropriate controls. Nadi's reagent for terpenoids (David and Carde 1964) was applied only in fresh material from *Octomeria crassifolia* and *Anathallis obovata*, being impossible to apply in the other species due to the constitutive purple color (anthocyanin) observed in their tissues.

Sections were viewed and digitally photographed with an Olympus BX53 compound microscope equipped with an Olympus Q-Color 5 digital camera with Image Pro Express 6.3 software.

Transmission electron microscopy (TEM)

Regions from sepals and nectaries were isolated and immediately fixed at room temperature in 3% glutaraldehyde and 0.2 M cacodylate buffer, pH 7.25 (Marques et al. 2013). After several washes with cacodylate buffer, the material was post-fixed in 1% OsO_4 for 2 h, dehydrated to 100% ethanol and then embedded in LR White embedding medium (Ted Pella Inc., USA). Thin sections were mounted on copper grids and viewed with Philips CM10 transmission electron microscope at 80kV.

Confocal analysis of nectar reabsorption

We performed the studies of nectar reabsorption in species which the exudate was positive

to the test with Glicofita Plus®, and the nectar was profuse enough for us to apply the technique with Lucifer Yellow CH. We followed the procedures detailed in Cardoso-Gustavson *et al.* (2013) and Cardoso-Gustavson and Davis (2014) (appendices 1 and 2, respectively) in the morning (0900) of the second day of anthesis. A 1% solution of Lucifer Yellow CH dilithium salt (Sigma-Aldrich) in distilled water was prepared in the dark at room temperature. 1µl of LYCH solution was applied to standing nectar using a micropipette. There was no direct injection of the solution into the nectary tissues; instead, the solution was added to the film layer of naturally accumulated nectar. Following each LYCH application, flowers were covered with aluminum foil and incubated for 24h. Control experiments were carried out using the same conditions (volume, number of applications, covering), but with distilled water alone.

After LYCH treatment, single nectaries were isolated, washed several times with distilled water and immediately fixed with Karnovsky's glutaraldehyde solution (modified by Kraus & Arduin 1997) for 24 h. LYCH does not spread to tissues during fixation. After fixation, material was washed several times in distilled water, free-hand sectioned and slides mounted in glycerin: distilled water (1:1, v:v). An inverted Zeiss LSM 510-Meta confocal laser scanning microscope was used to image the results with LYCH using Zeiss water immersion objectives (10 and 25×). Due to the difficulty to hand cut the small labellum of *A. obovata*, we observed the paradermal multi-depth images of this structure, each one of 10 µm thickness. The 405 nm line of a diode laser was used to excite the LYCH-treated samples, as well as the control samples and observations were taken of the emission bands from 449 to 695 nm (diode laser), with emission filter BP 530–600.

Collection and characterization of flower volatiles

The volatile analysis from *Octomeria crassifolia*, *Echinosepala aspasicensis*, *Acianthera fenestrata*, *Zootrophion atropurpureum* and *Phloeophila nummularia* were performed in the

second day of anthesis, from 9am to 6pm, in sampling periods of 1h 30 min. Flowers were kept apart from their vegetative constituents with aluminum foil and introduced in small Teflon bags with side vents for air input and output, ensuring the maintenance of gas exchange. The volatiles were collected through cartridges containing 100 mg of Tenax TA and 50 mg Carbopack absorbents (Supelco, mesh 60/80), with one end fixed in a bag opening and the other connected to a suction pump, ensuring the passage of the air inside the cartridge. The flow of the sampled air was 180 ml min^{-1} and the air inserted into the bags of 240 ml min^{-1}.

Volatile compounds were analyzed by gas chromatography-mass spectrometry (GC-MS) (MSD 5973; Agilent GC 6890). Trapped compounds were desorbed with a thermal desorption unit (Perkin-Elmer ATD400 Automatic Thermal Desorption system; Perkin Elmer, Waltham, MA, USA) at 250 °C for 10 min, cryofocused at -30 °C and injected onto an HP-5 capillary column (50 m × 0.2 mm i.d. × 0.5 µm film thickness; Hewlett-Packard) with helium as a carrier gas. The oven temperature program was held at 40 °C for 1 min and then raised to 210 °C at a rate of 5 °C min^{-1}, and finally further to 250 °C at a rate of 20 °C min^{-1}. The compounds were identified by comparing their mass spectra with those of compounds in the retention index libraries.

Presentation of the results and terminology

The results were described following the optimized phylogeny described in the chapter 1. We followed the simplified terminology of Nepi (2007) to describe the general anatomy of floral nectaries examined here, and the terminology of trichomes proposed by Theobald et al. (1979).

Results

Osmophores

Octomeria crassifolia and *Anathallis obovata* flowers exhibited secretory unicellular trichomes with ornamented cuticle all over both surfaces of the sepals (Figs. 2A, D). These trichomes remained intact during the anthesis, with no distension or cuticle rupture, becoming

plasmolysed close to the end of the anthesis (Fig. 2D). Dome-shaped unicellular trichomes from both species presented many starch grains and lipid droplets (Figs. 2B, E, F). Only the trichomes of *O. crassifolia* exhibited an intense blue content with Nadi's reagent, indicative of positive reaction to terpenoids (Fig. 2C).

The flowers of *Echinosepala aspasicensis* presented elevated clusters of secretory unicellular trichomes from the base to the median region of the sepals' adaxial surface (Fig. 2H), while the abaxial one exhibited non-secretory trichomes. The secretory trichomes did not show ornamentation but the cuticle was distended (Fig. 2I) and ruptured (Fig. 2H) during the period of anthesis. They presented a large vacuole and lipid droplets in the periphery of the gland (Fig. 2I). Under transmission electron microscope (TEM), many osmiophilic bodies of several electron-densities were observed inside plastids close to smooth endoplasmic reticulum (SER), and also vesicles toward the cell membrane (Fig. 2J).

All the inner region of *Acianthera aphthosa* flowers exhibit a very strong purple color (Fig. 1C). In this species two distinct osmophore morphologies were recognized, one in the sepals and another at the base of the labellum. Saliences were observed from the median to the apical region of the adaxial surface of sepals, easily recognized in the flowers at anthesis (Fig. 1C). These salient structures exhibited a stomata pore at their apices visible under SEM, while the abaxial surface of the sepals presented non-secretory trichomes (Figs. 2K, L). The secretory cells that constitute the saliences were rich in starch grains and lipid droplets (Figs. 2M, N). The basal portion of the labellum presented ornamented secretory unicellular trichomes (Figs. 3A, B), in which the cuticle showed no rupture or plasmolysis, but it was detached at the second day of anthesis (Fig. 3C), although no content between the cuticle and cell wall was observed. Secretory trichomes and adjacent parenchyma cells exhibited a dense protoplast, many lipid droplets and

starch grains, and several vacuoles of different sizes (Figs. 3C, D), some of them possessing phenolic compounds (Fig. 3E).

Acianthera fenestrata flowers exhibited ornamented secretory trichomes in the adaxial surface of sepals and at the base of the labellum. These unicellular trichomes possessed a spherical shape in the sepals (Fig. 3F), in which the cuticle exhibited no distention or rupture. The secretory trichomes and adjacent parenchyma cells exhibited an amorphous content of lipophilic nature (Fig. 3G). Non-secretory trichomes were observed at the abaxial surface of the sepals (Fig. 3F). The secretory unicellular trichomes from labellum, on the other hand, exhibited an acute shape (Fig. 3H–J). The cuticle was distended since the first day of anthesis, but no content was visualized in the subcuticular space, while vacuome were observed immersed in a dense protoplast (Fig. 3I). It is interesting to point that no content was visualized inside the vacuome with the Toluidine blue O (TBO) stain, contrasting to the use of TBO and p-phenylenediamine in the sepals in which the lipophilic content of vacuole was visible. Under TEM, the epicuticular ornamentations and also other interesting features, as a light region between the cuticle and cell wall, electron-dense regions of the cuticle close to cell wall, and osmiophilic bodies towards the cell wall were visualized (Fig. 3J).

Secretory unicellular trichomes presenting a tracery aspect and regions where the cuticle was ruptured were observed in the central region of the labellum of *Zootrophion atropurpureum* (Figs. 4A, B). These glands presented a detached cuticle and the protoplast exhibited a large vacuole and several lipid droplets (Figs. 4C, D). Small vacuoles co-occurring with a large one were observed inside the secretory trichomes after treatment with Sudan black B stain (Fig. 4D). Small osmiophilic bodies associated to SER and two other larger ones with no evidenced boundaries were visualized at the protoplast of the trichome (Fig. 4E).

The sepals of the tiny flowers of *Phloeophila nummularia* presented non-secretory trichomes in the abaxial surface, while the adaxial one exhibited secretory unicellular trichomes (Fig. 4F). These glands presented several starch grains (Fig. 4G). Osmiophilic bodies of several different electron-densities associated to SER were observed under TEM (Fig. 4H). Acuminate projections were observed all over the labellum of this species, and a smoother region consisting of secretory emergences was observed at the base of the structure (Fig. 4I). These glands presented a dense protoplast with few small starch grains (Figs. 4J, K). Starch grains, osmiophilic bodies close to SER and flocculent material in the vacuole were visualized under TEM (Fig. 4L).

Floral nectaries

During greenhouse observations, we detected glucose in the secretion of the labellum from *Octomeria crassifolia* and *Anathallis obovata* using Glicofita Plus®. The exudate was concentrated at the adaxial base of the labellum in *O. crassifolia*, where a purple spot is observed (Fig. 1A) and appeared as a thin layer all over the labellum of *A. obovata*, being more profuse in both species during the morning.

Floral nectaries of *O. crassifolia* were visualized as striated unicellular trichomes at the base up to the median region of the labellum (Fig. 5A, see details in the *inset*). Structurally, the square-shape nectary trichomes presented no cuticle distension or rupture, a large vacuole and the protoplast are confined to the periphery of the cell (Fig. 5B). Under TEM, the protoplast of the nectary trichome presented starch grains, osmiophilic bodies associated to SER, and several vesicles originated from dictyosomes (Fig. 5C).

The nectary unicellular trichomes of *A. obovata* occurred all over the adaxial surface of the labellum and exhibited a conical shape (Fig. 5D, see details in the *inset*). In contrast to *O. crassifolia*, the nectary trichomes exhibited a smaller vacuole and a dense protoplast with several

lipid droplets (Fig. 5E). The ultrastructure of the protoplast of both species essentially presented the same features. The nectar was observed as a thin film all over the surface of the labellum, and profuse enough to perform the reabsorption studies. The study with LYCH revealed the ability of the nectary trichomes to reabsorb the released nectar: the multi-depth images of the labellum under confocal microscope indicated that nectar reabsorption occurred mainly at the apex of the labellum, being the nectar able to reach the vascular traces after 24h of the application of LYCH (Fig. 5F).

Sticky-exudate glands

The apex of the adaxial surface of the lateral sepals and the labellum of *Echinosepala aspasicensis* presented a thin layer of a very shiny and sticky exudate (Fig. 1B) that did not react with Glicofita Plus® and was visualized all over the day.

In the sepals, the secretory region was characterized as smooth elevated undulations with several extensions of cuticle disruption (Fig. 6A). Structurally, these undulations were observed as different lengths of the uniseriated palisade epidermis and differential proliferation of secretory parenchyma (Fig. 6B), while the cuticle ruptures were a final process initiated by its detachment (Fig. 6C). The protoplast of the palisade epidermis was dense, presenting a large vacuole, lipid droplets (Fig. 6C) and small starch grains (Fig. 6D). The secretory parenchyma did not present conspicuous features except from the diminute size of the cells, otherwise structurally resembling the ordinary parenchyma, with a large vacuole (Fig. 6B).

The secretory tissues of the labellum exhibited similarities with the ones of the lateral sepals, with the taller palisade epidermis forming high elevations of lower width comparing to the sepals (Fig. 6E). Both secretory palisade epidermis and parenchyma cells, in contrast to those of the sepals, exhibited dense protoplast and large vacuole (Fig.6F).

The cell wall of the secretory palisade epidermis was thick and the protoplast dense, exhibiting strong affinity to TBO and PAS reaction + p-phenylenediamine, and Sudan black B stain, and many lipid droplets were observed in the protoplast and also over the cuticle (Figs. 6G–I). The cuticle was detached in some regions (Figs. 6G, H). The intensity of the PAS reaction was higher in the cell wall, decreasing as close to the cuticle limit, where a pale color exhibited by an amorphous secretory content was visualized (Fig. 6H).

Secretory residues were present on the cuticle of the labellum, and exhibited a granulate aspect, in which the granules presenting different electron-densities are immerse in an amorphous matrix (Fig. 6J). A system constituted by smooth endoplasmic reticulum (SER) and flocculate material apparently delimited by a membrane was observed in the palisade epidermal secretory cells. Some vesicles with flocculate material content seemed to leave the region of the SER system facing the cell wall, toward the cell membrane (Fig. 6K). A large vesicle of fibrillar content and several small ones merging to the plasmatic membrane were also co-observed in the same cells; in addition, an amorphous content was observed in the extracytoplasmic space (Fig. 6K). In the region of the cell close to the adjacent secretory parenchyma, lipid droplets were observed inside a plastid (Fig. 6L).

Floral volatiles

The results showed in table 1 summarize the pivotal compounds detected from the flowers' headspace of *Octomeria crassifolia*, *Echinosepala aspasicensis*, *Acianthera fenestrata*, *Anathallis obovata*, *Zootrophion atropurpureum* and *Phloeophila nummularia*. A number of unidentified compounds were also detected, and those in which the identification by the mass spectral and retention index libraries was below 80% of confidence were not considered for description.

Table 1. Chemical compounds (separated by mainly classes) identified from the volatiles emitted by flowers at second day of anthesis.

Compounds	RT*	Relative abundance (%)					
		*Octomeria crassifolia***	*Echinosepala aspasicensis*	*Acianthera fenestrata*	*Anathallis obovata***	*Zootrophion atropurpureum*	*Phloeophila nummularia*
Alkanes							
undecane	1278		0.349			0.106	0.145
dodecane	1578	0.215	1.16	1.998	0.384	0.856	2.008
undecane 2,6-dimethyl	1621						0.299
dodecane 2-methyl	1768			1.386			0.669
heptadecane 2,6,10,14- tetramethyl	1795			3.341			3.781
tridecane	1871	0.656	4.825	11.01	1.209	9.193	8.818
tetradecane, 2, 6,10-trimethyl	2050		0.868	3.341	0.335		
tridecane 3-methyl	2072	0.095		1.134			
tetradecane, 3-methyl	2086	0.239					
dodecane 2,6,10-trimethyl	2089			1.452			0.101
tetradecane	2151	2.36	7.291		4.7	2.756	3.524
tetradecane,2,6,10-trimethyl	2317	0.374	0.884				
hexadecane	2666				0.361		
octane, 1,1' oxybis	2823		0.677				
heptacosane	2905		0.268	1.969			
Cycloalkanes							
heptacyclohexane	2002			2.041			0.625
cyclopentene, undecyl	2809						0.899
Aldehydes							
4-pentenal-2-methyl	0498						
nonanal	1292		1.547		1.119	1.177	0.065
decanal	1597	0.707	1.673		1.077		0.982
Alcohols							
3-hexen-1-ol	0607				0.334		
1-hexanol, 2-ethyl	1064	0.707			0.685	0.546	2.1

1-dodecanol 3,7,11-trimethyl	1218						0.673
1,6 octadien-3-ol, 3, 7-dimethyl	1279	2.576					
Hexadecanol	1893			1.003			
1 hexadecanol, 2-methyl	2130		1.846	0.915			
n-nonadecanol-1	2395		0.786				2.524
behenic alcohol	2555						1.67
2-hexadecanol	2810		0.522				0.738
1-hexadecanol-2-methyl	2821	0.089	0.464				1.475
2-methyl-E-E-3,13- octadien-1-ol	3165						0.07
Carboxylic acids							
10,13-octadienoic acid, methyl ester	1352	0.28***					
2,4,6-octatrienoic acid	1644				0.541		
dodecanoic acid	2581			0.676			
oleic acid	3052						0.42
9-hexadecanoic acid	3444						0.124
n-hexadecanoic acid	3493						0.275
trans-13-octadecanoic acid	1848			0.592			
Esters							
triacetin	2012				0.429		
hexadecane, 1,1 bis(dodecyloxy)	2175	0.101				0.782	
hedione	2811				1.238		
Ketones							
5,9-undecadien 2-one, 6,10-dimethyl	2299					0.726	0.669
ß-Iraldeine	2853				1.476		
2,5-methyl-6-noneicosen,11-one	2873		0.159				
Cyclic ketone							
isophorone	1352						
Aromatic compounds							
benzaldehyde	0877	0.53	1.442		4.797	2.624	0.989
phenol	0922	0.17			0.182	1.127	0.585
benzyl alcohol	1081	0.304					
benzaldehyde,2-hydroxy	1116						0.321

acetophenone	1184	0.323***	1.138	0.299	1.985	0.727
benzenemethanol, α-α- dimethyl	1241					
benzoic acid, methyl ester	1270			0.474		
benzoic acid	1479			1.964		
E-2-hexenyl-benzoate	1470					0.327
benzoic acid	1503	1.217	1.69		10.683	1.247
methyl salicilate	1575	0.881		1.041		
benzene, 1-ethylbutyl	1610			1.675		
ethanol, 2 phenoxy	1645	0.3				
ethanol, 2-phenoxy	1646		1.024			1.186
3-phenylpropanol	1675	0.444				
cinnamaldehyde, (E)	1798	2.807				
benzaldehyde, 4-propyl	1804		0.457			
1-indanone	1830	0.146***				
cinnamyl alcohol	1899	6.085***				
eugenol	2046			1.01		
benzaldehyde,4-butyl	2259			6.583		
cinnamyl acetate	2280	1.378				
lilyal	2503			1.063		
versalide	3278			0.827		
Monoterpenes						
D-limonene	1072				1.983	
trans-linalool oxide	1202	6.744***				
cis-p-mentha-2,8-dien-1-ol	1219	0.194***				
β-linalool	1280	6.704				
3,7-octadiene,2,6-diol,2,6-dimethyl	1292	0.883***				
hotrienol	1294	4.319				
2,6-dimethyl-1,2,5,7-octatetraene, E, E	1374	0.15				
limonen-6-ol	2409	0.102				
Sesquiterpenes						
nerol oxide	1760	4.86***				
α-farnesene	2408	13.686				

α-patchoulene	2667	0.637					
Nitrogen containing compounds							
Amides							
nonanediamide, N, N'-di-benzyloxy	1467		1.209				
octanediamide, N, N'-dibenzyloxy	1469		1.209				
hexanediamide, N, N'-dibenzyloxy	1491		1.857				
decanediamide, N, N'-dibenzyloxy	1492						0.946
heptanediamide, N, N'-dibenzyloxy	1493				1.406	3.135	1.239
formamide,N,N'-dibutyl	1889		0.25		0.199		
Amine							
Indole	1865				6.337		
Chloroalkane							
1-chlorooctadecane	2016			1.084			

*Each compound identified by mass spectral and retention index libraries.
** Higher emissions during the morning (0800 to 1200).
*** Compounds detected only at the second sampling (between 0930 –1100).

Fig. 1. Flowers of Pleurothallidinae species at anthesis. (A) *Octomeria crassifolia*. Note the purple spot at the base of the labellum (arrow). (B) *Echinosepala aspasicensis*; note the shiny sticky exudate over the lateral sepals and labellum (*). (C) *Acianthera aphthosa*; note the rounded elevations in the apex of the sepals' adaxial surface (dashed circle). (D) *Acianthera fenestrata*. (E) *Anathallis obovata*. (F) *Zootrophion atropurureum*. (G) *Phloeophila nummularia*. Arrows (D, F) indicate the opening of the lateral sepals ("lateral windows"). Scale bars: 1 cm.


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Fig. 2. Osmophores in the sepals of Pleurothallidinae species. (A–G) Secretory unicellular trichomes with ornamented cuticle of *Octomeria crassifolia* (A–C) and *Anathallis obovata* (D–G); note the occurrence of plasmolysed cells at third day of anthesis (red arrowheads in D). Lipid droplets (B, E, F, black arrowheads), starch grains (G, short arrows) and blue content of the trichomes (C). (H) Elevated clusters of secretory unicellular trichomes with ruptured cuticle (red arrows) at the base of the adaxial surface of the sepals of *Echinosepala aspasicensis*. (I) Gland with detached cuticle (arrow), lipid droplets (arrowheads) and a large vacuole. (J) Many osmiophilic bodies (ob) of different electron-densities inside plastids and close to smooth endoplasmic reticulum (SER), vesicles toward the cell membrane (arrowheads) (cw, cell wall; m, mitochondria; sg, starch grain; vac, vacuole). (K–N) Elevated rounded structures at the apex of the adaxial surface of the sepal of *Acianthera aphthosa*; note the stomata pore at the apex (L, arrow in M), several starch grains (M) and lipid droplets (N, arrowheads) in the secretory cells. (A, D, H, K, L) Scanning electron microscopy (SEM). (B–C, E–G, I, M, N) Light microscopy (LM). (J) Transmitting electron microscopy (TEM). (B, E, I, N) Toluidine blue + p-phenylenediamine (TBO+phe). (C) Nadi's reagent. (F) Sudan black B. (G, M) PAS reaction.. Scale bars: 100 µm (H, K), 75 µm (J, M), 50 µm (B, C, E–G, I), 10 µm (A, D, L), 1 µm (J).

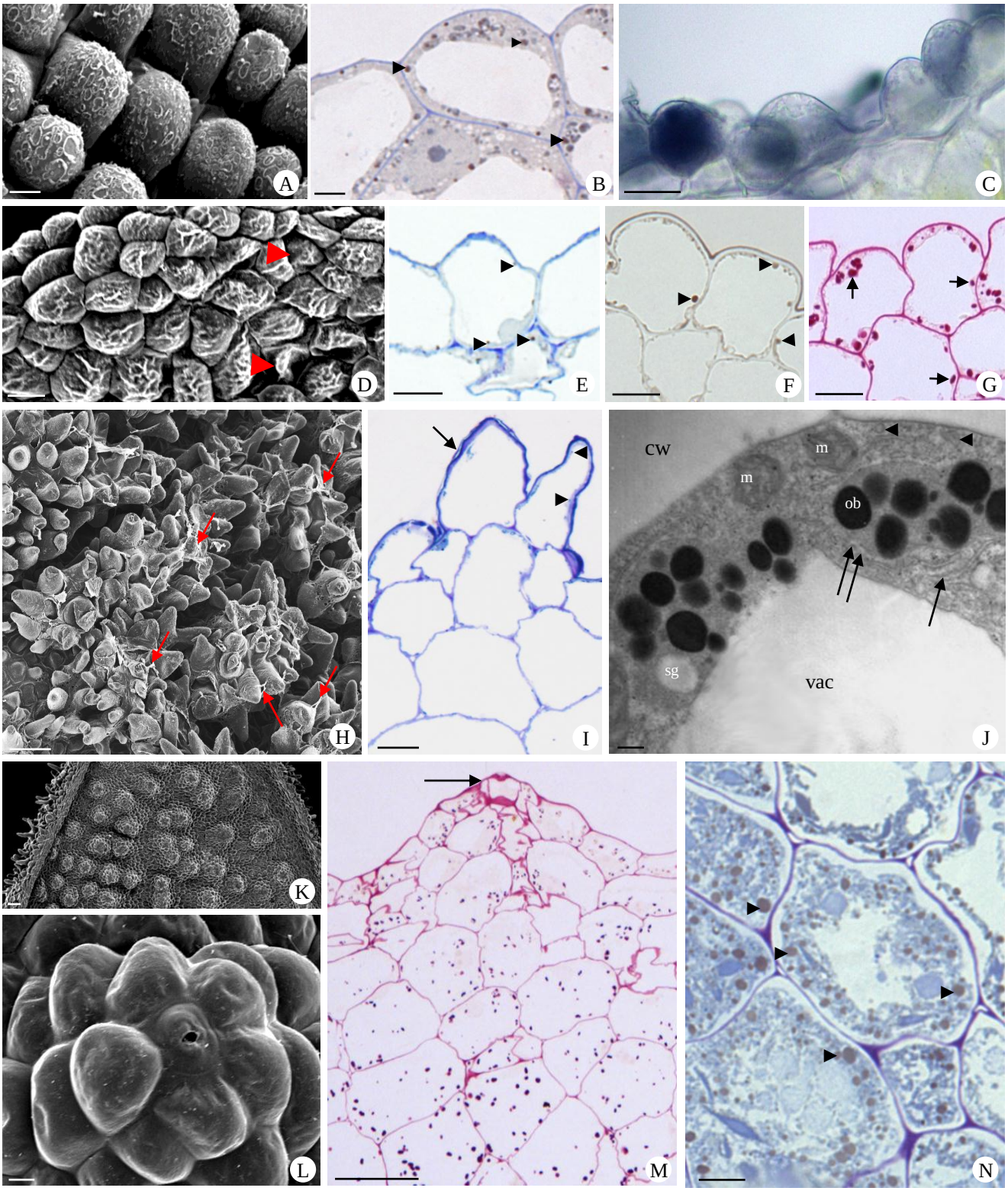
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Fig. 3. Osmophores in Pleurothallidinae species. (A–E) Secretory unicellular trichomes of *Acianthera aphthosa* restricted to the base of the labellum (A, dashed square); note the epicuticular ornamentations of the glands (B). The trichomes' cuticle is detached at anthesis (black arrow in C), and both secretory trichomes and adjacent parenchyma present dense protoplast with several lipid droplets (C and E, red arrowheads), starch grains (D), and small vacuoles (C, blue arrows), some of them with phenolic compounds content (E, white arrowheads). (F–J) Secretory unicellular trichomes of *Acianthera fenestrata* sepal (F–G) and labellum (H–J). (F–G) Non-secretory trichomes at the abaxial surface (upper), and secretory trichomes (bottom) at the adaxial one; these glands and secretory adjacent parenchyma present a lipophilic (*) content (G). (H–J) Secretory unicellular trichomes at the base of the labellum (H, dashed square); note the detached cuticle (I, arrow) and the occurrence of vacuome (I, *). (J) Detail of the trichome, showing the projections of the epicuticular layer (epi), and both thick cuticle (cut) and cell wall (cw). Note a light region between the cuticle and cell wall (black arrow), and electron-dense regions of the cuticle (white arrow); osmiophilic bodies towards the cell wall (black arrowheads) and cuticle (white arrowhead). (A, B, F, H) SEM. (C–E, G, I) LM. (J) TEM. (C,G) TBO+phe. (I) TBO. (D, J) PAS reaction. (E) Sudan black B. Scale bars: 100 µm (A, H, F), 75 µm (C, D, G), 50 µm (E, I, J), 10 µm (B), 0.5 µm.

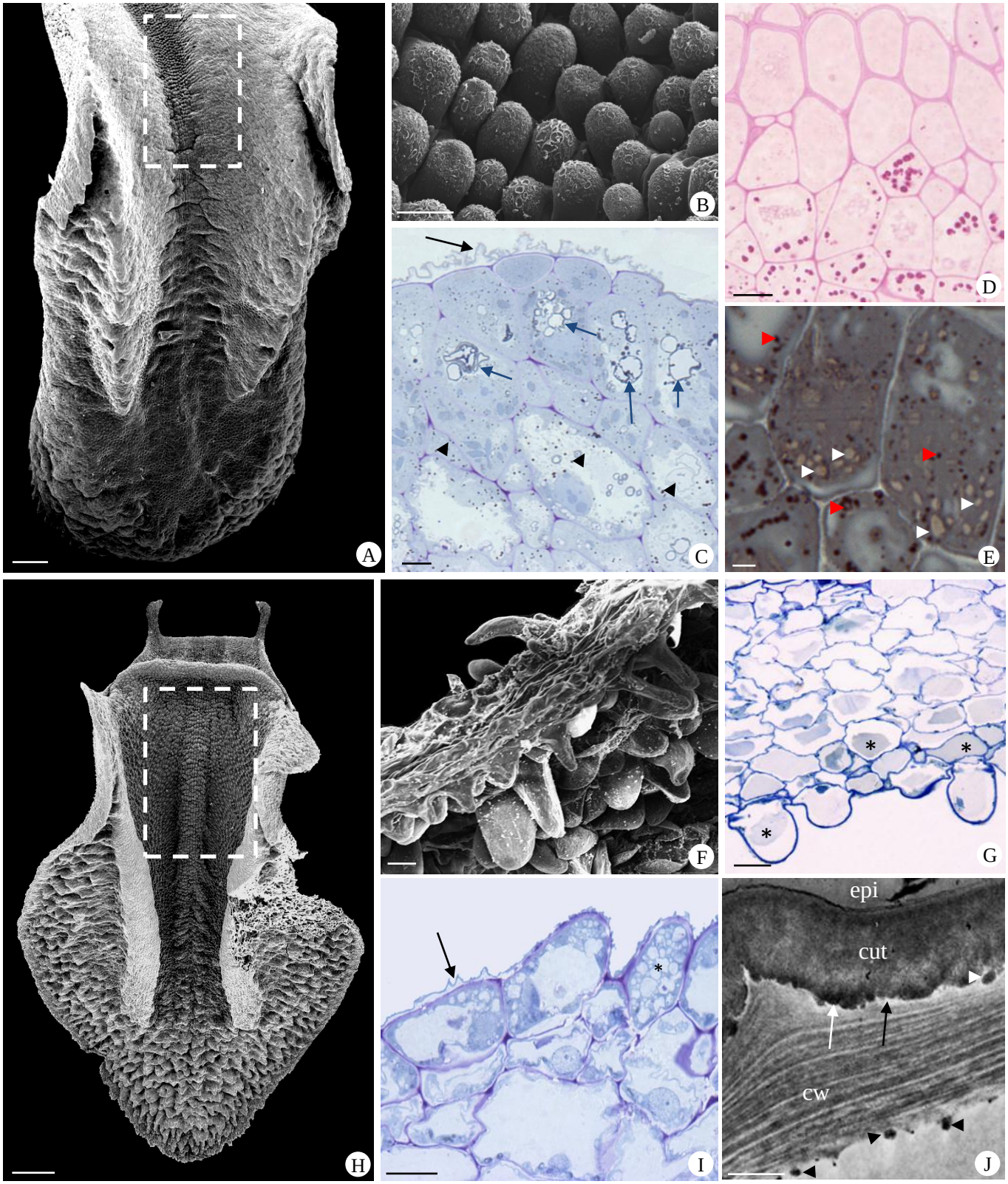
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Fig. 4. Osmophores in Pleurothallidinae species. (A–E) Secretory unicellular trichomes of *Zootrophion atropurpureum* since the base up to the middle of the labellum (A, dashed squares); note the ornamentation of these glands and regions of ruptured cuticle (B, arrows). Secretory trichomes exhibit cuticle distension (C, arrow), small vacuoles (D, double arrows), and lipid droplets (C, D, arrowheads). Occurrence of many small osmiophilic bodies close to SER (smooth endoplasmic reticulum), and two larger ones with no clear boundaries. (cw, cell wall; m, mitochondria). (F–L) Secretory unicellular trichomes in the sepals (F–H) and emergences and labellum (I–L) of *Phloeophila nummularia.* (F–H) Secretory trichomes in the adaxial surface and non-secretory trichomes (upper left) in the abaxial one (F); these glands and the adjacent secretory tissue present many starch grains (G). (H) Osmiophilic bodies of different sizes associated to SER (arrowheads): note that the larger ones do not exhibit evident boundaries (upper arrowhead). (I–K) Secretory emergences are located at the base of the labellum (I, dashed square); secretory epidermis and adjacent parenchyma of these glands exhibit a dense protoplast (J) and few small starch grains (K, arrow). (L) Note the occurrence of flocculent material in the vacuole (*) and osmiophilic bodies associated to SER (white arrowheads). (A, B, F, I) SEM. (C, D, G, J, K) LM. (H, L) TEM. (C) TBO+phe. (J) TBO. (G, K) PAS reaction. (D) Sudan black B. Scale bars: 100 µm (A, I, F), 75 µm (J, K), 50 µm (C, D, G), 10 µm (B), 0.1 µm (E, H, L).

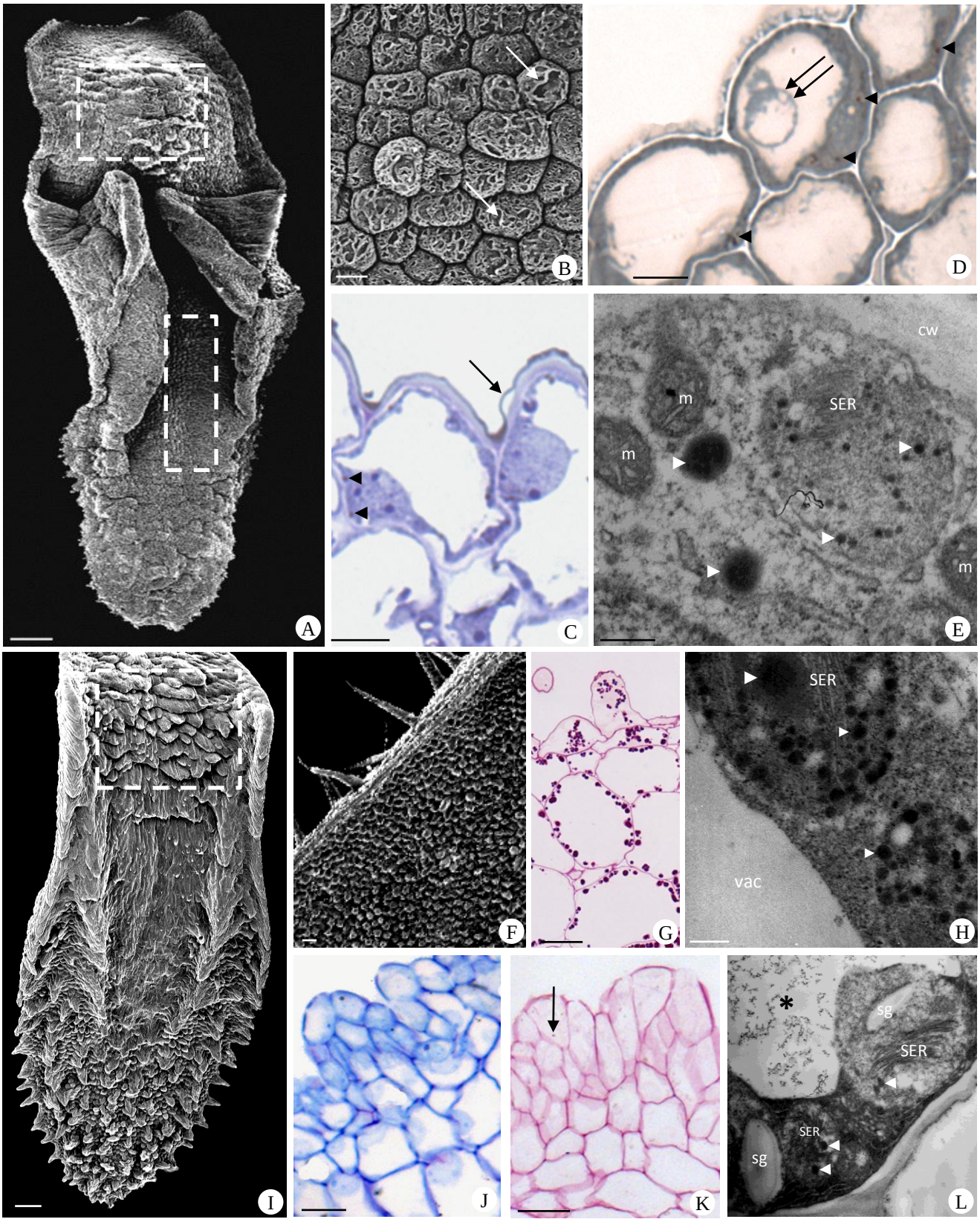
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Fig. 5. Nectary unicellular trichomes in the labellum of *Octomeria crassifolia* (A–C) and *Anathallis obovata* (D–F). (A–B) Dome-shaped trichomes (A, dashed square) exhibit epicuticular striations (*inset)*. Nectary trichomes and parenchyma present dense protoplast confined to the periphery of the cells (B) and a large vacuole. (C) Detail of the protoplast: note the occurrence of vesicles (*) originated from dictyosomes (d) close to the cell wall, and osmiophilic bodies close to SER (smooth endoplasmic reticulum; arrowheads). (cw, cell wall; m, mitochondria; sg, starch grain). (D–E) Nectary trichomes all over the labellum; note the ornamentation of the conical glands (D, *inset*) and the occurrence of several lipid droplets in a dense protoplast (E, arrowheads). (F) Multi-depth paradermic confocal images (from top to base and from left to right) of Lucifer Yellow CH uptake; note the emission from nectary trichomes, parenchyma and vascular traces. (A, D) SEM. (B, E) LM. (C) TEM. (B) TBO. (E) TBO+phe. Scale bars: 100 µm (A, D), 50 µm (B, E), 10 µm (*inset* in A, D), 0.5 µm (C).

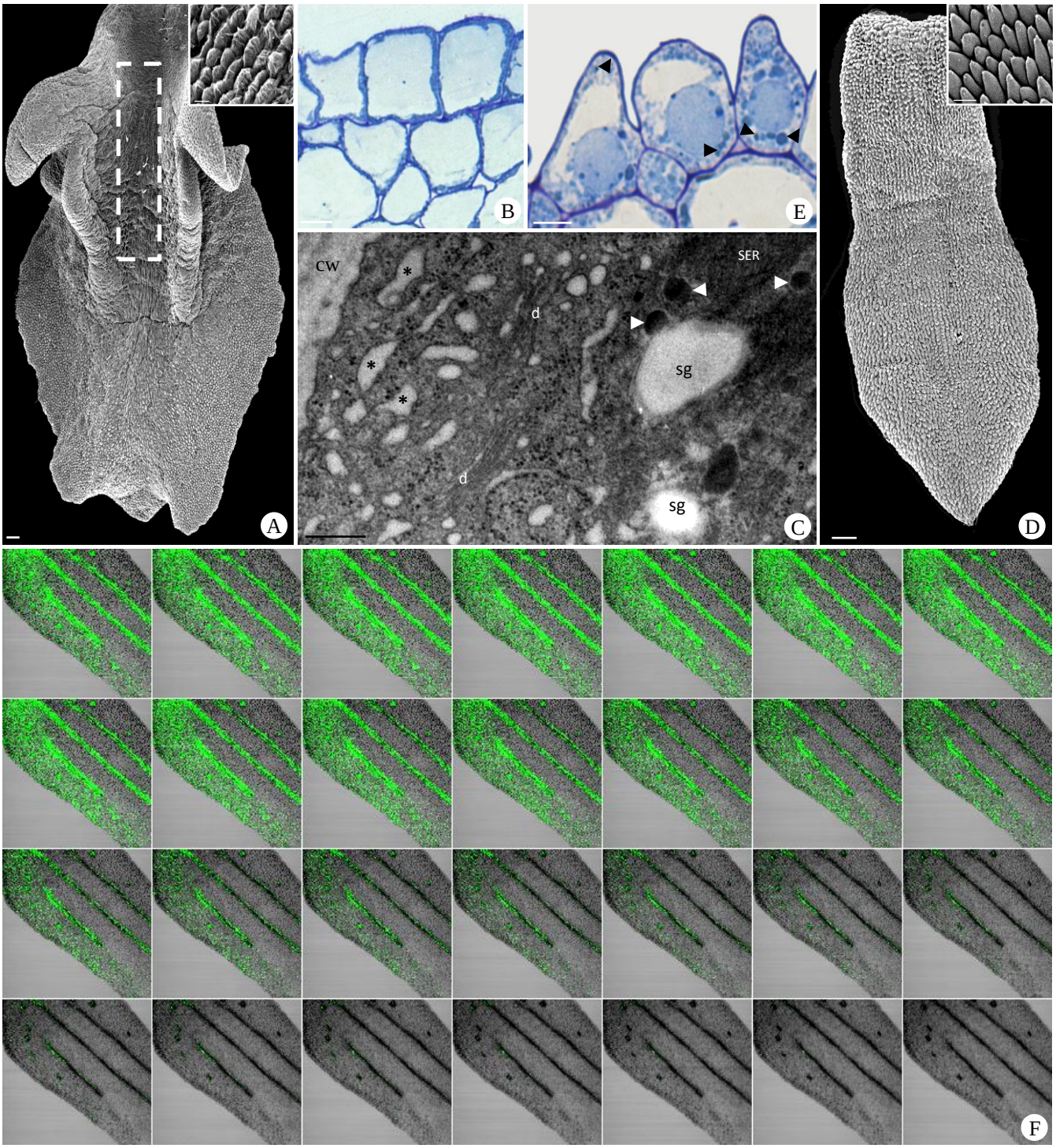
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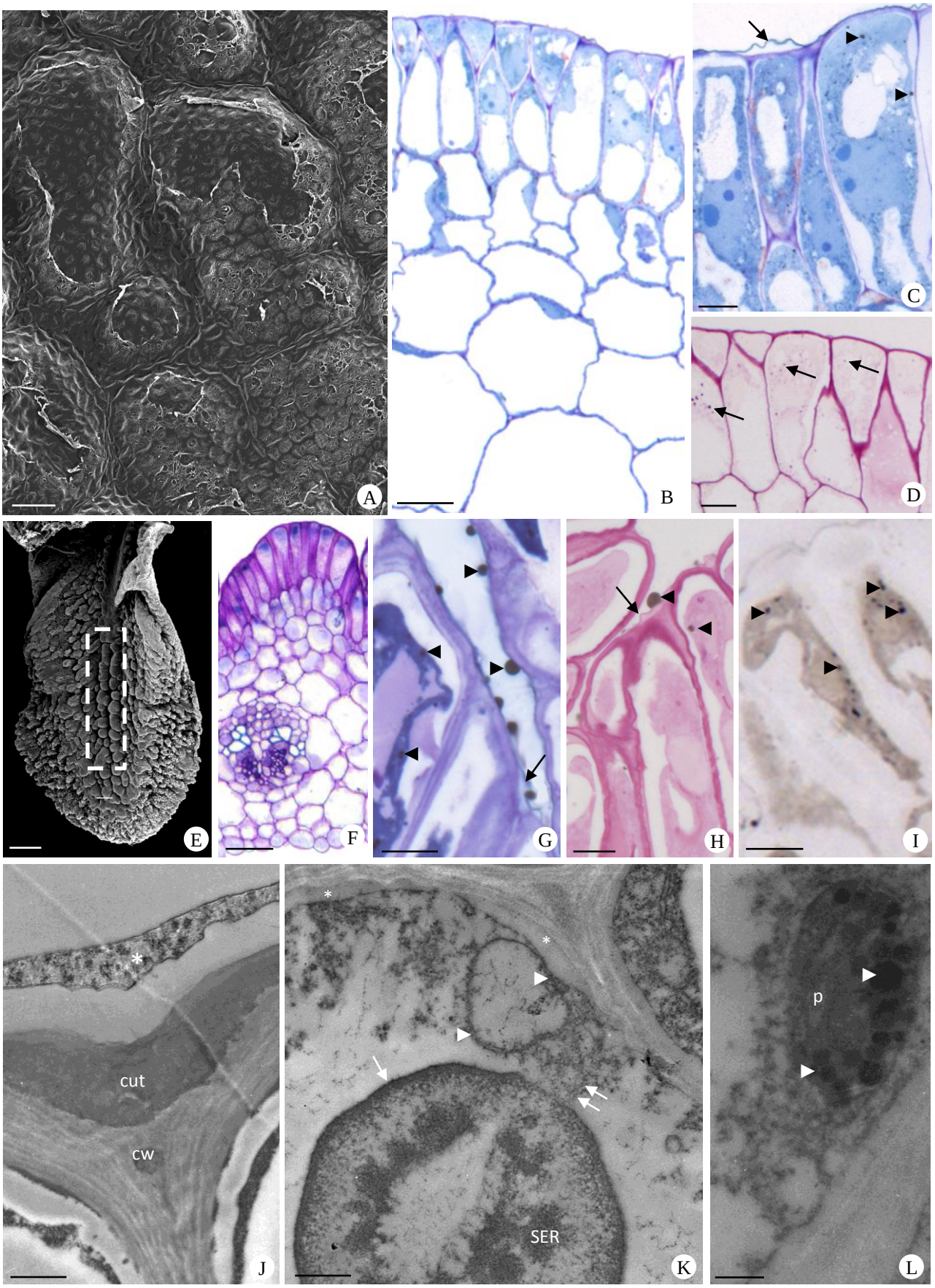
Fig. 6. Sticky-exudate glands of *Echinosepala aspasicensis.* Saliences formed by the elevations of the secretory palisade epidermis and the adjacent secretory parenchyma in lateral sepals (A–D) and labellum (E–I). (A–D) The cuticle is ruptured over the elevations (A); these glands are constituted by a palisade epidermis with different length and differential proliferation of adjacent parenchyma (B); the cuticle of the palisade epidermis detaches at anthesis (arrow in C), and the protoplast of these cells exhibits several lipid droplets (arrowheads in C) and small starch grains (arrows in D). (E–G) Glands occur in the central region of the labellum (E, dashed square); the palisade epidermis and adjacent parenchyma present dense protoplast (F) with several lipid droplets, also observed over the cuticle (G–I, arrowheads) that is detached in some regions (G, arrow). (H–I) The subcuticular secretion (H, arrow) exhibits a clearer intensity with PAS reaction comparing to the adjacent cell wall; note the higher affinity of the protoplast to PAS reaction (H) and to Sudan black B dye (I). (J) Secretion (*) on the surface of the cuticle (cut): an amorphous matrix and granules exhibiting different electron-densities. (K) A system constituted by SER and flocculate material associated is apparently delimited by a membrane (arrow); the flocculate material seems to leave this system toward the cell wall (double arrows). Note a larger vesicle and small ones (arrowheads) with fibrillar material merging to the extracytoplasmic space close to cell membrane and the existence of an amorphous content in the extracytoplasmic space (*). (L) Lipid droplets (arrowheads) inside a plastid (p) in the region of the same cell close to adjacent secretory parenchyma. (A, E) SEM. (B–D, F–I) LM. (J–L) TEM. (B, C, G) TBO+phe. (F) TBO. (D) PAS reaction. (H) PAS reaction+phe. Scale bars: 100 µm (E, F), 75 µm (B), 50 µm (C, D, G–I), 10 µm (A), 0.5 µm (J–L).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Discussion

Osmophores

The results showed in table 2 present a synthesis of the glands identified on sepals and labellum of the species examined here. The outstanding feature observed is the predominance of osmophores in the sepals or labellum or both, according to the species, that exhibit different morphologies as unicellular trichomes, elevations with a pore, and emergences. Both Pleurothallidinae and the Pantropical genera *Bulbophyllum* are considered the two largest non-related groups of myophilous orchids (Dressler 1993, Barbosa et al. 2009, Borba et al. 2011). Until now, there was no description of the occurrence of osmophores in the labellum of Pleurothallidinae species, in contrast with *Bulbophyllum* spp. where these glands are usually situated (Teixeira et al. 2004, Melo et al. 2010).

Table 2. Glands features in the sepals and labellum of Pleurothallidinae species.

Species	Sepals		Labellum
	adaxial surface	abaxial surface	
Octomeria crassifolia	Trichome osmophores	Trichome osmophores	Nectary trichomes
Echinosepala aspasicensis	Trichome osmophores at the base of sepals; sticky-exudate glands at the apex of lateral sepals	Non-secretory trichomes	Sticky-exudate glands
Acianthera aphthosa	Elevations with stomata pore osmophores	Non-secretory trichomes	Trichome osmophores
Acianthera fenestrata	Trichome osmophores	Non-secretory trichomes	Trichome osmophores
Anathallis obovata	Trichome osmophores	Trichome osmophores	Nectary trichomes
Zootrophion atropurpureum		Colleters*	Trichome osmophores
Phloeophila nummularia	Trichome osmophores	Non-secretory trichomes	Emergence osmophores

*Cardoso-Gustavson et al. 2014.

Ornamentations in the cuticle were observed in all unicellular trichome osmophores, more pronounced in the glands of the labellum. These ornamentations exhibit tactile stimulants that can be perceived by the pollinator; in addition, they can also mimic the body texture of the female insect, as already suggested by other sexually deceptive orchids (Singer et al. 2004, Woodcock et al. 2014).

As illustrated by the papillae of *Acianthera fenestrata*, the osmiophilic bodies can be visualized toward the cell wall, and the notable absence of vesicles suggest the secretion of volatile chemical compounds to occur by eccrine processes, being a constant feature in Pleurothallidinae species (Pridgeon and Stern 1983, 1985; Melo et al. 2010), although unusual in other orchid groups where granulocrine secretion is observed in the osmophores (Vogel 1990, Stpiczyńska 1993, 2001). Concomitantly to the visualization of osmiophilic bodies towards the cell wall, a more intense electron-dense region of the cuticle is probably where the volatiles progressively adhere or pass through it, and the beginning of the cuticle detachment is observed as a less electron-dense region of the same cuticle. In species that the volatiles are confined to the subcuticular space (although not visualized), is possible to suggest that the lipid bodies close to cell wall go through chemical reactions at the plasmatic membrane that increase the pressure of vapor of their constituent molecules, so the volatile fraction promotes the cuticle detachment and is progressively released to the environment by cuticular channels or promoting cuticle rupture.

The volatiles can be constantly emitted through cuticular diffusion or stomata, as observed in the sepals of *Octomeria crassifolia*, *Anathallis obovata*, *Acianthera aphthosa*, *A. fenestrata* and also in the labellum of *Phloeophila nummularia*, or they can be confined in the subcuticular region, causing its detachment. In the latter, the detachment can be followed by a cuticle rupture as in the glands present in the base of the sepals of *Echinosepala aspasicensis* and in the labellum of *Zootrophion atropurpureum*, or the compounds can cross the cuticle by diffusion, as in the

labellum of *Acianthera aphthosa* and *A. fenestrata*. In Pleurothallidinae, osmophore emission of volatiles by cuticular diffusion has been described in species of *Restrepia* (Pridgeon and Stern 1983), *Scaphosepalum* (Pridgeon and Stern 1985) and associated with the presence of stomata in species of *Acianthera* (Melo et al. 2010).

The main compounds that constitute the volatiles of the species examined here are hydrocarbons (mainly alkanes), carbonyl derivatives, aromatic compounds, nitrogen containing compounds (except *O. crassifolia* and *Acianthera fenestrata*), and terpenes (only in *O. crassifolia* and *Z. atropurpureum*). Alkanes and carbonyl derivatives are initially produced in the ER or Golgi and the last stages of their synthesis occur in the cytosol (Knust and Samuels 2009, McFarlane et al. 2014). Phenolic compounds are produced in the dictyosomes or endoplasmic reticulum and the polar ones (anthocyanin, flavonols, phenolic acids) are stored in the vacuole, while the nonpolar ones are present as oil bodies in the cytosol (Poustka et al. 2007, Solovchenko 2010). The synthesis of terpenes is initiated at the cytosol, where sesquiterpenes are completely synthesized; precursors and final synthesis of monoterpenes are observed in the plastids (Tholl et al. 2004, Chen et al. 2011). Lipid bodies are often intimately associated with and probably formed by the SER (Davies et al. 2003). According to our studies, lipid droplets (the osmiophilic bodies visible at LM; Davies et al. 2003, 2014) were observed in both TBO + p-phenylenediamine and Sudan black B[1] treatments, and under TEM in the cytosol associated to SER and inside plastids. Thus, by the methodology applied here, it is impossible to distinguish between the classes of compounds identified in the volatiles that were indeed observed under LM and TEM.

A large vacuole was observed in all the osmophores. In species that exhibit sapromyophilous features, this large vacuole is filled with anthocyanin, the phenolic compound

[1] Both Sudan black B (SBB) and p-phenylenediamine (p-phe) identify lipophilic compounds, with different mechanisms of reaction. The advantage of using p-phe over SBB is that the latter often yield suboptimal results that are difficult to interpret because of extraneous dye precipitate in plastic embedded sections (Anderson and Schochet 1982).

responsible for the purple color of these structures (Poustka et al. 2007), since no other class of volatile detected by GC-MS reported here can be stored in this organelle (e.g., mono and sesquiterpenes; De 2000). This large anthocyanin-vacuole is also observed in the nectary trichomes of *O. crassifolia* that are situated precisely in the regions of the purple spot. In the sepals of both *O. crassifolia* and *A. obovata*, the content of the large vacuole is probably related to the phenolic acids produced by these glands that are also constituents of the volatile emitted by them.

Floral nectaries

Nectar is usually offered as a reward to anthophilous species of Diptera (Singer and Cocucci 1999). Nectaries were observed as unicellular trichomes on the labellum of *Octomeria crassifolia* and *Anathallis obovata.* In both species, the secretion was profuse and its sugary composition indicated by the use of Glicofita Plus®. The nectar visualization and volatiles emission were higher during the morning in accordance with the period in which the pollination preferentially occurs by both male and female of *Bradysia* sp. (Diptera: Sciaridae) seeking for nectar in *O. crassifolia* (Barbosa et al. 2009; Borba et al. 2011). Although the breeding system of some species of *Anathallis* is well characterized (Gontijo et al. 2010), their effective pollinators are still unknown.

The presence of exposed nectaries on the labellum seems to be common in myophilous orchids (Borba and Semir 1998, 2001; Melo et al. 2010). Structurally, some floral nectaries exhibit many dictyosomes with abundant secretory vesicles (Stpiczyńska et al. 2003, Wist and Davis 2006, Nepi 2007), contrasting to elaiophores (Davies et al. 2014) and osmophores (Stpiczyńska 1993, and also see the aforementioned ultrastructure of osmophores). The protoplast of both *O. crassifolia* and *A. obovata* exhibit many vesicles of dictyosomes' origin towards the cell wall, what would characterize the secretion mode of these nectaries as granulocrine, in which

vesicles fuse to the plasma membrane (Fahn 1979). This process has been also observed in the nectaries of *Acianthera prolifera* (Melo et al. 2010) and other orchids (Stpiczyńska 1997, Stpiczyńska et al. 2005). However, current studies verified that this organelle (or endoplasmic reticulum) and its vesicles are not involved in the transport of nectar sugar, thus the specific function of the endomembrane system in nectary cells remains unknown (Vassilyev 2010). This highlights the fact that despite the ecological and evolutionary importance of nectar, mechanisms controlling its synthesis and secretion remain largely unknown (Orona-Tamayo et al. 2013).

At anthesis, the apex of the labellum of *A. obovata* is curved down, in a way that the nectar is concentrated at this region, which explains the higher reabsorption of the nectar observed at the tip of the labellum. We demonstrated using Lucifer Yellow CH that the nectar is reabsorbed 24 h after the application of the solution. Since we performed the experiment at the second day of anthesis, is plausible to assume that this event occurs concomitantly to nectar release, another indicative that the reabsorption is a phase of nectar dynamics that occurs currently with secretion (Nepi and Stpiczyńska 2008, Cardoso-Gustavson and Davis 2014 – appendix 2). Nectar reabsorption was verified by microautoradiography techniques from unicellular trichomes present in the spur, reaching other tissues of the flower as ovary wall and even the ovules of *Platanthera chlorantha* (Orchidaceae: Orchidoideae); contrasting to *A. obovata*, this event occurs in the end of the anthesis (Stpiczyńska 2003; Nepi and Stpiczyńska 2007, 2008). It is also interesting to note that reabsorption have been already described only in flowers in which the glands are somehow hide (Stpiczyńska 2003; Nepi and Stpiczyńska 2007, 2008; Cardoso-Gustavson et al. 2014), and its noteworthy that sugar reabsorption – and not only the evaporation of the aqueous fraction of the nectar – can occur even on species that present a small quantity of nectar in an "open" structure, as the labellum of *A. obovata.*

Lipophilic droplets and osmiophilic bodies associated with SER were visualized in the protoplast of the *O crassifolia* and *A. obovata* nectary trichomes, respectively, an indicative that these cells are also involved in the production and release of nectar volatiles. Lipids are common in the nectar of fly-pollinated flowers, in which the flies presumably have digestive lipases or esterases to break them down (Woodcock et al. 2014 and references therein). The scent of nectar itself is an honest signal to nectar-feeding animals (Raguso 2004, Heil 2011); the profile of volatiles emitted by the nectar can differ from those emitted by the osmophores to guide the pollinator conducting them to this reward.

Sticky-exudate glands

The adaxial surface of the labellum and apices of lateral sepals of *Echinosepala aspasicensis* present a glistering sticky-exudate. Contrasting to all the remaining species examined here, the sticky-exudate glands exhibit unique features. Regarding the anatomy and ultrastructure of the secretory cells, the resemblance of epidermal elaiophores[2] present on melittophilous orchid species is remarkable, as the observation of the exudate in the subcuticular space causing its detachment and posterior rupture, lipid droplets in the protoplast, a predominance of SER and osmiophilic bodies inside plastids (Davies and Stpiczyńska 2008; Davies et al. 2003, 2014). In fact, a system composed by SER organelles was observed in the epidermal cells, indicating an intense synthesis of oil lipids (Davies et al. 2003).

Conversely, structural aspects also resemble glands that produce mucilage (*e.g.*, colleters and mucilage cells of the transmitting tissue), as a strong result with PAS reaction in the exudate present at the subcuticular space and in the protoplast, an amorphous content in the extracytoplasmic space and small vacuoles with a fibrillar content (Miguel et al. 2006, chapter 4

[2] Elaiophores are glands generally present in surface areas of the labellum that produce wax-like materials and oils (Vogel 1990). These glands appear as trichomes, palisade-like epidermis or both (Pansarin et al. 2009).

and references therein). Thus, the glands of *E. aspasicensis* present features that allowed us to characterize them as glands of heterogeneous exudate (with lipids and polysaccharides). A strong indicative of this heterogeneous nature is the secretion observed under TEM, in which an amorphous matrix and many granules of different electron-densities are observed; the preservation and visualization of the exudate over the glands are indeed possible in both elaiophores and mucilaginous glands (Davies et al. 2014; chapter 4 and references therein).

A shiny appearance of a secretion may be attractive to some food-deceptive flies, as observed over the staminode of *Paphiopedilum villosum* (Orchidaceae: Cypripedioideae) that is shiny and slippery, composed by fatty oil (Bänziger 1996). It is also known that food/brood-deceptive pollinators are attracted by the unpleasant odor and the aspect of decaying (Jersáková et al. 2006, Urru et al. 2011). In *E. aspasicensis*, the sticky-exudate has a heterogeneous (lipids and polysaccharides) constitution. Our prediction is that the pollinator would be attracted initially by the odor emitted by the unicellular trichomes osmophores present at the base of the sepals that emit unpleasant odor rich in alkanes (dodecane, tridecane, tetradecane), phenolic compounds (benzene-derivatives) and amides, classes of chemical compounds also emitted by decaying organisms, feces and some mushrooms (Garner et al. 2007, Urru et al. 2011, Siddiquee et al. 2012, Woodcock et al. 2014). Thus, this shiny sticky-secretion associated to the unpleasant odor may possibly be related to food/brood deception pollination.

Floral volatiles: ecological and phylogenetic considerations

Concerning the attraction of pollinators, the floral fragrances of six orchids from different Epidendroideae subtribes (*Stanhopea lietzei*, *S. insignis*, *Polystachya estrellensis*, *Psilochilus modestus*, *Epidendrum secundum* and *E. paniculatum*) were described and compared to the offered rewards (pollen, nectar, pseudopollen and fragrant oil), so that the authors concluded that

in most pollinations, the attraction could not be assigned to one compound or class of compounds but to the "bouquet" that is an ensemble of compounds belonging to different compound classes (Reis et al. 2004). Thus, the compounds of floral bouquet of the species analyzed here have been compared to data available in the literature as regard to their odor, presence in other sapromyophilous taxa, possible role as semiochemicals[3], and presence in decaying organic matter. However, the prediction of pollinators as well as their behavior in the flower based solely on the volatiles emitted is completely speculative. Besides, the floral biology of the species analyzed here is beyond the scope of the present study.

Volatiles are used as semiochemicals by Diptera species; at close range, odor can act similarly to visual nectar guides, by directing the visitor to the resources, and position them appropriately to contact sexual structures and perform pollination (Woodcock et al. 2014). *Echinosepala aspasicensis*, *Acianthera fenestrata*, *A. aphthosa*, *Zootrophion atropurpureum* and *Phloeophila nummularia* exhibit features typical of sapromyophilous flowers, as a strong purple and yellow color, with purple lines and dots, unpleasant volatiles, and absence of nectar (Humeau et al. 2011, Woodcock et al. 2014). The “lateral windows”, regions of detachment of the coherent sepals (chapter 1), observed in *A. fenestrata* and *Z. atropurpureum* are also linked to this pollination syndrome, since it acts as attractants and direct the movements at close range of positively phototactic visitors (Woodcock et al. 2014 and references therein). The absence of nectar in sapromyophilous systems is effective in trap-like mechanisms, as exhibited by some *Bulbophyllum* species (Teixeira et al. 2004), and it is related to pollinators that are attracted for oviposition rather than food instinct (Woodcock et al. 2014). Only the basal *O. crassifolia* and *A. obovata* exhibit myophilous features as clear color, pleasant odor, and nectar reward (Jersáková et al. 2006, Woodcock et al. 2014).

[3] Semiochemicals are chemicals that mediate interactions between organisms. Semiochemicals are subdivided into allelochemicals and pheromones depending on whether the interactions are interspecific or intraspecific, respectively (Reddy and Guerrero 2004).

Contrasting to the other species, the floral bouquet of *Acianthera fenestrata* was the simplest one, composed mostly by alkanes and also by some alcohols, carboxylic acids and a chloroalkane, all of them related to fungal fecal-like odor (Wood et al. 2003). Although we found compounds related to unpleasant odors in *E. aspasicensis*, *Z. atropurpureum* and *P. nummularia*, in which alkanes were the higher compounds emitted (also benzoic acid in *Z. atropurpureum*), in these three species a peak related to nonanal is also verified. This compound exhibits a pleasant odor (Jørgensen et al. 2000). The occurrence of a lower concentration of a pleasant compound among unpleasant ones, as nonanal and also D-limonene (monoterpene) in *Z. atropurpureum*, is also related to the deceptive pollination. The flowers attract pollinators by their form and odor, acting as a sensor trap upon flies searching for food or brood site when neither of these are present. In addition, a pleasant scent arising from the center of the flower not previously reported because it was hidden by the stench may deter the flies from laying doomed eggs (Woodcock et al. 2014).

Some Diptera can obtain rewards from flowers in the form of chemical precursors to sexual attractants or phenylpropanoid derivatives as defensive compounds against predators (Woodcock et al. 2014). Except from *A. fenestrata*, the remaining species present these derivatives also in a higher concentration as *Z. atropurpureum* and *A. obovata.*

The flies usually identified as a pollinator in Pleurothallidinae species are *Bradysia* sp. commonly known as fungus gnats because they are almost always dependent upon fungal substrates for larval development (Duque-Buitrago et al. 2014). Nitrogen containing compounds are the main constituents of the fecal-like odor emitted by fungi (Wood et al. 2003, Saddiquee et al. 2012) being also detected in other non-related sapromyophilous species as *Caralluma europaea* (Apocynaceae; Formisano et al. 2009), *Bulbophyllum variegatum* (Humeau et al. 2011), and *Astragalus* species (Fabaceae; Platikanov et al. 2005). These compounds were detected in all

species here examined but *O. crassifolia* and *A. fenestrata*; in these species, the other classes of compounds also present representatives that exhibit unpleasant odor. Also interesting, this absence of nitrogen containing compounds in *A. fenestrata* contrast to the results already described for *Acianthera* species in which nitrogen compounds are usually directly attributed to the strong odor emitted by these flowers (Melo et al. 2010).

Regarding the myophilous species *O. crassifolia*, nectar and higher emission of volatiles were observed during the morning, coinciding with the period of higher frequency of flower visitation and pollination in the field, that occurs between 0600 and 0800 hours, with a second peak between 0900 and 1000 hours (Barbosa et al. 2009; Borba et al. 2011). Also, our volatile analysis (Table 1) detected some compounds as carboxylic acids, aromatic alcohols, ketones, mono and sesquiterpenes only in the second period of the sampling, which we assume to be directly related to the specific pollinator attraction of *O. crassifolia*, both male and female of *Bradysia* sp. (Diptera: Sciaridae) seeking for nectar (Barbosa et al. 2009). No data concerning the differential attraction of male or female or even of different pollinators caused by the compounds specifically emitted during the second sample was observed to date. Another possibility to this differential detection could be associate to the repellence of nectar robbers, as observed in *Osmanthus fragrans* (Oleaceae) in which the floral bouquet discourages the forage behavior of a butterfly *Pieris rapae* (Omura et al. 2000).

Contrasting to *O. crassifolia*, no differences between the chemical compounds emitted by the flowers of *A. obovata* were observed. While the volatile's profile of *O. crassifolia* is rich in mono and sesquiterpenes, the one from *A. obovata* present several compounds associated to sapromyophily, as benzene-derivatives, amides, and indole. Benzene-derivatives and amides are usually described in sapromyophilous species (Formisano et al. 2009, Urru et al. 2011). Indole is found in human feces and is reported to have intense fecal odors at high concentrations,

becoming pleasant in very dilute solutions (Wood et al. 2003), in which assumes odor of mothball, floral and sweet (Formisano et al. 2009, Bryant and McClung 2011). Despite the occurrence of these compounds, an agreeable odor was perceived from these flowers at anthesis, caused by the occurrence of nonanal, eugenol, cynnamyl acetate, lilyal and versalide. These aldehydes and aromatic compounds are related to pleasant odors even in low concentrations (Dötterl et al. 2005, Wolski et al. 2006, Tan et al. 2006). Due to the several differences found comparing the profile from both species, we presume they must present different species (maybe genera) of pollinators, although presenting the same search for nectar.

Interestingly, methyl salicylate (MeSa) was detected only in the volatile of both species. MeSa is known to mediate relationship between plants and negatively affects the attraction of parasitoids in herbivore-induced plants (Snoeren et al. 2010), and may, at least in *O. crassifolia*, avoid the attraction of *Bradysia* parasitoids. This indication is plausible since parasitoids use floral and fruit chemical cues to locate their hosts (Stuhl et al. 2011).

The use of floral fragrance variation in evolutionary and ecological studies was successfully applied in eight taxa (including two putative varieties) of *Cypripedium* (Orchidaceae: Cypripedioideae), in which the authors observed the formation of clusters that indicated a high degree of biogenetic uniformity among some species as well as substantial relative divergence between others, resulting in good parameter to establish phylogenetic groups and also to estimate pollinators' relationships (Barkman et al. 1997).

Even on species of the pantropical *Bulbophyllum*, genus that present strong convergence with the neotropical Pleurothallidinae (Borba et al. 2011), the highest compounds are related to ketones, while in Pleurothallidinae are clearly the alkanes, present in all species examined here. Further, in data available for *Bulbophyllum* spp. alkanes are absent (Kaiser 1993, Keng-Hong and Nishida 2005, Tan et al. 2006, Humeau et al. 2011). In Pleurothallidinae, even when these

compounds are at lower concentration, there are several kinds of alkanes present in floral bouquet, thus their sum being at least qualitatively expressive. In this context, we can hypothesize that the alkanes would be a chemical marker of Pleurothallidinae. Unfortunately, the volatile data are still really scarce, but another hypothesis can be brought analyzing the chemical profile already described in Laeliinae species, sister group of Pleurothallidinae, where alkanes are also absent (Hills et al. 1968, Williams 1981, Kaiser 1993, Higgins 2003). Therefore, we hypothesize the occurrence of alkanes in the floral bouquet as another synapomorphy of Pleurothallidinae.

Borba et al. (2011) by proposing the split of the currently circumscription of Pleurothallidinae, also hypothesized that myophily (flies seeking for nectar) is a plesiomorphic characteristic in the myophilous clade of the subtribe in spite of the most common occurrence of sapromyophily (pollination by female flies by deceit) and may represent a transition from rewarding melittophily to deceptive sapromyophily. The morphology and occurrence of terpenes in the flower volatiles as identified in *O. crassifolia*, sister group of the remaining Pleurothallidinae (Pridgeon et al. 2001, 2010; Borba et al. 2011), seemed coherent with mellitophylous pollination; in fact, this syndrome was proposed to this species before the evaluation of fly-pollination (see details and discussions in Barbosa et al. 2009, Borba et al. 2011). Not surprisingly, alkanes are described as volatile compounds from non-bee sources (Smith et al. 2002).

It is also noteworthy that both machinery to produce carbohydrates and lipophilic compounds are present in the nectaries of *O. crassifolia* and *A. obovata* and also in the sticky-glands of *E. aspasicensis*. Considering the phylogeny of the species analyzed here (chapter 1), it is clear that the location where nectaries/heterogeneous sticky-glands are observed is replaced by osmophores in early-divergent species. We hypothesize that the occurrence of osmophores in the

labellum may be a case of homeosis[4], starting from a homeoheterotopic change from producing simple sugars (as nectaries of *O. crassifolia* and *A. obovata*), and heterogeneous secretion (sticky-exudate glands of *E. aspasicensis*) to completely lost of carbohydrate production remaining only the machinery to synthesize volatiles (osmophores in sapromyophilous), reinforcing the hypothesis of Borba et al. (2011) of the transition to deceptive sapromyophily in Pleurothallidinae.

Finally, the volatile analysis of the small ornithophylous-clade containing the genera genera *Dilomilis*, *Neocogniauxia*, and *Tomzanonia* presently included in Pleurothallidinae (Pridgeon et al. 2001, 2010) is needy and if the lack of alkanes-predominance were observed, this would strengthen our hypothesis that this chemical class is a synapomorphy of this subtribe.

[4] Heterotopy is any evolutionary change in the spatial location of a developmental program. It can be distinguished in two kinds: neoheterotopy, when a structure is generated in a novel location, and homeoheterotopy, in which genetic identity is transferred from a donor structure to another, pre-existing recipient structure. Homeosis is defined as a special case of homeoheterotopy in which all aspects of genetic identity are conferred upon the recipient structure (Baum and Donoghue 2002).

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Capítulo 4

The rostellum, stigma, style and ovarian transmitting tissue in Pleurothallidinae (Orchidaceae, Epidendroideae)

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(a ser submetido para Botany)

Abstract

Premise of research. The Pleurothallidinae is recognized as the largest orchid group pollinated by Diptera. The floral biology and mating systems of its species have been recently assessed; thus, a description of secretory tissues of the gynostemium and ovary will provide important insights to the knowledge of the events of pollination. We describe the ontogeny and structure of secretory tissues from species representing both early- and late-divergent clades of this subtribe.

Methodology. The gynostemium and ovary at several stages of development from species belonging to seven genera of Pleurothallidinae were examined using light, scanning and transmission electron microscopy.

Pivotal results. The rostellum differentiates and initiates its mucilaginous secretory phase early in flower development. Stigma transmitting tissue (TT) cells are arranged in a transversal secretory gradient (in relation to the gynostemium's length), composed of cells in transition zone (Tz), secretory (Sc), free-floating (Ff) and post-secretory (Ps) states. Tz cells are involved in the production and sending of precursors to Sc, the latter recognized by their angular detachment and mucilage secretion. The second phase of secretion, initiated at Ff, is constituted by lipophilic compounds, while Ps cells reside in the stigma's central region. Stylar TT cells resemble those of the stigma TT, except for the absence of detached cells. Ovarian TT, papillate obturator and absence of a specialized ovarian tissue acting as obturator, were observed. With respect to the papillate obturator, two different cell types with distinct modes of secretion were recognized.

Conclusions. The rostellum acts in pre-anthesis events, secreting a mucilaginous exudate involved in TT differentiation and secretion. A transversal analysis of the secretory cells constituting the stigma explains secretion dynamics in this wet, detached stigma type. The presence of lipophilic compounds in the cuticle and stigma can be related to self-incompatibility sites described in some Pleurothallidinae species.

Introduction

The Pleurothallidinae (Epidendoideae, Epidendreae) is one of the largest subtribes of the Orchidaceae and has been taxonomically reformulated due to the occurrence of poly and paraphyletic genera (Pridgeon and Chase 2001, Pridgeon et al. 2001, 2010). This subtribe is recognized as the largest orchid group pollinated by Diptera (Borba et al. 2011), although studies on floral biology and morphology are restricted to some self-incompatible species of *Anathallis* and *Octomeria* (Barbosa et al. 2009, 2013; Gontijo et al. 2010). Also, there is a lack of knowledge regarding the structure of secretory tissues present in the gynostemium and ovary that are directly involved in the pollination and fertilization processes of Pleurothallidinae, despite some of these processes being unique in this subtribe.

The rostellum and stigmatic, stylar and ovarian transmitting tissue are the secretory tissues present on the orchid gynostemium involved in the processes of pollen reception, adherence, penetration and growth of pollen tubes, anterior to the micropyle (van der Pijl and Dodson 1966, Clifford and Owens 1990, Arditti 1992, Dressler 1993). The rostellum is a structure whose origin has been discussed intensively (see van der Pijl and Dodson 1966, Arditti and Flick 1974, Dressler 1981, 1993, Rasmussen 1982, 1985, 1986, Arditti 1992, Yam et al. 2009). Currently (and in the present study), the rostellum is accepted as a modified (distal) portion of the median stigma lobe (Kurzweil and Kocyan 2002, Gamisch et al. 2013, Rudall et al. 2013). Functionally, it is a versatile physical barrier between the anther and stigma of the same flower that releases an adhesive, viscous exudate aiding pollinia adherence (van der Pijl and Dodson 1966, Arditti and Flick 1974, Arditti 1992, Dressler 1993, Gamisch et al. 2013). Generally, the rostellum is described as unsuitable for pollen germination, although pollen penetration through the rostellum was recently reported in *Bulbophyllum bicoloratum* (Gamisch et al. 2013). The rostellum is also a dynamic structure actively controlling many post-pollination

phenomena (Arditti and Flick 1974, 1976). Its secretory nature is defined as mainly mucilaginous, since it resembles the stigmatic exudate (Arditti and Flick 1974); structural aspects of the rostellum, however, remain underexplored.

Here we adopted the definition of Tilton and Horner (1980) in which the transmitting tissue refers to the one along which or whereby pollen tubes grow to reach the micropyle. It extends from the stigma to the ovary, may be partially or fully secretory, and consists of three regions: stigmatic, stylar and ovarian transmitting tissues. Stigma characters are useful in taxonomic studies (Heslop-Harrison 1981), and in orchids they display remarkable uniformity at subfamily, tribe and subtribe levels (Dannenbaum et al. 1989). Pleurothallidinae species have the IIIb stigma type (Dannenbaum et al. 1989), also named wet detached cell stigma (Calder and Slater 1985, Slater and Calder 1990), characterized by a cuticle covering the stigmatic cavity that consists of detached cells suspended in a mucilaginous matrix (Calder and Slater 1985, Dannenbaum et al. 1989). These floating cells were also referred to as eleutherocytes in *Dendrobium* by Johansen (1990), who also suggested that the incompatibility response found in this genus is probably controlled by these cells. There are few reports of the structural aspects of the transmitting tissue in the style, but all studies indicate that the secretory cells are similar to those of the stigma (Clifford and Owens 1990, Leitão and Cortelazzo 2010).

Ovarian transmitting tissue or the obturator is an anatomical modification of the transmitting tissue in the ovary region whose function is to direct pollen tubes toward the micropyle (Tilton and Horner 1980). Anatomical descriptions of the obturator in monocots are well documented in families such as Liliaceae (Tilton and Horner 1980), Rapateaceae (Venturelli 1988), Pontederiaceae (Scribailo and Barrett 1991), Cyperaceae (Coan et al. 2008), Bromeliaceae (Fagundes and Mariath 2010) and Mayacaceae (Oriani and Scatena 2012). The occurrence of intra-ovarian mucilage trichomes also acting as obturator is reported to Acoraceae,

Asteliaceae, Araceae and Hanguanaceae by Rudall et al. (1998), who present a revision of the families where these trichomes are reported. To date, references to the presence of this tissue in Orchidaceae are restricted to species of subtribe Oncidiinae (Clifford and Owens 1990, Leitão and Cortelazzo 2010). In both taxa, the obturator is a continuation of the transmitting tissue from the style, although less differentiated in the ovary of *Rodriguezia venusta*.

This investigation concerns the gynostemium and ovary structure of seven species of Pleurothallidinae. Our study provides a deeper comprehension of pollination mechanisms that involve the pollinia and the need for specific pollinators that may limit the reproductive success of these species. Selected species chosen for this study represent both early- (*Octomeria crassifolia* from *Brachionidium-Octomeria* clade) and late-divergent clades (*Echinosepala aspasicensis* from *Echinosepala-Barbosella* clade; *Acianthera aphthosa*, *Acianthera (Criptophoranthae) fenestrata* from *Acianthera* clade; *Anathallis obovata* and *Zootrophion atropurpureum* from *Zootrophion-Trichosalpinx* clade; *Phloeophila nummularia* representing the remaining Pleurothallidinae clade), according to the phylogenetic relationship described in Pridgeon et al. (2001, 2010).

The aims of this study are to: (1) describe the ontogeny of the gynostemium and ovary of Pleurothallidinae species to determine the origin and pre-anthesis events of rostellum and transmitting tissues; (2) characterize the secretory phases of the tissues by light and transmission electron microscopy; and (3) verify the existence of structural patterns of the rostellum and transmitting tissue in this subtribe. The ontogeny and comparison between gynostemium morphology of different genera from Pleurothallidinae also provide useful information on the assessment of homologies in the subtribe.

Material and Methods

Plant material

Flowers were collected from plants cultivated in the living orchid collection of the Núcleo de Pesquisa Orquidário do Estado, Instituto de Botânica (São Paulo, Brazil). All were collected in Brazil and are listed below with provenance and accession numbers. *Acianthera fenestrata* ("number pending") was recently incorporated to the collection.

Acianthera aphthosa (Lindl.) Pridgeon & M.W.Chase: São Paulo, Reserva Ecológica do Morro do Diabo, 14854, 14855; Minas Gerais, Camanducaia, P1082.

Anathallis obovata (Lindl.) Pridgeon & M.W.Chase: Guaira, Região de Sete Quedas, 12599, 12611, 13299; Reserva Ecológica do Morro do Diabo, 14263, 14294; Boracéia , 14359; Carangola, 14961.

Acianthera fenestrata (Barb.Rodr.)Pridgeon & M.W.Chase , number pending.

Echinosepala aspasicensis (Rchb.f.) Pridgeon & M.W.Chase: Serra do Sincorá, Ibiacoara, 12306; Serra da Neblina, 16306.

Octomeria crassifolia Lindl.: Serra da Piedade, 5666; Campos do Jordão, 12096; Carangola, 14866.

Phloeophila nummularia (Rchb.f.) Garay: Bananal, 18235.

Zootrophion atropurpureum (Lindl.) Luer: Serra do Mar, 678; Carangola, 14998; Boracéia, 16936.

The material was fixed in Karnovsky solution (Karnovsky 1965, modified by Kraus and Arduin 1997) for 24 h for morphological and anatomical purposes. The samples were washed, dehydrated and stored in 70% ethanol.

Scanning electron microscopy (SEM)

The gynostemium and ovary were isolated from flowers, further dehydrated to 100% ethanol and rinsed in a hexamethyldisilazane (HMDS) series (33.3, 50.0, and 66.6% v/v in 100% ethanol) and then three times in 100% HMDS for 1min each (Jeger et al. 2009) to dry the material. Samples were mounted on stubs, coated with gold palladium in a Hummer 6.2 sputtering system (Anatech, Union City, CA, USA) and viewed with a JSM-541OLV SEM (JEOL, Tokyo, Japan) at 10 kV. Digital images were edited using Adobe Photoshop version 7.0.

Light microscopy

The material was processed using standard methods for Paraplast® (Fisher Healthcare, Houston, Texas, USA) or Leica Historesin® (Heraeus Kulzer, Hanau, Germany) embedding, then serially sectioned at 5 (Paraplast® sections) or 1.5–2 µm (Historesin®) thickness. Sections prepared with Paraplast® were stained with safranin and astra blue (Gerlach 1969) and mounted in Permount® (Fisher Scientific, Pittsburgh, Pennsylvania, USA); sections prepared with resin were stained with toluidine blue O (TBO) (Sakai 1973) and mounted in Neomount resin.
Histochemical tests were performed on both structural and semi-thin sections: TBO and p-phenylenediamine were used for metachromasy and lipid localization (Kivimäempää et al. 2004); Sudan black B for total lipids and starch (Bronner 1975); PAS reaction for total polysaccharides (McManus 1948); and ruthenium red (Gregory and Baas 1989) and coriphosphine (observed under blue light; Weiss et al. 1988) for pectin. Appropriate controls were performed simultaneously.

Sections were viewed and digitally photographed with an Olympus BX53 compound microscope equipped with an Olympus Q-Color 5 digital camera with Image Pro Express 6.3 software using either diascopic and episcopic (fluorescence) light illumination according to the

stain procedure.

Transmission electron microscopy (TEM)

Gynostemia and ovaries were isolated and immediately fixed at room temperature in 3% glutaraldehyde and 0.2 M cacodylate buffer, pH 7.25 (Marques et al. 2013). After several washes with cacodylate buffer, the material was post-fixed in 1% OsO_4 for 2 h, dehydrated to 100% ethanol and then embedded in LR White embedding medium (Ted Pella Inc., USA). Semi-thin sections (0.5 μm) were cut using glass knives and stained in 1% TBO in distilled water. Thin sections were mounted on copper grids and contrasted 20 min in 0.5% uranyl acetate, followed by 5 min staining in 1% lead citrate. Grids were viewed with JEM 1200 EXII and Philips CM10 transmission electron microscopes at 80kV.

Results

Morphological aspects

All the Pleurothallidinae species examined here exhibited an inferior ovary, a labellum free and articulated to the gynostemium by its foot, and two small petals (Fig. 1A). The gynostemium is characterized by more or less conspicuous lateral wings (according to species), an evident stigmatic cavity, a prominent rostellum and the anther cap, below which the pollinia are observed (Fig. 1B).

Micromorphology of the rostellum, stigma, style, and ovary

At anthesis, all species showed a prominent rostellum curved downward toward the stigmatic cavity, preventing visualization of the median lobe of the stigma in frontal view, but not blocking the observation of the confluent lateral lobes (fig. 2*A*–*G*). The stigma is concave, except for *Acianthera fenestrata* in which the cells formed a convex shape with a cavity apparent

in the central region (fig. 2*D*).

The rostellum exudate was copious and viscous (fig. 3*C*), resembling the stigmatic secretion (fig. 2*E*). Pollinia with growing pollen tubes were observed attached to the rostellum and stigma surface in some open flowers of *A. fenestrata* (fig. 3*E*), indicating that the exudate from both structures assisted adherence of the pollinia and the germination and growth of pollen tubes. A cuticle with variable thickness and a ridged nature covered the stigmatic cavity (fig. 2*B*, 3*B*), below which detached cells of the stigma occurred (fig. 3*A*–*B*). The transmitting tissue started from the stigma and lined the style (fig. 3*A*, *D*). Numerous pollen tubes that had grown close to the ovules were evident in the ovary locule (fig. 3*F*).

Development of the secretory tissues of the gynostemium

Folding of carpel primordia in the very young flower bud led to formation of the stylar canal and ovary cavity (fig. 4*A*). Protodermal cells internalized by folding of the carpel did not merge, giving rise to the stylar canal. Differentiation of the glandular rostellum occurred early in flower development and its secretory phase began while the gynostemium was still immature. The copious exudate reached the developing ovary, lining the entire morphological surface of the pollen tube transmitting tract (fig. 4*B*). Although in this bud stage the stigma and transmitting tissue cells were not completely differentiated, secretory cells were easily recognized. At the pre-anthesis stage, median and lateral lobes of the stigma were evident, as well as loosely attached cells filling the stylar canal and limiting the stigmatic cavity (fig. 4*C*). The rostellum possessed a secretory palisade epidermis whose cells had dense protoplasts with nuclei oriented to the outer secretory surface (fig. 4*D*). The adjacent secretory cells were smaller and presented dense protoplasts; no vascular trace directly supplied this gland (fig. 4*D*). There was no indication of cuticle distension or rupturing.

Flowers at anthesis: structure, ultrastructure and histochemistry of the secretory cells

Stigma and style transmitting tissue

With light microscopy (LM), it was difficult to recognize which cells from the stigma and style (besides detached cells) were secretory, because their protoplasts were not easily visualized even from semi-thin sections. Thus, we linked the results from LM and transmission electron microscopy (TEM) to present a panorama of the secretory cells in these tissues.

Except for species-specific occurrences of detached stigmatic cells and the relative thickness of the cuticle that covered those cells (fig. 5*A*), the secretory cells of the stigma and style presented common features. Ordinary parenchyma cells constituted the cortex of stigma and style (fig. 5*B*). Adjacent to these parenchyma cells, there was a zone of small-diameter cells – eventually cells with the same size and with no clear distinction from ordinary parenchyma – often referred to in the literature as a "transition zone" (fig. 5*B*). As this term is well known, we maintain its nomenclature, but to include "cells" to recognize it as a specific tissue. Transition zone cells contained starch grains (fig. 5*C*) and mucilage (fig. 5*A*).

"Secretory cells" were those that effectively released the mucilaginous secretion to the extracellular medium (fig. 6*A*). Countless plasmodesmata connected the junctions of secretory cells to those of the transition zone (fig. 6*B*). Cells of the secretory stage were also recognized by the presence of a thin cell wall and very abundant mucilaginous exudate in the extracytoplasmic space, promoting a retraction of the protoplast (fig. 6*A*). Vesicles with fibrillar content were seen in the protoplast of secretory cells; some other small vesicles, with different content from the larger ones, were evident toward the cell wall. These vesicles appeared to merge into a larger vesicle whose content is merging in the cell wall, indicating a granulocrine secretion (fig. 6*B*, *inset*).

Close to the secretory cells, cells at a "free-floating" stage were evident in the

mucilaginous matrix. Under LM, these cells were distinguished by their conspicuous protoplast, large vacuome and lipid droplets (fig. 5*D*). Under TEM, these cells were finishing the phase of mucilage secretion, in which this exudate in the extracytoplasmic space gradually decreased, remaining visible as a diminished quantity near their junctions (fig. 6*A*, *C*). Large lipid droplets of uncertain origin occurred in the cytoplasm of secretory cells at this stage, and small granules were seen toward the cell wall, and also around the secretory cell, while the cell wall itself appeared randomly thickened and disorganized (fig. 6*C*). In the very inner region of the stigma, the detached cells were plasmolysed and devoid of secretory activity (fig. 6*D*). These cells dominated the "post-secretory" phase; no evidence of cell death was detected. In some sites without cells in the extracellular matrix, the exudate consisted of a dense lipophilic and an opaque polysaccharidic fraction (fig. 6*E*).

The fibrillar mucilage from the cytoplasm, the extracytoplasmic space and from the extracellular matrix between secretory cells showed different aspects under TEM. This difference was also dependent on the region of the transmitting tissue, wherein the mucilage changes toward the inner region of the stigma (fig. 6*A*, *C*) or style.

Under LM, the mucilage between the attached secretory cells and the extracellular matrix had different metachromasy by TBO (fig. 5*B*), although the same pectic and polysaccharidic nature was demonstrated by ruthenium red and the PAS reaction, respectively (fig. 7*A*, *B*). The exudate consisted not only of polysaccharides but also had a lipophilic fraction, as indicated by staining with Sudan black B (fig. 7*C*). It was also interesting that both ruthenium red stain and the PAS reaction resulted in a strong color within the exudate between secretory cells, weakening in the central region of the style, while the opposite occurred with Sudan black B.

Obturator

All species except for *Acianthera aphthosa* (fig. 8*A*) are characterized by ovarian tissue functioning as an obturator, which, in some species, reaches the placenta. Secretion from the obturator cells was detected in flowers at anthesis, regardless of their pollination status. In *Echinosepala aspasicensis* and *Zootrophion atropurpureum* (fig. 8*B*, *C*), the obturator consisted of a continuum of the stylar transmitting tissue, with active secretory cells connected by their angulations and an absence of free-floating detached cells. This tissue was visualized prior to anthesis but was conspicuous in the ovary locules within flowers at anthesis, developing in the inner ovary wall and at the placenta base. At least in *E. aspasicensis*, there was a profuse exudate in the ovary locule with a different metachromasy compared to the region where secretory cells were active (fig. 8*B*).

In *A. aphthosa* and species that possess papillae, the transmitting tissue ends at the ovary entrance. Papillae are recognized in *Acianthera fenestrata*, *Anathallis obovata*, *Octomeria crassifolia*, and *Phloeophila nummularia* but are active only within flowers at anthesis (fig. 8*D*–*G*). Again, the structure and secretory nature of the papillae were elucidated by TEM. *Phloeophila nummularia* papillae possessed a thick cuticle and cell wall, in which the fibrils appeared loosely assembled and porous (fig. 9*A*). No wall ingrowths typical of transfer cells were observed. The fibrillar mucilage found in the protoplast and inside the vesicles resembles that of the secretory cells from the stylar transmitting tissue. The occurrence of vesicles near the cell wall may be indicative of a granulocrine mode of secretion. Granular lipophilic material in vesicles of uncertain origin was also identified in the protoplast of the papillae (fig. 9*B*).

The obturator papillae of *Acianthera fenestrata*, *Anathallis obovata* and *Octomeria crassifolia* had thin outer walls with thin cuticle that became detached during mucilage secretion. The exudate accumulated in the subcuticular space and was released to the extracellular space by

cuticle disruption (fig. 9*C*). There was no difference between the appearance of fibrillar mucilage and granular electron-dense material from the protoplast and subcuticular space. The lipophilic nature of these granules was also verified by Sudan black B (fig. 10*A*), as well as the cuticle of the papillae being thicker than the ordinary epidermis. The pectic nature of the exudate in the subcuticular space was confirmed by ruthenium red and coriphosphine; its presence in the subcuticular space promoted a smoky appearance to the cell wall of papillae (fig. 10*B*, *C*). In *Acianthera fenestrata*, developing pollen tubes were evident in the ovary locule. Beyond the secretory product itself, the lipophilic and hydrophilic constituents of pollen tubes in the locule were also evident from the histochemical reagents (fig. 10*A*–*C*).

The mucilaginous secretion was still available in the locule during seed development within the fruit of *Echinosepala aspasicensis* (fig. 10*D*).

Fig 1. Morphology of *Acianthera fenestrata* flower at anthesis. (A) Overview of the flower with sepals excised; note the gynostemium (gyn), petals (pet) and labellum. (B) Detail of the gynostemium, highlighting the lateral wings (*), stigmatic surface (arrow), rostellum (r) and anther cap, below which the yellow pollinia are visible. Scale bars: 0.5 cm (A), 0.3 cm (B).

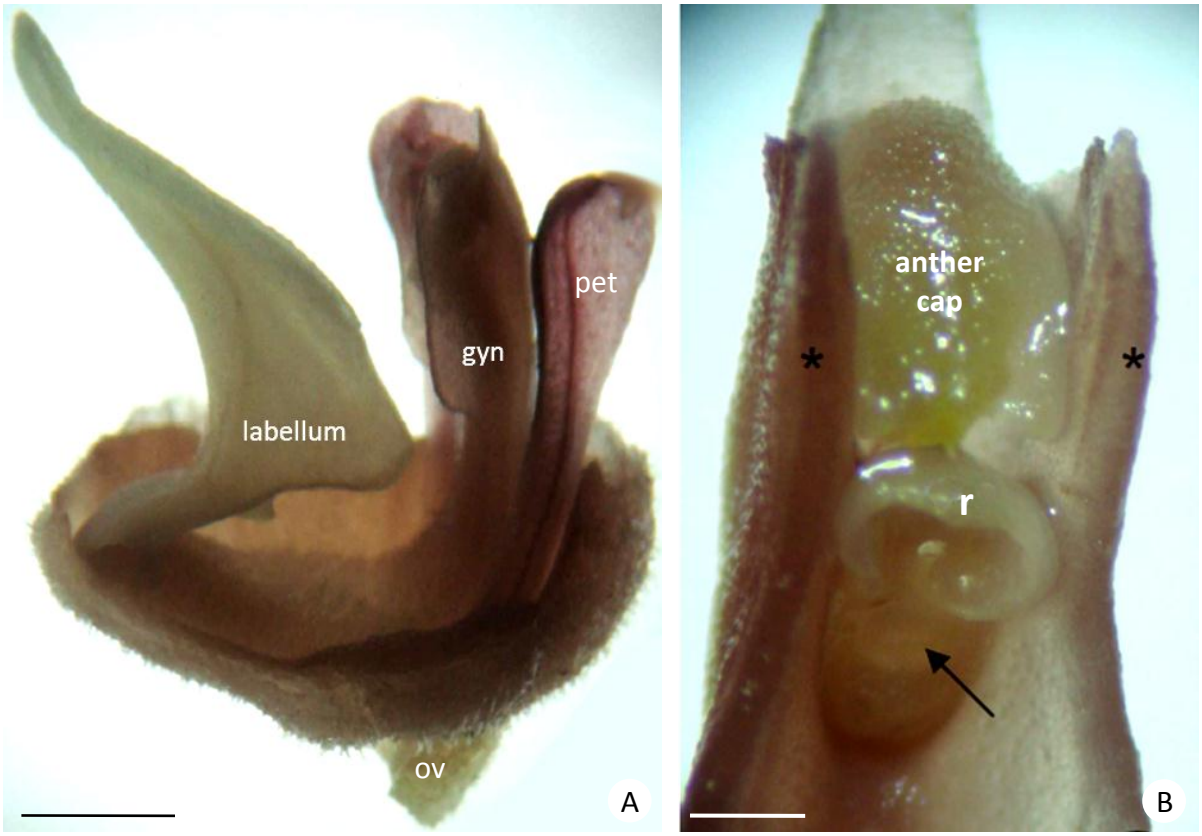
pet
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Fig. 2. SEM of rostellum and stigma of Pleurothallidinae species. (A) *Octomeria crassifolia*. (B) *Acianthera aphthosa*. (C) *Echinosepala aspasicensis*. (D) *Acianthera fenestrata*. (E) *Anathallis obovata*. (F) *Zootrophion atropurpureum*. (G) *Phloeophila nummularia*. Note cuticle covering the stigmatic cavity (thick arrow), evident in Fig. 2A, B, plus profuse secretion from stigma (thin arrow in E). ls, fused lateral stigma; r, rostellum. Scale bars: 120 µm.

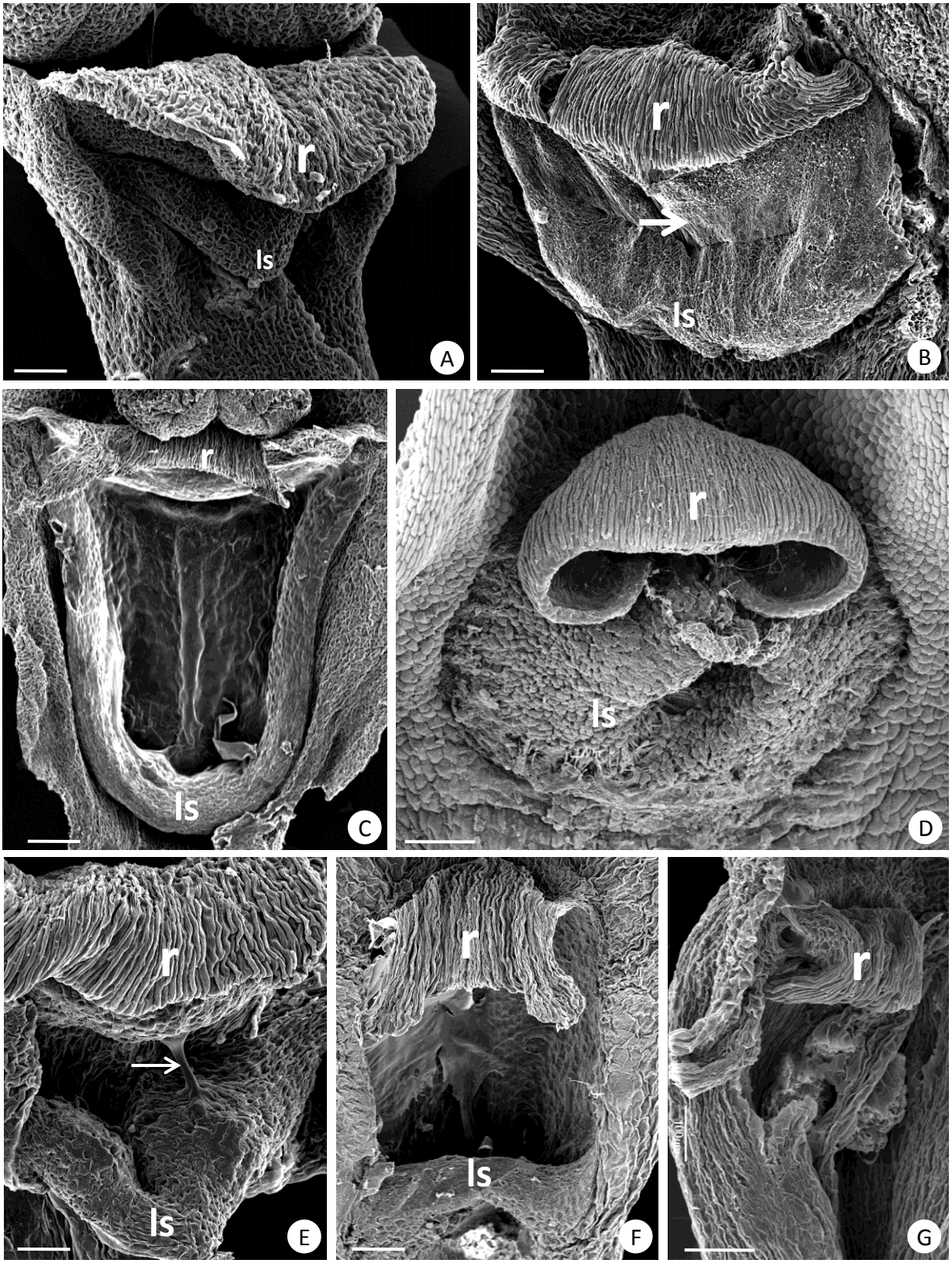
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Fig. 3. SEM of transmitting tissue and growth of pollen tubes in Pleurothallidinae species. (A–B) *Acianthera aphthosa*. (C) *Octomeria crassifolia*. (D–F) *Acianthera fenestrata*. (A) Longisection of gynostemium: transmitting tissue with detached secretory cells (dashed box). (B) Detail of stigma: cuticle (thick arrow) covering cavity containing detached cells. (C) Profuse secretion (*) produced by the rostellum. (D) The rostellum (thin arrow) and stigmatic cells (star). (E) A pollinium attached to the stigma below the rostellum (thin arrow). (F) Dissection of the ovary revealing arrival of many pollen tubes (long arrow) within the locule. Hundreds of ovules at the right. Scale bars: 100 µm (A), 50 µm (B–F).

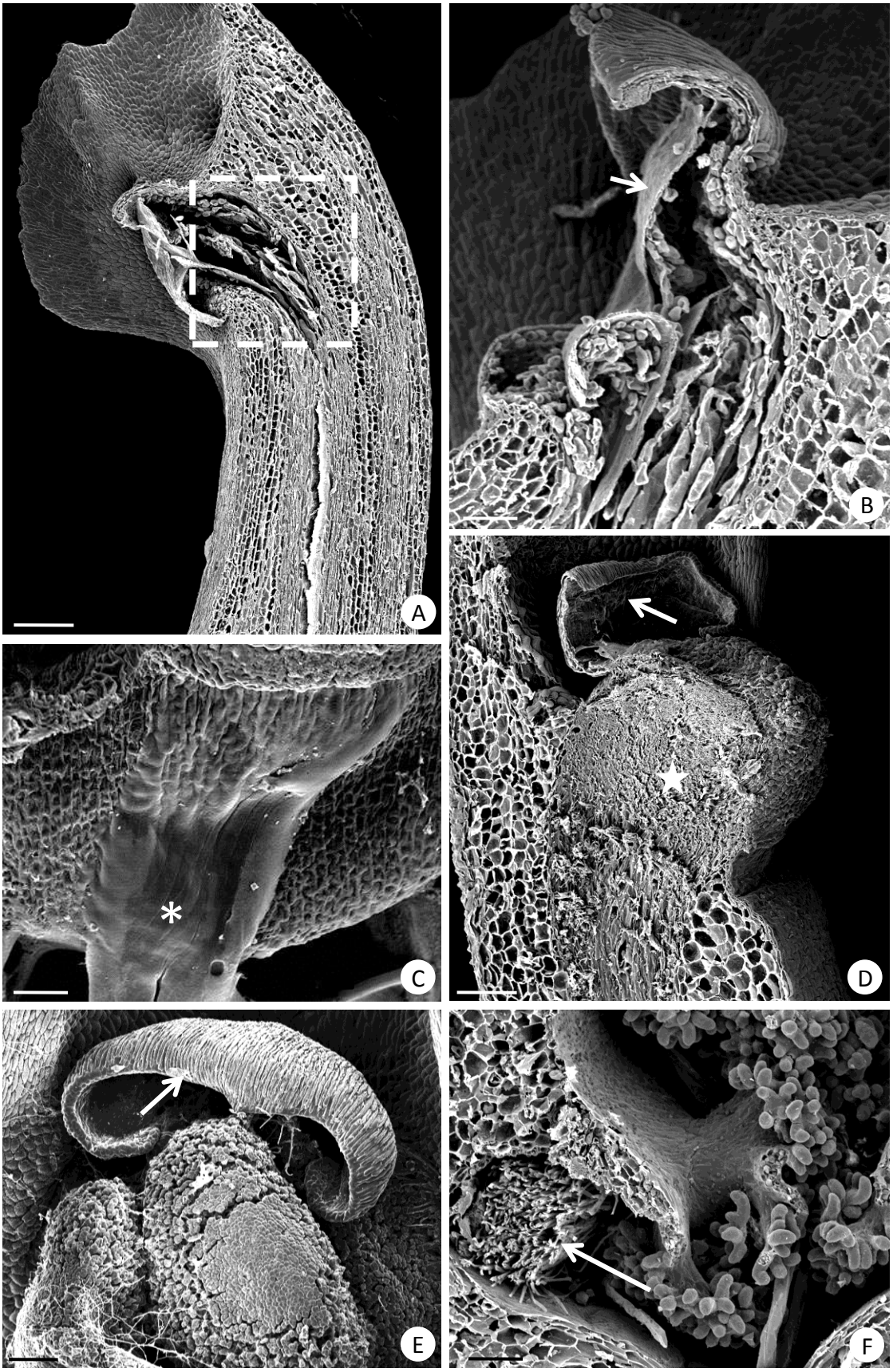
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Fig. 4. Development of the rostellum and transmitting tissue in Pleurothallidinae floral buds. (A–B) *Zootrophion atropurpureum*. (A) Gynostemium tissues under differentiation. (B) A profuse exudate (*) from the early differentiated rostellum (r) extends to the ovary (ova), while style secretory cells (arrowhead) are developing. (C–D) *Acianthera aphthosa* (C) Flower at pre-anthesis, showing stigma cavity (sc) that consists of loosely attached cells. (D) The rostellum possesses a secretory palisade epidermis that releases a mucilage-rich exudate (*). an, anther; ms, median stigma; ls, lateral stigma. Scale bars: 150 µm (B), 75 µm (A, C), 50 µm (D).

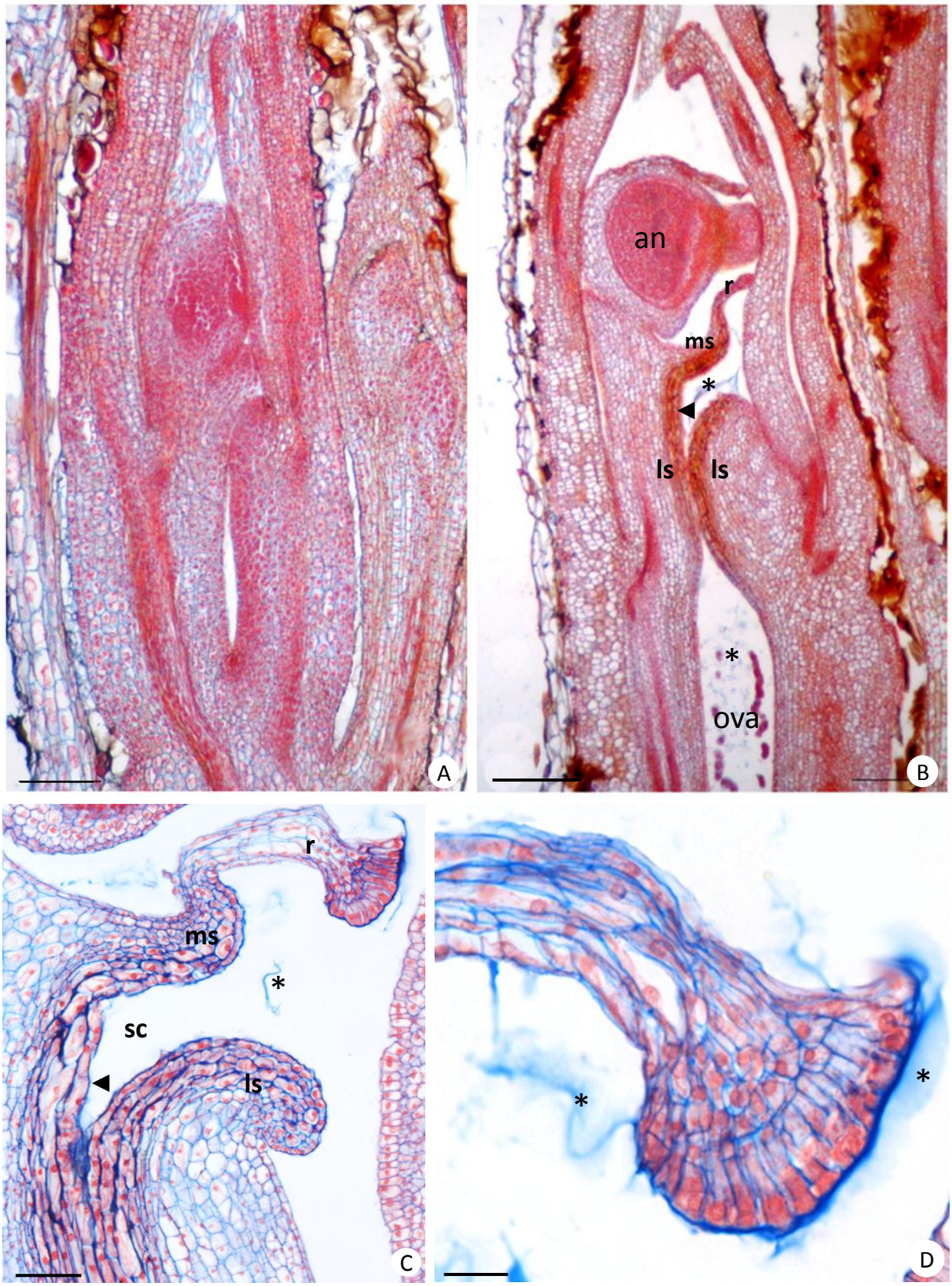
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Fig. 5. Stigma and style of Pleurothallidinae species. (A–C) Cortex (c) of parenchyma cells, transition zone cells (tz), secretory cells (sc) connected by their angulations, and free-floating cells (ff). (A) *Acianthera fenestrata* stigma; note the covering cuticle (long arrow). (B) *Echinosepala aspasicensis* style; the profuse secretion that fills the style cavity (**) presents different metachromasy compared to the exudate between secretory cells (*). Note the absence of free-floating cells. (C) *Phloeophila nummularia* stigma; PAS reaction identifies polysaccharidic exudate between secretory cells and starch grains mainly inside transition zone cells. (D) *Anathallis obovata* stigma; free-floating cells with lipophilic content (arrowheads). (A, D) TBO and p-phenylenediamine. (B) TBO. Scale bars: 75 µm (B, C), 50 µm (A, D).

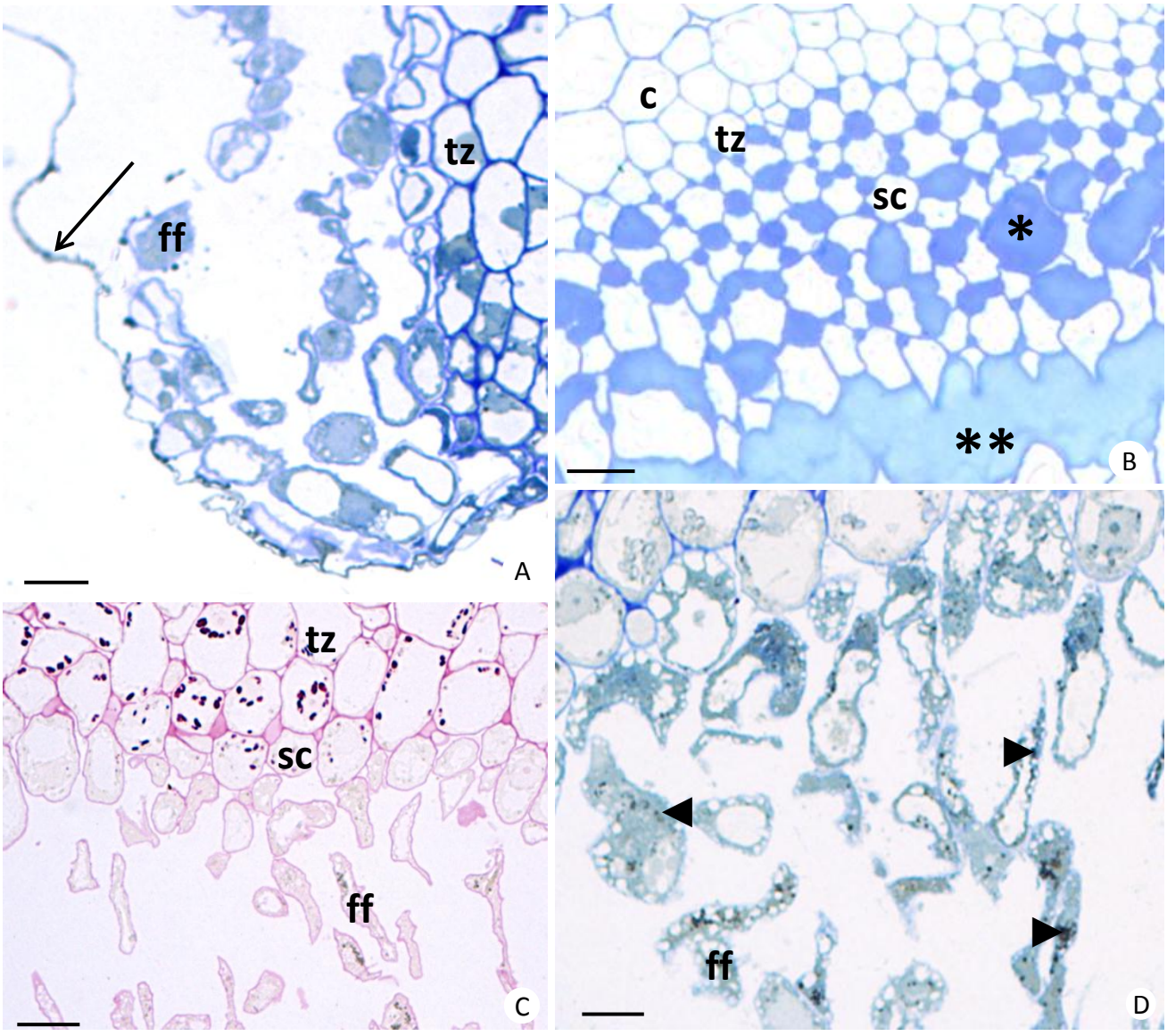
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Fig. 6. Ultrastructure of stigmatic cells from *Echinosepala aspasicensis*. (A) Overview of the secretory process. Secretory cells (sc) possess thin wall (arrow) and retracted protoplast due to the presence of mucilage in the extracytoplasmic space. Transition zone (tz) and free-floating cells (ff) occur left and right of the secretory cells, respectively, which are distinguished by the occurrence of extracytoplasmic mucilage and plasmodesmata where contact is maintained at cell junctions. Free-floating cells are at the end of the mucilage secretory phase, noted by the sparse exudate in extracytoplasmic space (*), and the dense protoplast with lipophilic droplets indicate the start of a secondary phase of secretion. Fibrillar mucilage in the protoplast, extracytoplasmic space (*) and in the mucilaginous matrix where the cells are floating (star) possess different aspects. (B) Cell junctions showing plasmodesmata (arrows) between transition zone and secretory cells and mucilage in the extracytoplasmic space. Several vesicles toward the cell wall (arrowheads) are indicative of granulocrine secretion. *Inset:* vesicles (arrows) merging in the cell wall; the content of the major vesicle is different from those exporting material to the extracytoplasmic space. (C) Free-floating cell at the end of its mucilage secretory phase: presence of large lipophilic droplets in the cytoplasm and several small granules towards the cell wall and around the cell; note the loose aspect of the cell wall (*). (D) Plasmolysed post-secretory (ps) cells in the inner region of the stigma. (E) Secretion in the extracellular matrix in the inner region of the stigma consists of a dense lipophilic and an opaque polysaccharidic fraction. Also note that the mucilage matrix from inner (C–E) and cortex (A–B) regions present different aspects.

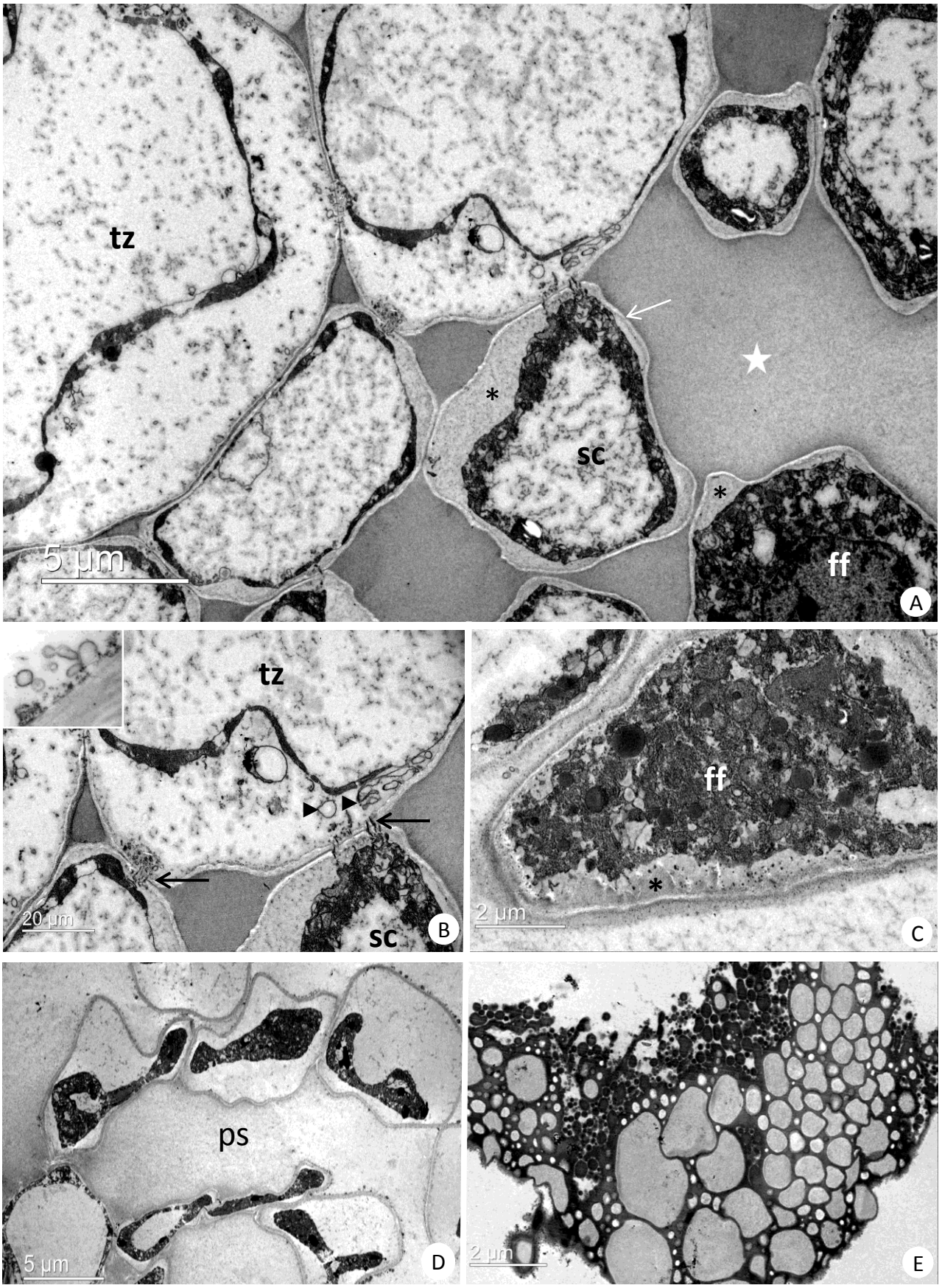
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Fig. 7. Histochemical tests performed in the *Octomeria crassifolia* style. (A–B) Median region of the style: polysaccharides and pectic compounds identified by PAS reaction (A) and ruthenium red (B), respectively. These compounds display a gradient, being more concentrated between secretory cells in relation to extracellular matrix in the inner region of the style. (C) Apical region of the style: the lipophilic content of the exudate verified by Sudan black B, exhibits an opposite pattern in relation to mucilaginous compounds, being more concentrated in the inner region of the style. Scale bars: 75 µm.

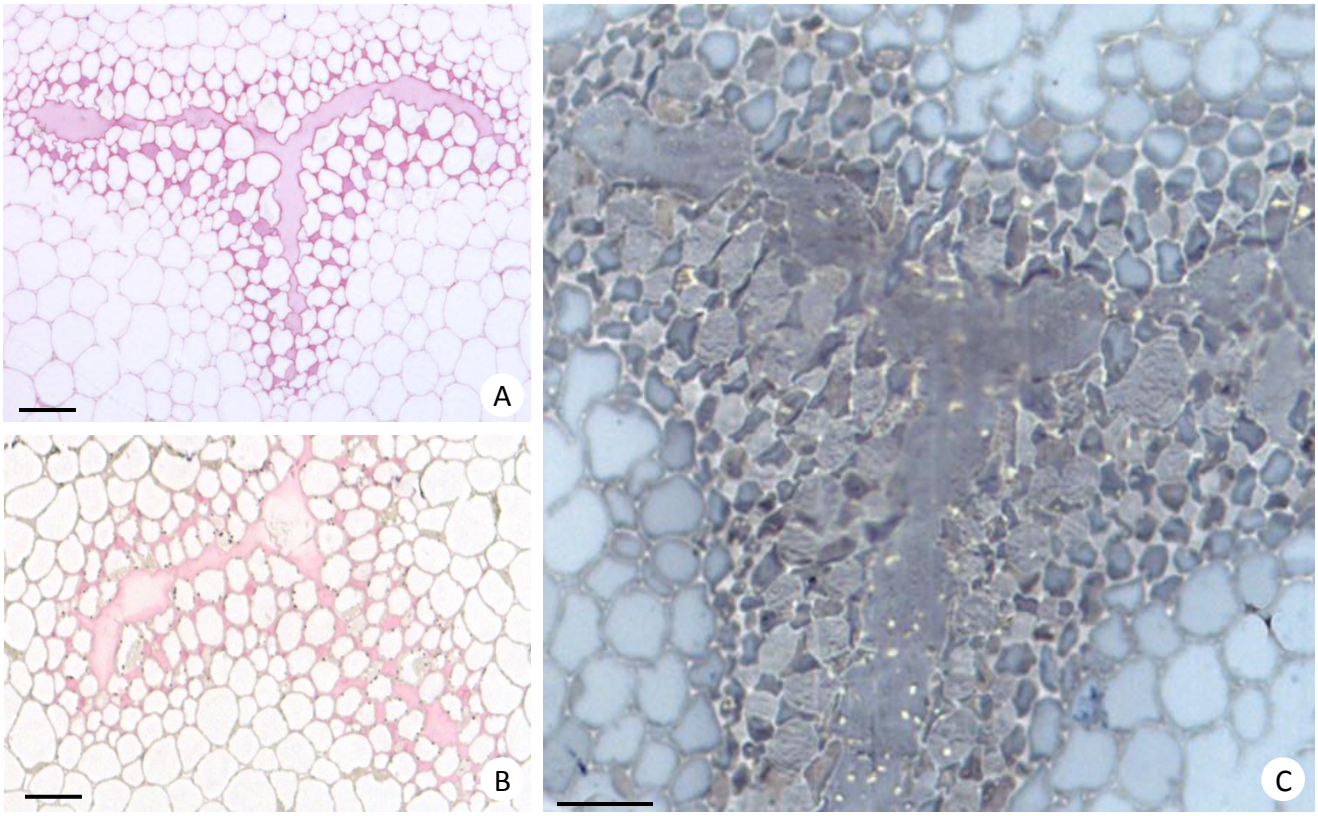
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Fig. 8. Morphological diversity of the obturator in Pleurothallidinae. (A) *Acianthera aphthosa*. (B) *Echinosepala aspasicensis.* (C) *Zootrophion atropurpureum.* (D) *Phloeophila nummularia.* (E) *Anathallis obovata.* (F) *Acianthera fenestrata.* (G) *Octomeria crassifolia.* (A) Absence of ovarian glandular tissue. (B, C) Ovarian transmitting tissue: a profuse exudate (*) is observed in the locule (B). (D–G) Papillae (arrows). Note the pollen tubes (PT) near the papillae lining the *A. fenestrata* locule (F). (A, D) TBO and p-phenylenediamine. (B–C, E–G) TBO. ov, ovules. Scale bars: 50 µm.

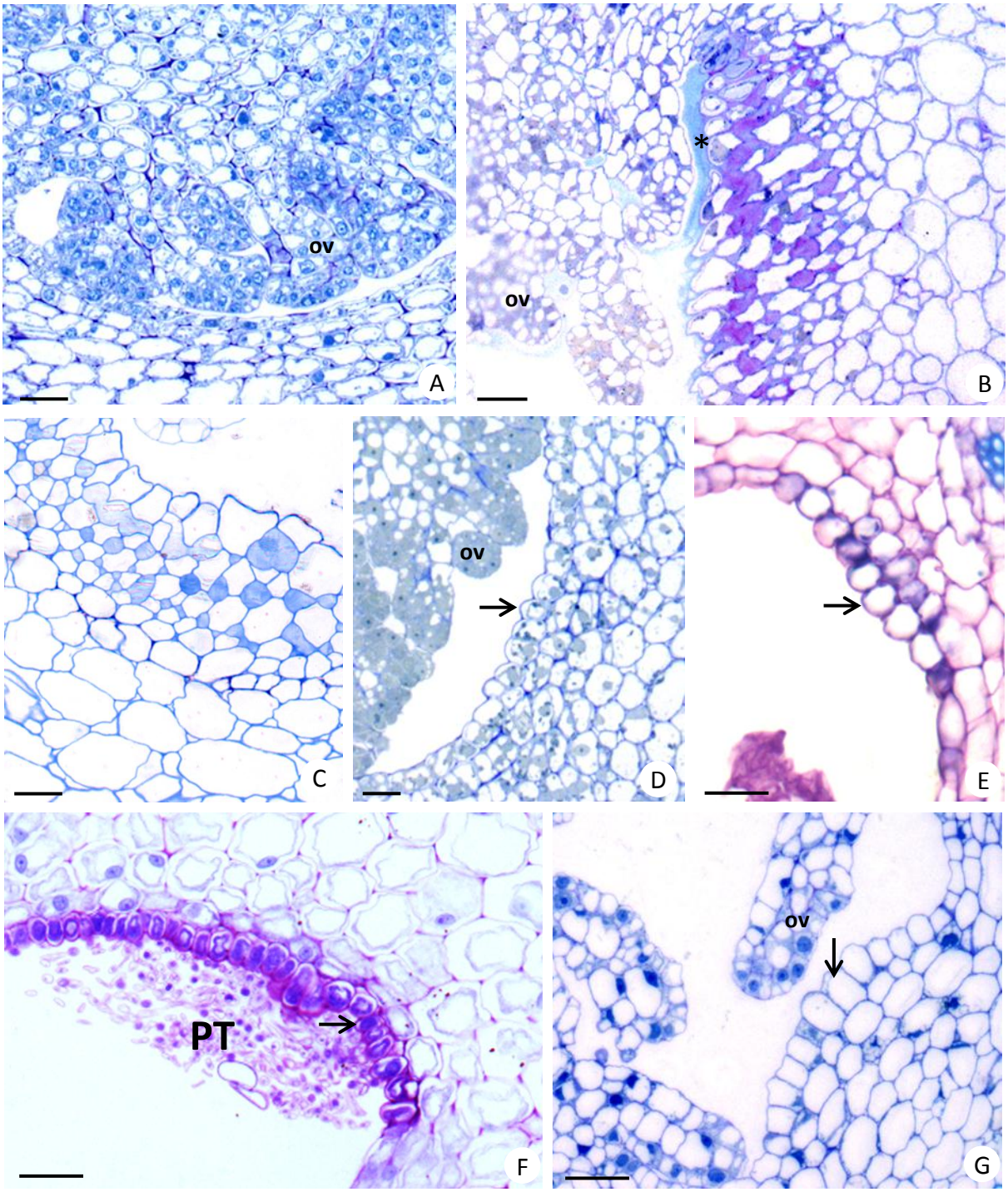
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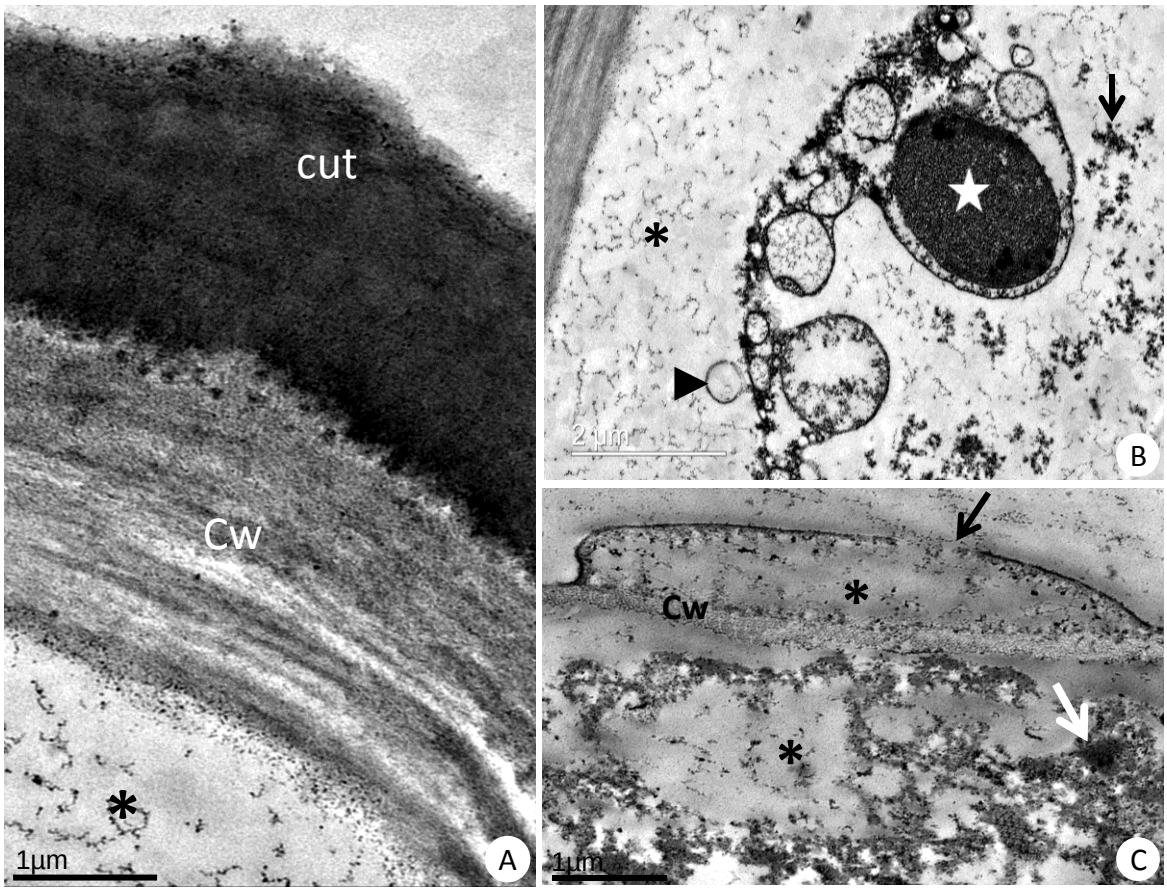
Fig. 9. Ultrastructure of papillate obturator in Pleurothallidinae species. (A–B) *Phloeophila nummularia* papillae. (A) Thick cuticle (cut) and cell wall (Cw), and fibrillar mucilage (*). (B) Vesicles with fibrillar mucilage (arrowhead) and granular lipophilic material (star), the latter also observed in the cytoplasm (arrow). (C) *Acianthera fenestrata* papilla at the secretory phase: note the thin detached and disrupted cuticle (black arrow), thin cell wall (Cw), fibrillar mucilage and granular electron-dense material (white arrow) in the cytoplasm and subcuticular space (*).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1µm
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1µm
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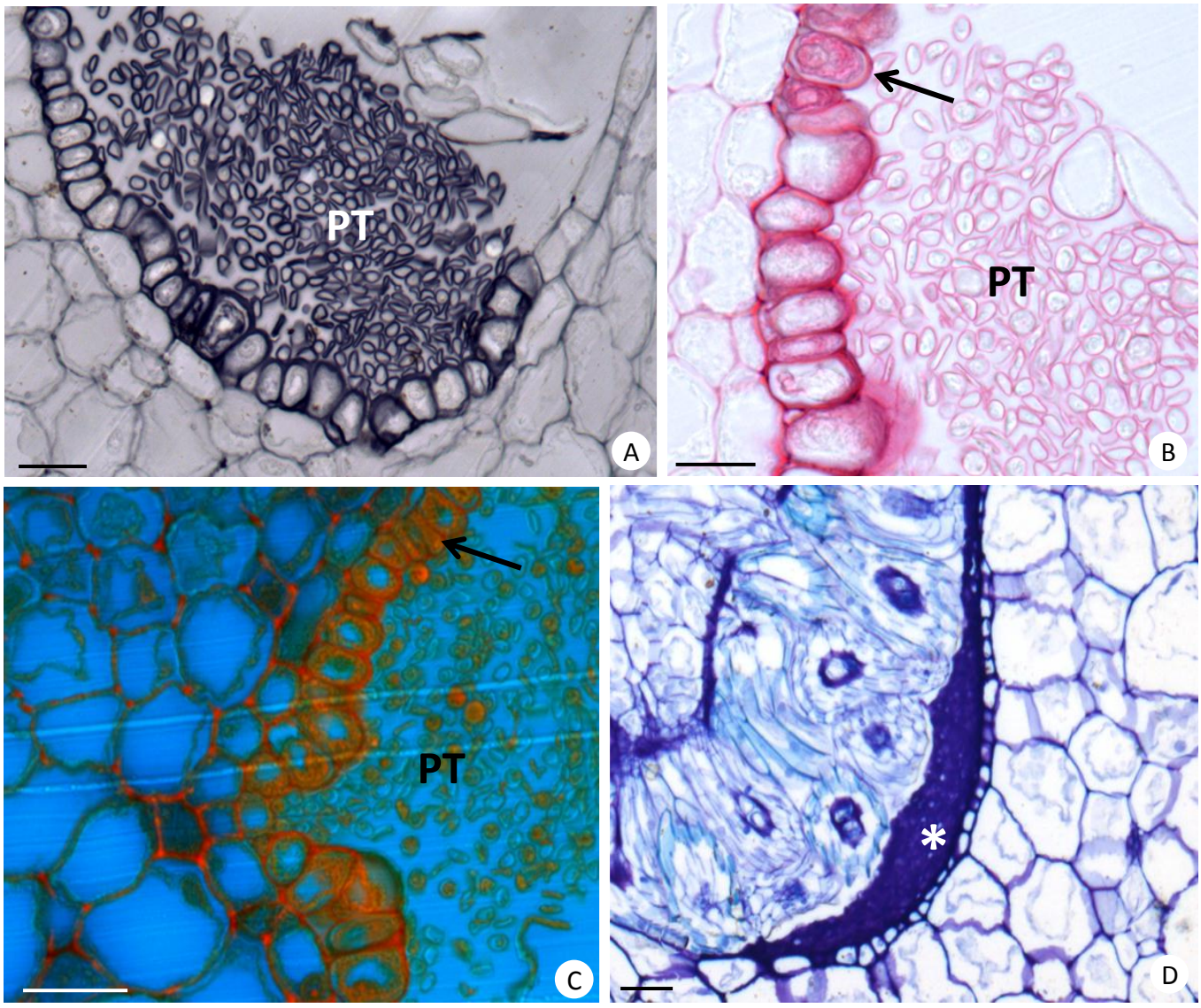
Fig. 10. Histochemical tests performed in the papillate obturator of *Acianthera fenestrata* (A-C) and fate of the mucilaginous exudate in the fruit of *Anathallis obovata* (D). (A) Sudan black B stains the cuticle and lipophilic content inside papillae. Note the differences of dye intensity between the glandular tissue and adjacent epidermal and subepidermal cells. (B–C) Ruthenium red (B) and coriphosphine (C) indicate the pectic nature of the secretion. (A–C) Tests also stain pollen tubes (PT) as well as the secretion in the locule. (D) TBO: presence of mucilage (*) in the locule during seed formation. Scale bars: 75 µm (A, D), 50 µm (B–C).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Discussion

Rostellum

Differentiation of the rostellum occurs early in flower development, while transmitting tissues are not fully developed. The origin of the rostellum in Pleurothallidinae lies on the carpel's median stigmatic lobe, that differentiates before the lateral ones (Kurzweil and Kocyan 2002). In this subtribe, the rostellum is not merely an expansion that keeps anther and stigma separated, but also presents a secretory palisade epidermis that releases a mainly mucilaginous exudate resembling the stigmatic one. The release of secretions from the rostellum occurs through the cell wall and cuticle, however it is not followed by any indication of rupturing or distension. This same pattern of exudate liberation is also identified in other mucilaginous-rich glands, such as some vegetative colleters of the Apocynaceae (Martins 2012).

We hypothesize that the secretion from the rostellum produces the extracellular mucilaginous medium, in which secretory cells of the transmitting tissue will develop, detach and float, not only by providing a mucilaginous medium, but probably by signaling cells to differentiate, analogous to mucilage signaling that leads to columella production during secretory cell differentiation in the *Arabidopsis* seed coat (Western et al. 2004). The secretion from the rostellum may create an osmotic gradient in which the mucilaginous extracellular matrix is less concentrated in relation to the transmitting tissue, facilitating release of exudate from the secretory cells. The role of mucilage in the management of osmosis is already described in plant responses to salt stress, and there are several evidences that extracellular mucilage plays an important role in maintenance of water potential in both aerial and underground tissues (see Ghanem et al. 2010 and references therein), which is essential to pollen development and functioning. The existence of several molecular mechanisms for pollen osmoregulation (Firon et al. 2012) reinforces the relevance of extracellular mucilage in the gynostemium of

Pleurothallidinae species.

Though not closely related to the Pleurothallidinae, orchids of *Bulbophyllum* share floral morphological features with this subtribe, owing to their common myophilous pollination system (Borba et al. 2011). Due to a recent report of the re-gained stigmatic function of the rostellum in *B. bicoloratum* (Gamisch et al. 2013), it is important to highlight that there was no structural indication of pollen penetration through rostellar tissue in the species studied here, even though some *Acianthera fenestrata* flowers were found with pollinia attached to both the rostellum and stigmatic surface. In the species examined here, the rostellum exhibits only its mucilaginous secretory function. In *Epidendrum ibaguense*, the rostellum also possesses a glandular aspect, but the nature of the secretion is lipophilic; its synthetic activity starts in early buds and ends before anthesis, in which the secretory product is stored in the intercellular spaces between cells, being fused together to form the viscidium by the disintegration of the secretory cells (Yeung 1987). This report highlights the differentiation and activity of the glandular rostellum in early phases of development, and also the changes in the secretion produced according to its function before, during and even after anthesis.

Stigma and style

Regardless of the species, there is a cuticle of variable thickness covering the stigmatic surface (concave, or even convex as in *Acianthera fenestrata*) below which detached cells are found. Clifford and Owens (1990) refer to possible functions of the cuticle including a reduction of water loss, a surface for pollinium adherence, or a barrier against invasion by foreign pollen. Also, they suggest that the interaction between the chemical constituents of the pollinium walls could elicit stigma closure, preventing entrance of more pollen to the stigma. Pleurothallidinae species present strong self-incompatibility (Barbosa et al. 2009, Borba et al. 2011), as reported by the absence or very low pollen grain germination with no penetration of pollen tubes from

self-pollinia in the stylar canal in *Octomeria crassifolia* (Barbosa et al. 2009). Considering that the cuticle is the first obstacle for a pollen tube, it is also relevant to point out another probable function for this structure as a first site for recognition of self-pollination in the Pleurothallidinae.

Our interpretations of the transmitting tissue cells present in stigma and style differ from previous descriptions of this tissue in other orchid flowers (Pais 1969, Calder and Slater 1985, Yeung 1988, Clifford and Owens 1990, Slater and Calder 1990, Prutsch and Schill 2001, Leitão and Cortelazzo 2010) characterized as type IIIb (Dannenbaum et al. 1989) or wet detached cell stigmas. There is a transversal organization of secretory cells with different functions and secretory phases, regardless of their position along the pistil (however some cells are absent in the stylar and ovarian transmitting tissue). In other words, these cells constitute a secretory system analogous to the pattern observed in highly specialized glands such as structured nectaries (Fahn 1979, 2000). The cells that compose the "transition zone" were located close to the parenchyma that constitute the gynostemium's cortex; in this region, the cells are very small, possess several vesicles evidently originating from dictyosomes, many plasmodesmata and starch grains – features also exhibited by these cells in other orchids (Clifford and Owens 1990, Slater and Calder 1990, Prutsch and Schill 2001, Leitão and Cortelazzo 2010). Leitão and Cortelazzo (2010) suggested that these cells could be involved in the process of secretion; indeed, the ultrastructural features displayed by transition zone cells indicate a function of pre-formation of mucilage, resembling cells of nectary parenchyma, a tissue that is involved in the formation of pre-nectar (Nepi 2007). The occurrence of numerous vesicles leaving transition zone cells towards secretory ones indicates that at least precursors are sent to the latter. Secretory cells are connected to each other and to transition zone cells only by plasmodesmata at their junctions, and are effectively at the mucilage secretory phase. In this scenario, transition cells filling the secretory ones with precursors of the mucilage present a function analogous to nectary

parenchyma, while secretory cells are analogous to the nectary epidermis, whose activity is to release the nectar (after major chemical modifications) outside the gland. Our analogy among these tissues that consist both structured nectaries and transmitting tissues of Pleurothallidinae is not so surprising if one considers the relationship between the evolution of sugary secretions produced by a stigma or by the micropylar terminus as a landing site for pollen in most gymnosperms (Nepi et al. 2009) and the pronectar produced by wet stigmas believed to be a relictual reward that evolved before the first nectar glands (Bernardello 2007).

Using LM, transition zone and secretory cells superficially resemble the secretory collenchymatous cells described in floral nectaries of the orchids *Maxillaria coccinea* (Stpiczyńska et al. 2003) and *Hexisea imbricata* (Stpiczyńska et al. 2005), due to the angular thickness in the walls that stigmatic cells exhibit. This resemblance, however, is clearly distinguished by the ultrastructural observations of these cells: while the nectary's secretory collenchymatous cells possess cellulose microfibrils resulting in thick walls (Stpiczyńska et al. 2003, 2005), stigmatic cells exhibit thinner walls, but massive mucilaginous secretion in the extracytoplasmic space, mainly in the angulations of the cells. Both glands, moreover, present innumerous plasmodesmata, which are directly related to a symplastic transport of the secretion products from precursor to secretory cells.

Concerning the free-floating cells restricted to the stigma, little or no mucilage was observed in the extracytoplasmic space, indicating that these cells are ending their mucilage-secreting phase. Instead, there is a secondary phase of secretion, now producing lipophilic compounds. The occurrence of two phases of secretion, the first consisting of mucilage and the second of lipophilic compounds, is reported for colleters (also mucilage-producing glands) of some Apocynaceae (Appezzato-da-Gloria and Estelita 2000). This secondary phase of lipophilic secretion restricted to free-floating cells explains the gradient of lipophilic compounds

concentrated more closely to these cells, opposite to the higher concentration of mucilage nearest the secretory cells, as identified by histochemical tests. Although the style also has the same secretory arrangement as the stigma, with detached cells united by their angulations, there are no floating cells in the central region of transmitting tissue at this level. Consequently, no lipid droplets were observed in the transmitting tissue cells along the stylar canal, and the staining intensity with Sudan black B decreases as it approaches the ovary.

A correlation between histochemical detection of lipids and transmitting tissue competence for tube growth seems to indicate an important role for lipids in the progamic phase (Dumas 1977, Graaf et al. 2001). Lipids possess important functions in pollen-stigma interaction, specially determining the temporal receptivity of the stigma (Sanzol et al. 2003, Leitão and Cortelazzo 2010) and can be involved in pollen recognition and germination processes. Also, microscopic investigations of the free-floating cells (also called eleutherocytes) after compatible and incompatible pollinations, suggest that the incompatibility response is probably controlled by these cells (Johansen 1990). Complete, strong or partial self-incompatibility is reported to occur in Pleurothallidinae, this process being indeed regarded as a biological synapomorphy of this clade (Barbosa et al. 2009, 2013; Borba et al. 2011; Melo et al. 2011). Two different sites of self-incompatibility, the stigma and the stylar canal, are recognized in this subtribe (Borba et al. 2011). Multiple genes are involved in lipid metabolism related to the stigmatic role in supporting pollen hydration and compatible pollination (Sankaranarayanan et al. 2013). Considering the occurrence of the cuticle and also of lipophilic compounds in the stigma until the beginning of the style, we infer that these compounds are related to incompatibility processes described in Pleurothallidinae.

Lipophilic material, observed as osmiophilic electron-dense granules by TEM, occurred both in the cytoplasm as well as outside the transmitting tract. The granules initially appeared

inside vesicles dispersed in the cytoplasm, but later as thin grains in and outside the cell wall. The multivesicular bodies in the extracytoplasmic space and granules external to the wall suggest that the secretion mode of these lipophilic compounds is also granulocrine. This same pattern of osmiophilic multivesicular bodies and external granules beyond the wall is described in secretory trichomes of *Fagonia* (Zygophyllaceae), where a granulocrine manner of secretion was also reported (Fahn and Shimony 1998). The region inward from the primary wall of free-floating cells exhibits a loose aspect, which could suggest that the extracytoplasmic space's matrix may actually represent porous wall material. We do not believe that this possibility occurs since the appearance of the extracytoplasmic secretion differs from that of the thin primary wall, and that the latter maintains its integrity even in cells at the post-secretory phase (fig. 6*D*). According to Slater and Calder (1990), the cell wall of the detached cells is not uniform across its width. Thus, we can also hypothesize that during the second phase of secretion, the cell wall can undergo biochemical changes to facilitate the permeability of the lipophilic compounds through it, then assuming a porous condition but without jeopardizing its integrity.

It is interesting to note the similarities between colleters and stigma cells (at least those of the wet, detached stigma type), since both release their mainly mucilaginous exudate to the environment (although in the stigma it is confined to a hollow), possess the same granulocrine mode of secretion, and produce lipophilic compounds, concomitant with the mucilaginous phase, in the beginning or throughout a second phase of secretion (Thomas and Dave 1989, Appezzato-da-Gloria and Estelita 2000). These similarities suggest the occurrence of a model of mucilage glands to release exudate outside the plant body (as colleters) or outside the cells in a hollow (as transmitting tissue) regardless of their position or ecological function in the plant, more related to the kind of exudate produced (mainly mucilaginous).

The extracellular mucilage among the secretory cells shows different metachromasy by

TBO in comparison to the inner regions of the stigma and style. As aforementioned, histochemical tests indicate a higher concentration of polysaccharides close to the secretory cells, and an opposite pattern from lipophilic compounds. A decrease in the metachromatic staining of extracellular secretion is reported by Leitão and Cortelazzo (2010) during the development of floral buds of *Rodriguezia venusta*. The authors associated the gradual loss of secretion metachromasy to chemical changes occurring in the mucilage during flower development, probably due to the occurrence of lipophilic compounds during the final stages of gynostemium development. This pattern is congruent with our results in the stigma. Also, the appearance under TEM of the mucilage inside the secretory cells and in the extracellular medium differs according to the site: elementary fibrils dispersed in an amorphous matrix were visualized in the cytoplasm of transition zone and secretory cells, and also inside the vesicles that carry the pre-secretory mucilage towards the extracytoplasmic space. In the extracytoplasmic space, the exudate assumes an amorphous aspect less electron-dense than the extracellular matrix. At high concentrations, mucilage fibrils aggregate to form a web-like network, while at lower concentrations, globular particles in the secretion aggregate in a linear fashion (Rudall et al. 1998). As the exudate in the extracellular medium is less concentrated than inside the secretory cells, the appearance of mucilage tends to be more amorphous. Thus, it indicates that chemical changes occur inside the vesicles until they reach the extracytoplasmic space – it is important to note that the fibrils inside the vesicles also differ from the mucilage cytoplasmic fibrils. The difference in electron density from mucilage inside and outside secretory cells can also be related to the occurrence of the secretion from the rostellum that, besides being also constituted by mucilage, can have differences in chemical constitution or in the relative concentration of its components.

In the post-secretory phase, after the secretion of lipophilic compounds, the floating cells

are plasmolysed. We refer to these plasmolysed cells as "post-secretory" since there was no indication of cell death; in fact, there are reports that these cells are still living and viable (Calder and Slater 1985, Slater and Calder 1990). The occurrence of plasmolysed cells floating in the inner region of the style seems to be ubiquitous in flowers at anthesis that possess the wet, detached cell type of stigma (Pais 1969, Calder and Slater 1985, Yeung 1988, Clifford and Owens 1990, Slater and Calder 1990, Prutsch and Schill 2001, Leitão and Cortelazzo 2010). This is another indication that all secretion phases – pre-secretory, secretory (mucilage and lipophilic compounds), and post-secretory – are present and can be visualized in a transversal gradient (in relation to the gynostemium) not only in the stigma of Pleurothallidinae species, but in all wet, detached cell stigmas.

Obturator

In many plants, secretion products are chemically different between stigma, stylar canal and obturator cells, indicating a difference in function (Rudall et al. 1998): stigmatic secretions may have a selective role in self-compatible crosses, whereas stylar and ovarian secretions guide the pollen tubes (Sanders and Lord 1992). Two types of obturator were found in the ovary of Pleurothallidinae species: a continuum of the transmitting tissue in *Echinosepala aspasicensis* and *Zootrophion atropurpureum*, and papillae in *Acianthera fenestrata*, *Anathallis obovata*, *Octomeria crassifolia* and *Phloeophila nummularia*. Only in the ovary of *Acianthera aphthosa* was there no indication of a tissue acting as obturator.

In species where the obturator is a continuum of the transmitting tissue, cells present the same pattern verified in the style, in which floating cells are absent. This pattern of ovarian transmitting tissue is also observed in *Lemboglossum* spp. (Clifford and Owens 1990) and *Rodriguezia venusta* (Leitão and Cortelazzo 2010), species of orchids in which ovarian

transmitting tissues have been described to date. Although we did not detect lipophilic compounds in the ovarian transmitting tissue, again a metachromatic distinction was observed in the exudate between secretory cells and in the inner region of the locule of *Echinosepala aspasicensis*, as described in the stigma and style, probably related to chemical differences in the exudate that the histochemical tests applied here were insufficient to assess.

The basipetal pattern of development of the transmitting tissue is clearly observed in this position, since it is the last portion that differentiates just prior to anthesis. Even the papillae are distinguishable and active only in flowers by anthesis. This period of late differentiation is probably related to the fact that the development of the ovules from orchids is initiated after pollination is successful (Yeung and Law 1989).

Although the papillae are indistinct by LM, strong differences were observed between the morphology and mode of secretion of *Phloeophila nummularia* papillae and those from *Acianthera fenestrata*, *Anathallis obovata* and *Octomeria crassifolia*. *Phloeophila nummularia* papillae possess a thick cuticle and outer cell walls; vesicles both with electron-dense and fibrillar aspects were observed in the cytoplasm, which possibly refers to lipophilic compounds and mucilage, respectively. At least the mucilage secretion appears to be granulocrine, since vesicles are observed shifting towards the cell wall. A disarrangement of the wall microfibrils of *P. nummularia* papillae seems to occur during the secretory phase: these fibrils appear porous and apparently allow secretions to pass through them (Bystedt and Vennigerholz 1990). On the other hand, the other papillate species present a very thin cuticle which detaches and breaks to release the secretion composed by both mucilage fibrils and dense granular material. Comparing the ultrastrutural aspects of the secretion from secretory cells of the transmitting tissues and the obturator papillae, they seem quite different: both papillae morphology seems to release fibrillar mucilage that resembles the one found in the cytoplasm of secretory cells before being sent to the

extracytoplasmic space. It reinforces that chemical modifications occur to the secretion inside the vesicles prior to their release to the extracytoplasmic space. Also, at least in the papillae in which the cuticle detaches, the granular lipophilic compounds are also freely sent to the locule, contrasting to the granulocrine manner of secretion observed in the stigma's free-floating cells.

The presence of mucilage-filled ovaries mainly occurs in aquatic plants or plants of moist habitats (Rudall et al. 1998 and references therein), the latter being the environment inhabited by all Pleurothallidinae examined here. The free space in the locule of *Acianthera aphthosa* is apparently smaller than the other Pleurothallidinae species; perhaps the secretion that comes from the stylar transmitting tissue is sufficient to fill the ovary locule and guide the pollen tubes to the micropyle of the ovules. According to Rudall et al. (1998), the occurrence of mucilage-filled ovaries is probably the plesiomorphic state for this character that evolved from secretory gynoecial epidermal cells possibly several times in the monocotyledons. Even in the relatively few species analyzed here, there seems to exist a diversity of obturator morphology in Pleurothallidinae. We were not able to trace an evolutionary pattern concerning the obturator in this subtribe's species to establish relationships, since papillae and ovarian transmitting tissues occur in both early- and recent-divergent species. However, species that share floral morphology, such as *Octomeria crassifolia* and *Anathallis obovata*, possess papillae in their obturator; perhaps these species have similar pollinators and are under the same selection pressure, which can be reflected by their obturator morphology.

Overall, our study shows that the rostellum acts effectively in pre-anthesis events, secreting a mucilaginous exudate closely related to the development and secretory phase of cells from the transmitting tissue. We propose a "transversal interpretation" of the secretory cells which constitute the stigma that may explain secretion dynamics in the wet, detached stigma type of these orchids. We present comparisons between secretory cells of transmitting tissue, colleters

and nectaries, showing the similarities between glands regarding their mucilaginous exudate (transmitting tissue and colleters) and the organization of secretory cells (transmitting tissue and nectaries), emphasizing how different glands can share features that allow insights to the interpretation of other secretory tissues. Finally, we describe the first occurrence of papillae in the locule acting as an obturator in Orchidaceae. In light of ongoing studies about the floral biology of the Pleurothallidinae, this study provides useful information regarding the pollination and mating system of this subtribe, as well as relevant information toward understanding the phylogeny of the Pleurothallidinae.

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Considerações finais

Os resultados obtidos durante o desenvolvimento da pesquisa permitiram responder satisfatoriamente às questões inicialmente levantadas; as perguntas e suas respectivas respostas são detalhadas adiante.

(1) As características do padrão de desenvolvimento floral de espécies de Pleurothallidinae apresentam apomorfias em relação ao modelo descrito em Epidendroideae (Kurzweil 1987, *Kurzweil & Kocyan* 2002)? O padrão de vascularização dos representantes desta subtribo obedece ao modelo descrito por Swamy (1948) para orquídeas monândras?

Nenhuma apomorfia foi observada em relação ao padrão de desenvolvimento, em que as etapas iniciais são constantes e idênticas às descritas em Epidendroideae *(*Kurzweil 1987, Kurzweil & Kocyan 2002), incluindo o surgimento dos primórdios de estaminódios. Ao longo do desenvolvimento, estas estruturas ou são completamente incorporados ao ginostêmio, que apresenta uma estrutura simples como em *Octomeria crassifolia* e *Anathallis obovata*, ou se manifestam como estruturas elaboradas denominadas asas laterais do ginostêmio.

Em relação ao padrão de vascularização, no entanto, houve alterações importantes em comparação ao descrito por Swamy (1948), como a existência de traços supranumerários no ovário, parte envolvida na vascularização direta da placenta em *Echinosepala aspasicensis* (embora esta característica tenha sido atribuída à retenção de caracteres ancestrais nesta espécie, *cf*. capítulo 1), a supressão do traço referente ao lobo mediano do estigma em todas as espécies (exceto em *E. aspasicensis*), e a supressão de todos os traços referentes aos lobos estigmáticos de *Acianthera apthosa*, cuja estrutura é vascularizada por apenas um traço (o da antera).

A diferença encontrada na vascularização do ginostêmio das espécies de Pleurothallidinae foi muito contrastante e difícil de ser interpretada considerando-se apenas os membros desta subtribo. Desta forma, analisamos adicionalmente duas espécies de representantes de Laellinae (subtribo-irmã de Pleurothallidinae; Pridgeon *et al.* 2001),

Encyclia patens var *patens* que também apresenta estaminódios observados como projeções laterais do ginostêmio, e *E. patens* var. *serroniana*, variedade teratológica que apresenta três estames férteis. O mesmo padrão de vascularização descrito para *E. aspasicensis* foi observado em ambas as variedades, comprovando que a redução no número de traços do ginostêmio descritos nas outras espécies de Pleurothallidinae é uma característica singular a esta subtribo.

(2) Quais são as estruturas secretoras presentes nos órgãos florais das espécies estudadas? É possível observar padrões evolutivos nestas glândulas?

Três capítulos da tese foram dedicados ao estudo das glândulas presentes nas flores de espécies de Pleurothallidinae, sendo estas: (*i*) **coletéres**, presentes nas brácteas (involucral e floral), sépalas (apenas em *Zootrophion atropurpureum*), e, ineditamente, nas invaginações da parede externa do ovário (capítulo 2); (*ii*) **osmóforos**, presentes nas sépalas de todas as flores e também no labelo de algumas espécies (*Acianthera apthosa*, *A. fenestrata*, *Zootrophion atropurpureum*, *Phloeophila nummularia*); (*iii*) **nectários florais**, presentes apenas no labelo de *Octomeria crassifolia* e *Anathallis obovata*; (*iv*) **glândulas de secreção heterogênea** (lipídios e polissacarídeos) localizadas nas sépalas laterais e labelo de *E. aspasicensis*; (*v*) **rostelo**, uma projeção glandular presente no ápice do lobo do estigma mediano produtora de mucilagem; (*vi*) **tecido transmissor**, que se estende desde o estigma até o lóculo do ovário (ausente apenas no lóculo de *A. aphthosa*) responsável pela produção de mucilagem e compostos lipídicos.

Em relação aos padrões evolutivos, estes foram propostos para os coléteres presentes nas invaginações da parede externa do ovário (Cardoso-Gustavson *et al.* 2014) e para os osmóforos presentes no labelo (capítulo 3). Os coléteres estão presentes na região ovariana em que comumente nectários extraflorais são encontrados em outros grupos de orquídeas. Hipotetizamos a ocorrência de coléteres nesta região como um caso de homeose, em que o

metabolismo das glândulas foi totalmente alterado da produção de açúcares simples para polissacarídeos complexos (mucilagem). Em relação aos osmóforos presentes no labelo, uma transição semelhante é observada quando comparamos a ocorrência de nectários nesta posição em *Octomeria crassifolia* (clado-irmão do restante das Pleurothallidinae; Pridgeon *et al.* 2001) e osmóforos em *Acianthera aphthosa*, *A. fenestrata*, *Z. atropurpureum* e em *P. nummularia* (espécies presentes em clados mais derivados), caracterizando outro caso de homeose.

(3) É possível hipotetizar sinapomorfias a partir do estudo morfológico e anatômico das flores?

Sim, porém apenas se considerarmos a proposta de divisão da atual circunscrição de Pleurothallidinae (Pridgeon *et al.* 2001, 2010) em duas subtribos, uma mantendo as delimitações prévias de Pleurothallidinae (Luer 1986) e outra compreendendo os gêneros recém incorporados (*Dilomilis*, *Neocogniauxia* e *Tomzanonia*; Borba *et al.* 2011). Caso a proposta seja aceita, três sinapomorfias poderão ser atribuídas às Pleurothallidinae: a articulação entre o pedicelo e o ovário (sinapomorfia morfológica; Luer 1986), auto-incompatibilidade e miiofilia (sinapomorfias biológicas; Borba *et al.* 2011). Seguindo esta proposta de divisão, é possível hipotetizar, com base nos resultados descritos nesta tese, outras duas sinapomorfias: a **redução** do número de traços que vascularizam o ginostêmio (capítulo 1), e a presença de **alcanos** como constitutivos ubíquos dos voláteis florais (capítulo 3).

É importante ressaltar que a análise química dos componentes do *bouquet* floral não constava nos objetivos iniciais desta tese; no entanto, esta análise forneceu informações úteis tanto para a interpretação dos resultados morfológicos e ultraestruturais quanto para sua própria descrição e constatação de alcanos como um marcador químico em Pleurothallidinae. Embora caracteres florais tenham sido descritos como homoplásicos nesta subtribo, de acordo com a filogenia vigente (Pridgeon *et al.* 2001), considerando a atual proposta de divisão de

Pleurothallidinae, todas as sinapomorfias (já propostas e aqui hipotetizadas) compreendem aspectos relacionados à flor.

Nota-se que as análises morfológicas de flores de apenas sete espécies de Pleurothallidinae foram suficientes para hipotetizar as sinaporfias e tendências evolutivas das glândulas. Torna-se necessário, a partir daqui, o estudo de uma amostragem maior de espécies – a subtribo apresenta cerca de 1400 espécies neotropicais (Pridgeon *et al.*2001) – que incluam aquelas recentemente incorporadas em Pleurothallidinae pertencentes aos gêneros *Dilomilis*, *Neocogniauxia* e *Tomzanonia* (Pridgeon *et al.* 2001, 2010), e de espécies de Laeliinae. Desta forma, será possível levantar mais características que auxiliem na melhor circunscrição da subtribo e testar as hipóteses filogenéticas sugeridas por Borba *et al.* (2011) e as levantadas nesta tese.

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Apêndice 1

A light in the shadow: the use of Lucifer Yellow technique to demonstrate nectar reabsorption

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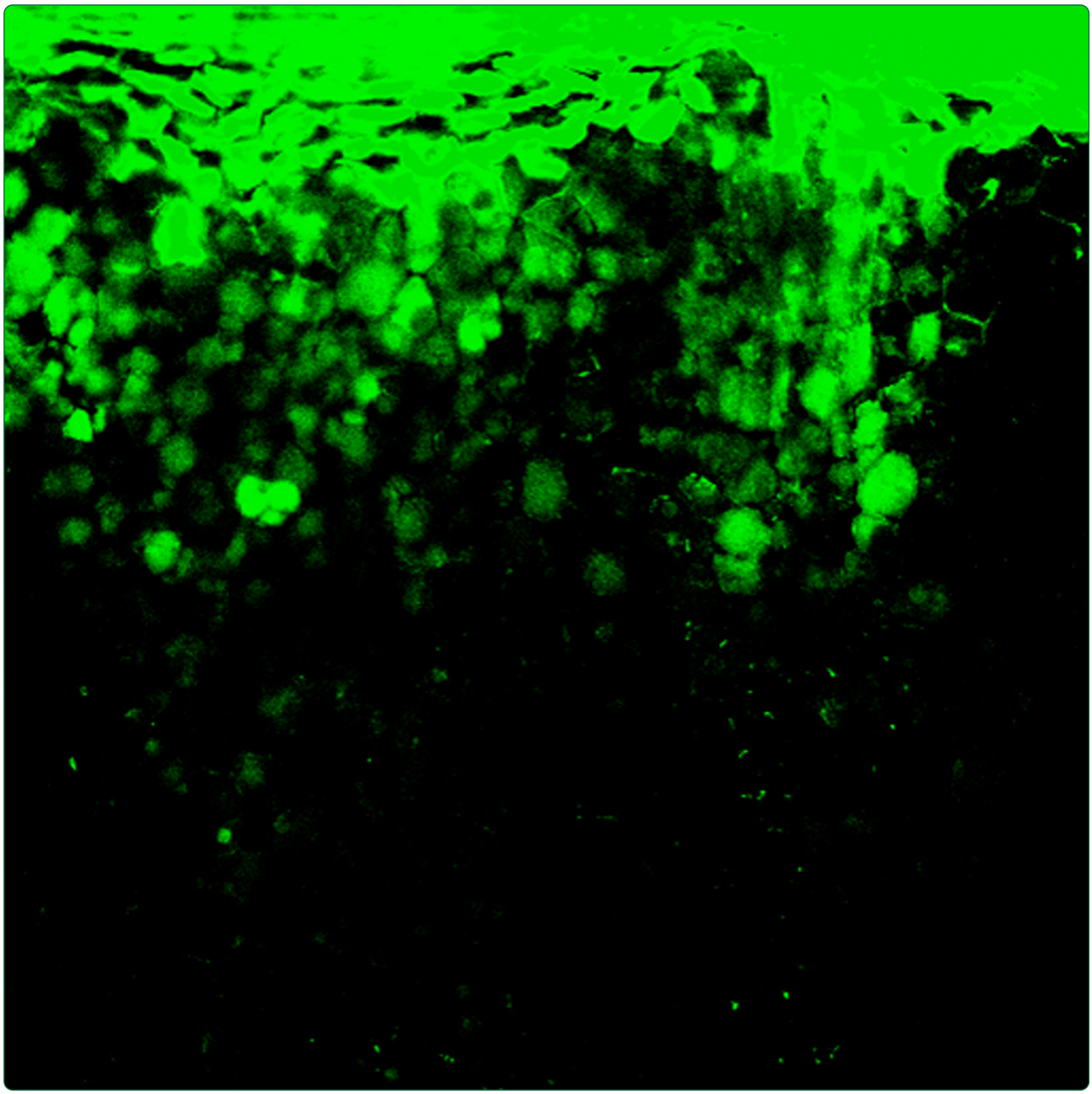
Plant Methods 9: 20

PLANT METHODS



A light in the shadow: the use of Lucifer Yellow technique to demonstrate nectar reabsorption

Cardoso-Gustavson *et al.*

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PLANT METHODS

METHODOLOGY Open Access

A light in the shadow: the use of Lucifer Yellow technique to demonstrate nectar reabsorption

Poliana Cardoso-Gustavson[1*], João Marcelo Robazzi Bignelli Valente Aguiar[2], Emerson Ricardo Pansarin[3] and Fábio de Barros[4]

Abstract

Background: Nectar reabsorption is a widely known phenomenon, related to the strategy of resource-recovery and also to maintain the nectar homeostasis at the nectary. The method currently performed to demonstrate nectar being reabsorbed involves the use of radioactive tracers applied to the nectary. Although this method works perfectly, it is complex and requires specific supplies and equipment. Therefore, here we propose an efficient method to obtain a visual demonstration of nectar reabsorption, adapting the use of Lucifer Yellow CH (LYCH), a fluorescent membrane-impermeable dye that can enter the vacuole by endocytosis.

Results: We applied a LYCH solution to the floral nectary (FN) of *Cucurbita pepo* L., which is a species known for its ability of nectar reabsorption, and to the extrafloral nectary (EFN) of *Passiflora edulis* Sims which does not reabsorb the secreted nectar. In all tests performed, we observed that LYCH stained the nectary tissues differentially according to the reabsorption ability of the nectary. The treated FN of *C. pepo* presented a concentrated fluorescence at the epidermis that decreased at the deeper nectary parenchyma, until reaching the vascular bundles, indicating nectar reabsorption in the flowers of the species. In contrast, treated EFN of *P. edulis* presented fluorescence only at the cuticle surface, indicating that nectar is not reabsorbed by that particular tissue.

Conclusion: LYCH is an efficient marker to demonstrate nectar reabsorption.

Keywords: *Cucurbita Pepo*, Fluorescence Microscopy, LYCH, Nectar Pathway, *Passiflora Edulis*

Background

Nectar reabsorption is a phenomenon known for many plant families, occurring as a resource-recovery strategy and also in order to maintain the nectar homeostasis at the nectary. Several direct and indirect demonstrations of nectar reabsorption have been published [see reviews in 1,2], highlighting the micro-radiography technique in which ^{14}C-labelled sucrose is incorporated by nectaries making it possible to follow the detailed rate of movement of radioactive tracer within the nectary and in other plant tissues [2 and references therein]. This technique, however, is complex and requires some specific laboratory equipment and hazardous supplies.

The substitution of floral nectar by a dilute solution of vital stains (e.g. neutral red) is not recommended, since vital dyes diffuse across the tissues without specificity. Although Schiff's reagent bonds to 1,2 – glicol (after a pretreatment with periodic acid), it shows a weak reaction with glucose and sucrose even in an analysis with purified sugars [3], being also not adequate to specifically track nectar itself through the plant tissues. In other words, to find a dye that can efficiently mark reabsorbed nectar in plant tissues, it is necessary that it somehow interacts with sugar molecules present within nectar itself and moves with them, signalizing the complete pathway.

Lucifer Yellow CH (LYCH), which is 6-amino-2,3-dihydro-1,3-dioxo-2-hydrazinocarbonylamino-1H-benz[d,e] isoquinoline-5,8-disulfonic-acid dilithium salt, and other fluorescent apoplastic tracers have been used to demonstrate the route of the absorbed water present in air humidity by the foliar epidermis [4-6]. These experiments were developed in a passive way, applying a solution of LYCH over the leaf surface and submitting the plant to dry conditions and later it was possible to visualize the apoplastic route of the absorbed solution. LYCH has been also extensively used as fluorescent tracer in studies of

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intercellular communication in plants and relationships between sucrose and phloem [7-9], since it is transported in parallel with sucrose, and can accumulate in the vacuole. An evidence of the interaction between LYCH and sucrose is that the uptake of the external dye marker occurs preferentially in the presence of this sugar [9,10]. This tracer has also been applied in studies of the process of nectar secretion [11,12]. In those studies, LYCH was injected into the nectary or nectar cells, forcing the dye to be incorporated by the plant.

The properties of LYCH can bring several advantages for the use as a marker for nectar reabsorption: a very high quantum yield resulting in easily visualized fluorescence after uptake by cells; small size of the LYCH fluorescent molecule, allowing passage through the cell wall; stability over a wide range of pH, from 2.0-10.0; and absence of toxicity to cells [7,13-15]. Considering that the reabsorption of nectar may occur through the apoplast or symplast pathways [16], the characteristics of LYCH allow tracing in these both pathways.

Our intention was to find a reliable and simpler method to visually demonstrate the nectar reabsorption phenomenon, in the most faithful way to what happens naturally. For that, we performed tests with LYCH dye, adapting its already known use in studies of nectar secretion and nectary structure [11,12], on the floral nectaries of *Cucurbita pepo* L. (Cucurbitaceae), a species recognized by its ability for nectar reabsorption [2,16,17], and extrafloral nectaries of *Passiflora edulis* Sims (Passifloraceae), where structural characteristics indicates that nectar reabsorption does not occur [18,19].

Results and discussion

In all tests performed it is unequivocal that LYCH marks the pathway of nectar according to the ability of the nectary to reabsorb it. LYCH does not spread to tissues during fixation; this implies that the movement of LYCH throughout the cytoplasm is due to the way in which it is taken into the cell and not to the fixation process [13,20]. The reaction of LYCH with fixative clearly

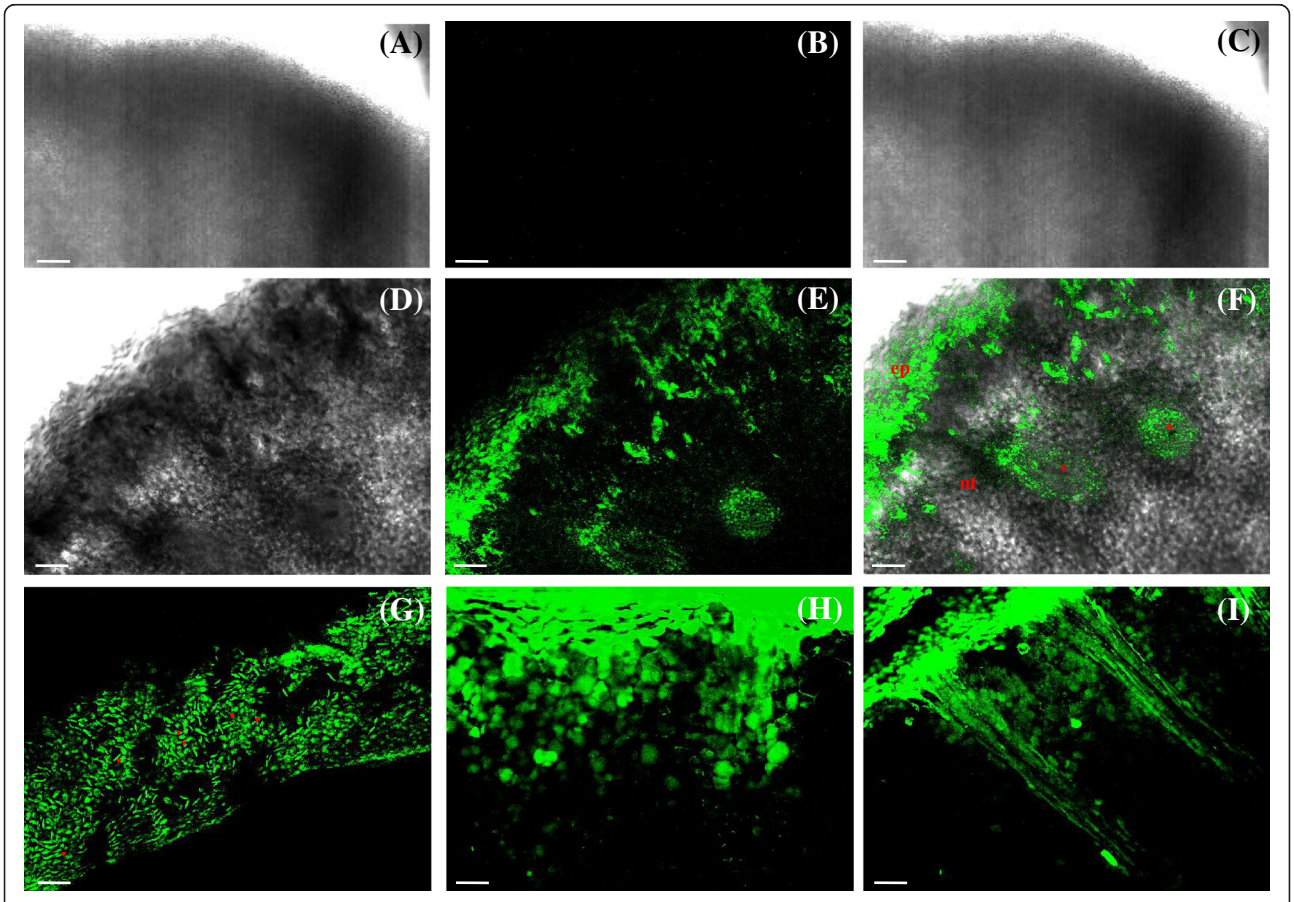

Figure 1 LYCH dye efficiently stains the reabsorbed nectar pathway through nectary tissues. Hand sections of floral nectaries (FN) of *Cucurbita pepo* 24 h after the introduction of the LYCH solution onto nectar standing at the flower base. **(A, D)** Transmission image. **(B, E, G, H)** LYCH fluorescence (485 nm excitation, 530 nm emission). **(C, F, I)** Overlay projection images of transmission and fluorescence. **(A-C)** FN treated with distillated water instead of LYCH solution. Note the absence of fluorescence (B,C). **(D-I)** Different regions of the same nectary: labelled cells in epidermis (ep) **(E-G)**, nectary tissue (nt), inner parenchyma and vascular bundles (*) **(F, H-I). (G)** Epidermis in frontal view; arrowhead, stomata. Scale bars: 25 μm.

avoids several problems associated with the localization of water-soluble dyes in plant tissues [21].

Reabsorption process is visualized on FN in male flowers of *Cucurbita pepo* L

Control treatment showed no fluorescence (Figure 1A-C). A very strong fluorescence is observed within the nectary epidermis illustrated in frontal view (Figure 1G) indicating the presence of LYCH over it. The structural description of the FN indicates that nectar exudes from the stomata and forms a continuous layer on the nectary surface [22]. LYCH fluorescence was concentrated in the epidermis and in the nectary parenchyma layers, decreasing in the deeper part of the parenchyma, reaching the vascular bundles (Figure 1E-I). This experiment revealed the same result reported with the use of ^{14}C-sucrose [17], confirming the use of LYCH as indicative for nectar reabsorption.

According to ultrastructural studies of nectar reabsorption in *Platanthera chlorantha* [23], numerous vesicles are associated with the plasmalemma during the stage of reabsorption, and it is likely that they are formed by endocytosis. Turgor pressure may prevent endocytosis, but evidence from studies on the uptake of membrane-impermeable molecules support the operation of endocytosis in plants and argue for the hypothesis that vesicles are produced by this process in *P. chlorantha* [23 and references therein]. Considering the properties of LYCH as a fluorescent membrane-impermeable dye, it is plausible to use it as a marker for nectar reabsorption.

No reabsorption process is observed on EFN of *Passiflora edulis* Sims

The tests with LYCH were positive only in some portions of the cuticle and cuticular flanges, indicating that there was no influx of nectar into the nectary (Figure 2E-I). We supposed that the aqueous portion of the nectar evaporated, leaving the sugar and LYCH over the EFN surface. Besides fixing the EFN, we also carried out the treatment

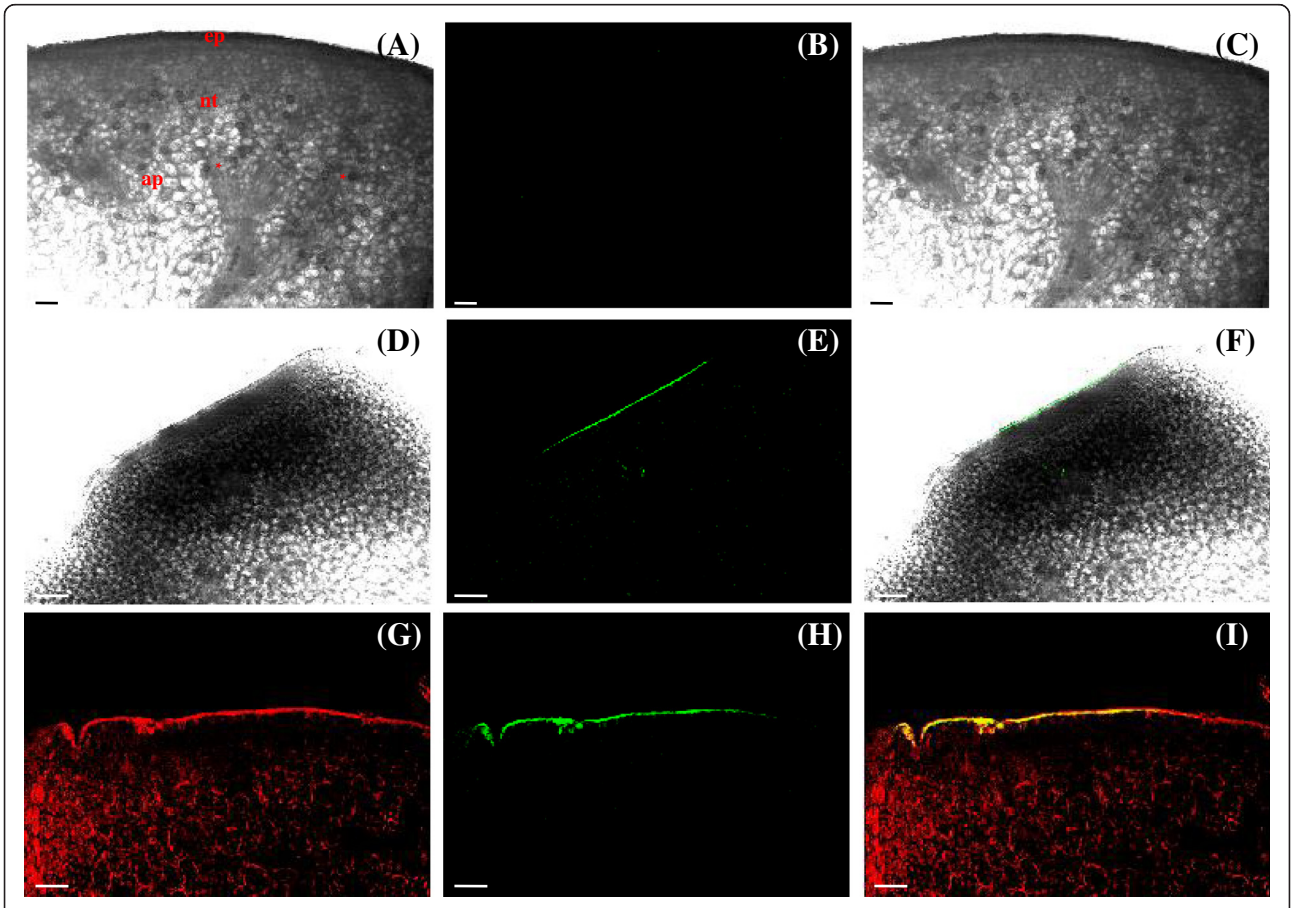

Figure 2 LYCH dye moves with the nectar. Hand sections of extrafloral nectary (EFN) of *Passiflora edulis* 24 h after the application of the LYCH solution. **(A, D, G)** Transmission image. **(B, E, H)** LYCH fluorescence (485 nm excitation, 530 nm emission). **(C, F, I)** Overlay projection images of transmission and fluorescence. **(A-C)** EFN treated with distillated water. Note the absence of fluorescence; ep, epidermis; nt, nectar tissue; ap, adjacent parenchyma, *, vascular terminations. **(E-I)** Cuticle labelled. **(G-I)** Live EFN. **(G)** Autofluorescence of the cuticle and chloroplasts present in nectary tissue and adjacent parenchyma. **(H, I)** Positive result in the cuticle when only the emission of LYCH is selected. Nectar traces remain attached to the cuticle and cuticular flanges. Scale bars: 25 μm.

with LYCH in fresh glands. It was possible to visualize the autofluorescence of the cuticle and chloroplasts (Figure 2G). After the selection of the LYCH-emission wavelength, we were able to visualize the same results observed in fixed material (Figure 2H-I). To avoid the interference of autofluorescence in results from fresh material, we strongly recommend the use of aldehyde-fixatives, especially if the visualization is under epifluorescence microscope, in which only the LYCH-emission wavelength cannot be selected. The control treatment showed no fluorescence (Figure 2A-C).

Since nectar of *Passiflora edulis* possesses glucose, fructose and sucrose [19], we associate the non-occurrence of reabsorption in EFN of *Passiflora* to the presence of a relative thick cuticle and cuticular flanges. The abundance of wax can vary widely and is an important factor for permeability: a cuticle rich in wax favors the passage of fat soluble substances but is a significant barrier for water soluble compounds [24]. There is probably little or no wax in the cuticle of nectaries of *Cucurbita pepo*, which also enables some nectar to emerge through ruptures or openings in the cuticle [22].

Unlike studies where LYCH was applied into the nectaries [11,12], we tried to keep the phenomenon of nectar reabsorption away from any surrounding interference, using the inert fluorescent dye to passively follow the natural pathway of the reabsorbed nectar in the tissues (or retained outside the gland, like at the *P. edulis* EFN) without cutting the flower parts or injecting LYCH directly into the tissues. That is also the reason why we applied the solution of LYCH directly over the nectar, unlike what is performed when applying radioactive labeled techniques, where the nectar is removed and replaced by the marked artificial nectar [16]. Thus, our experiment was performed in order to preserve as much as possible the structure of the nectary, maintaining its natural capacity of reabsorption. Here, we demonstrate the efficiency on the use of LYCH as a fluorescent tracer to observe nectar reabsorption by contrasting glands that reabsorb/not reabsorb to emphasize that this is a trustful technique. Nectar reabsorption is generally accepted as a commonly-occurring phenomenon although the actual process of nectar reabsorption is understudied [2]. Hence, this paper provides a method toward visualization of this process, hoping it can help on future studies that aim to clear the cytological mechanisms of nectar reabsorption.

Methods

Plant material

Floral nectaries (FN) of male flowers of Cucurbita pepo L

Flowers from three plants cultivated in the greenhouse of "Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto" from the University of São Paulo were tested (Figure 3A). Nectar of *C. pepo* is sucrose dominant, regardless of the sex and age of the flower [17]. Relevant morphological and structural aspects [16,22] are detailed: the nectary can be found at a cavity in the base of the partially fused filaments and is accessible through pores of the nectary (Figure 3D). The nectary is composed of an epidermis with stomata from which nectar exudes, and a nectar producing parenchyma (Figure 3E) that receives xylem vessels and phloem sieve tubes.

Extrafloral nectaries (EFN) of Passiflora edulis Sims

Nectar glands present on petioles from second and third visible nodes of three individuals cultivated at the Institute of Botany from São Paulo were tested. The base of branches was isolated with Tanglefood® grease to avoid the removal of the nectar by ants or other insects. These

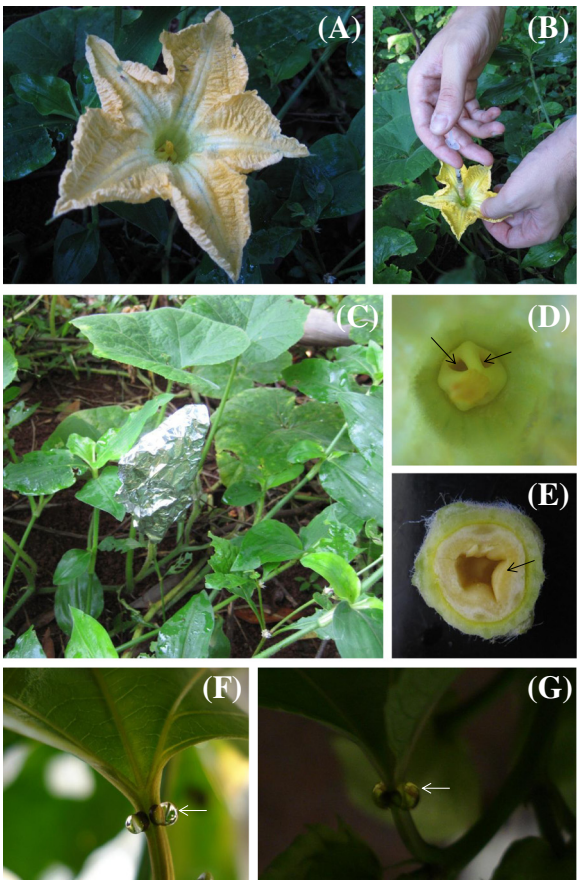

Figure 3 Field procedures and morphological aspects of the nectaries. (A) Floral nectary of a male flower of *Cucurbita pepo* **(B)** Application of 0.3 ml of 1% LYCH onto the accumulated floral nectar. **(C)** Flower immediately wrapped with aluminum foil to avoid sunlight. **(D)** Detail of the androecium. Note the pores of the nectary (arrows). **(E)** Transversal section of the nectary. Note the nectary tissues (arrow). **(F, G)** Extrafloral nectaries (EFN) in the petiole of *Passiflora edulis* Sims. **(F)** Nectar over the surface of the EFN (arrow). **(G)** Plant kept in the dark immediately after the application of a drop of LYCH over the nectar. Note the yellow aspect of the drop after the LYCH application (arrow).

glands exude profuse nectar available during daytime (Figure 3F). The nectar of *P. edulis* is composed by sucrose, glucose and fructose [19]. Some relevant anatomical details are pertinent [19]: the multiple epidermis is covered by a cuticle and cuticular flanges extend up to the inner periclinal wall of the first cell layer. The nectar rising out of the epidermis promotes the cuticle detachment, although cuticle disruption is observed only when ants have access to the EFN. Nectary tissue and the adjacent parenchyma are distinct and separate from each other by vascular terminations.

Field and laboratory procedures

A 1% (w/v) solution of Lucifer Yellow CH dilithium salt (Sigma-Aldrich) in distilled water was prepared in the dark at room temperature.

In the morning (ca. 10 am), 0.3 ml of the solution was applied to the nectary of freshly opened male flowers of *C. pepo* (Figure 3B). Note that no previous manipulations were performed and the stain was mixed to the available floral nectar. The flowers were immediately covered with an aluminum foil (Figure 3C) and kept in the dark during 24 h. In the subsequent morning, the androecium (Figure 3D) of each flower was removed and the FN was isolated (Figure 3E). Nectar glands were washed several times with distilled water and immediately fixed with Karnovsky's glutaraldehyde solution [25] for 24 h. After the fixation the material was washed several times in distilled water, free-hand sectioned and slides mounted in water.

A drop of LYCH was applied over the nectar of each EFN of *Passiflora edulis* (Figure 3G) in the morning (9 am) and the vases were kept in the dark during 24 h. The same procedure was performed with treated flowers of *Cucurbita pepo.* Also, we avoided the fixation step for a pair of glands to compare the results from fresh material to fixed ones. All glands (fixed and fresh) were free-hand sectioned and slides mounted in water.

Control treatments were carried out simultaneously to each LYCH test, submitting FN and EFN to the same procedures with distilled water (Figures 1A-C and 2A-C).

A Zeiss LSM 510-Meta laser scanning confocal microscope was used to obtain the images of the LYCH stained tissues and controls. LYCH was excited by 488 nm and a 530 nm emission filter. In some instances, the tissue was photographed using a combination of emitted and transmitted lights. The resultant images could be overlaid to determine the location of the fluorescent signal.

Abbreviations

LYCH: Lucifer yellow dye; FN: Floral nectaries; EFN: Extrafloral nectaries.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PCG planned the research, carried out the techniques, and drafted the manuscript. JMRBVA also participated in the techniques and in the manuscript writing. ERP and FB contributed with critical review of the manuscript, provided lab support and orientation during the development of this work. All authors read and approved the final manuscript.

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Apêndice 2

Is nectar reabsorption restricted by the stalk cells of floral and extrafloral nectary trichomes?

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RESEARCH PAPER

Is nectar reabsorption restricted by the stalk cells of floral and extrafloral nectary trichomes?

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Keywords

Centranthus ruber; extrafloral nectaries; floral nectaries; *Gossypium hirsutum*; *Hibiscus sinensis*; lucifer yellow CH; neutral red; reabsorption; *Vicia faba*.

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ABSTRACT

Reabsorption is a phase of nectar dynamics that occurs concurrently with secretion; it has been described in floral nectaries that exude nectar through stomata or unicellular trichomes, but has not yet been recorded in extrafloral glands. Apparently, nectar reabsorption does not occur in multicellular secretory trichomes (MST) due to the presence of lipophilic impregnations – which resemble Casparian strips – in the anticlinal walls of the stalk cells. It has been assumed that these impregnations restrict solute movement within MST to occur unidirectionally and exclusively by the symplast, thereby preventing nectar reflux toward the underlying nectary tissues. We hypothesised that reabsorption is absent in nectaries possessing MST. The fluorochrome lucifer yellow (LYCH) was applied to standing nectar of two floral and extrafloral glands of distantly related species, and then emission spectra from nectary sections were systematically analysed using confocal microscopy. Passive uptake of LYCH *via* the stalk cells to the nectary tissues occurred in all MST examined. Moreover, we present evidence of nectar reabsorption in extrafloral nectaries, demonstrating that LYCH passed the stalk cells of MST, although it did not reach the deepest nectary tissues. Identical (control) experiments performed with neutral red (NR) demonstrated no uptake of this stain by actively secreting MST, whereas diffusion of NR did occur in plasmolysed MST of floral nectaries at the post-secretory phase, indicating that nectar reabsorption by MST is governed by stalk cell physiology. Interestingly, non-secretory trichomes failed to reabsorb nectar. The role of various nectary components is discussed in relation to the control of nectar reabsorption by secretory trichomes.

INTRODUCTION

The first studies of *in situ* visualisation of nectar reabsorption involved the uptake of C^{14}-labelled sucrose by alfalfa nectaries (Pedersen *et al.* 1958). Thereafter, studies using sucrose as well as other radioactive sugars (Shuel 1961; Bieleski & Redgwell 1980; Stpiczyńska 2003a,b; Nepi & Stpiczyńska 2007) demonstrated that sucrose, fructose and/or glucose are reabsorbed by glands, even while they actively secrete nectar. Nectar reabsorption is a common process in floral nectaries, occurring regardless of the age, sex or pollination status of the flower, or nectary structure (Nepi & Stpiczyńska 2008). The major functions (not mutually exclusive) attributed to this event are the recovery of resources invested in nectar production and a homeostatic mechanism during nectar secretion and presentation (Pacini & Nepi 2007; Nepi & Stpiczyńska 2008). Although this event is well reported in floral nectaries (Burquez & Corbet 1993), direct observation of nectar reabsorption has not yet been recorded in extrafloral nectaries.

Floral nectaries occur inside the flower and are directly associated with the process of pollination (Fahn 2002). Extrafloral nectaries are less well defined (cf. Schmid 1988), it being simplest to consider these glands as those nectar-secreting structures not directly involved with pollination, regardless of whether they occur on vegetative or reproductive organs (Fahn 1979, 2000, 2002; Gaffal 2012). Irrespective of its origin, nectar can be released through stomata, trichomes (uni- or multicellular) or non-specialised epidermal cells (Fahn 2002), and reabsorption theoretically can occur through the same route (Nepi & Stpiczyńska 2008). However, anatomical details of the reabsorption process itself are scarcely reported, being restricted to a few studies that demonstrate the absorption route in floral nectaries with stomata or unicellular trichomes (Stpiczyńska 2003a, b; Nepi & Stpiczyńska 2007; Cardoso-Gustavson *et al.* 2013).

Multicellular trichomes from floral and extrafloral nectaries generally possess impregnations of lipophilic compounds in the anticlinal walls of their stalk cells (Figier 1971; Gunning & Hughes 1976; Eleftheriou & Hall 1983a; Davis *et al.* 1988; Sawidis 1991; Zellnig *et al.* 1991; Paiva 2009). These basal impregnations are advocated to restrict solute movement into trichomes exclusively by the symplast, promoting a unidirectional transport of solute (Christensen *et al.* 2009), and hypothetically preventing reflux of the accumulated secretion (Lűttge 1971; Paiva 2009). However, Steer (1981) postulated that such cutinised walls may prevent water loss from the apoplast of neighbouring tissues that otherwise could occur due to osmotic attraction of the secreted product. These impregnations also occur at the base in some nectary

unicellular trichomes (Fahn 1979), but not in others, thereby allowing the nectar to be reabsorbed (Stpiczyńska 2003a,b; Nepi & Stpiczyńska 2007). This apoplastic barrier (Gunning & Hughes 1976) at the base of nectary multicellular trichomes has been referred to as cutinised (Eleftheriou & Hall 1983a; Sawidis *et al.* 1987) or lignified (Davis *et al.* 1988; Paiva 2009), and also occurs on the stalk cells of water absorbing multicellular trichomes (North *et al.* 2013). Water absorption by leaves is a well-known strategy performed by any plant regardless of species, phylogenetic lineage or ecosystem type (Limm *et al.* 2009; Qiu *et al.* 2010). Both non-secretory hairs, as commonly found on bromeliads (Pierce *et al.* 2001 and references therein) and glandular multicellular trichomes (Pridgeon 1981) may play the dominant role in water absorption (Lyshede 1978), in which water moves inward from absorbing trichomes through the apoplastic and symplastic routes to the innermost tissues until reaching the xylem (North *et al.* 2013). Although both water absorbing and nectary multicellular trichomes share the same structural characteristics, in the literature it has been attributed to nectary secretory trichomes that their basal lipophilic impregnations impede nectar uptake.

That nectary multicellular secretory trichomes are unable to reabsorb nectar due to the presence of lipophilic impregnations in the anticlinal walls of their stalk cells is investigated here. Species representing various types of nectary trichomes (Nepi 2007) were selected to apply a protocol recently developed to visualise *in situ* reabsorption of nectar using lucifer yellow CH (LYCH) – 6-amino-2,3-dihydro-1,3-dioxo-2-hydrazinocarbonylamino-1H-benz(d,e)isoquinoline-5,8-disulphonic-acid dilithium salt – as a confocal probe (Cardoso-Gustavson *et al.* 2013). The structure and secretory activity of the trichomes investigated here are well documented: unicellular capitate trichomes are present in floral spurs of *Centranthus ruber* (Wagenitz & Laing 1984; Mack 2013), whereas elongate multicellular trichomes typical of the Malvaceae (Janda 1937; Vogel 2000) constitute both the floral and extrafloral nectaries of *Hibiscus sinensis* (Sawidis *et al.* 1987, 1989; Sawidis 1991) and *Gossypium hirsutum* (Wergin *et al.* 1975; Eleftheriou & Hall 1983a,b), respectively; and multicellular capitate trichomes occur on stipules of *Vicia faba* (Bhattacharyya & Maheshwari 1971; Figier 1971; Fahn 1979; Davis *et al.* 1988).

It is demonstrated here that nectar reabsorption indeed occurs in unicellular and multicellular secretory trichomes, and the first direct evidence of this event is provided for extrafloral nectaries. We discuss the possible nectary components involved in this process and mechanisms that may regulate it. Control experiments with neutral red stain, as well as without any dye, also were performed to compare to the uptake of LYCH solution. Finally, data are given for the differential emission of fluorescent constituents of nectary tissues, and the usefulness of confocal microscopy to distinguish between several emissions that can be visualised within nectaries is discussed.

MATERIAL AND METHODS

Plant material

All experiments were carried out on potted plants of *Hibiscus sinensis* Mill. (Malvaceae), Snowcloud variety (Thompson & Morgan, Ipswich, UK) of *Centranthus ruber* (L.) DC. (Valerianaceae), *Gossypium hirsutum* L. (Malvaceae) and *Vicia faba* L. (Fabaceae) grown in the greenhouse at the Department of Biology, University of Saskatchewan. Plants were maintained to prevent access of arthropod visitors to the floral nectaries of *H. sinensis* (Fig. 1A and B) and *C. ruber* (Fig. 1C and D) and the extrafloral nectaries of *G. hirsutum* (Fig. 1E) and *V. faba* (Fig. 1F).

Rationale for use of lucifer yellow CH (LYCH) and neutral red (NR) dye

Lucifer yellow CH has been successfully used to visualise nectar reabsorption as an alternative to the use of radioactive tracers, which are complex and costly (Cardoso-Gustavson *et al.* 2013). The uptake of this salt occurs preferentially in the presence of sucrose, being transported in parallel with this sugar, and it can accumulate in the vacuole (Botha *et al.* 2000; Exteberria *et al.* 2005; and discussion in Cardoso-Gustavson *et al.* 2013). On the other hand, vital stains have been applied to visualise nectar and water absorption by glandular trichomes (Pridgeon 1981; Nepi *et al.* 1996). However, their use is not recommended since diluted stains can diffuse across the tissues without specificity (Cardoso-Gustavson *et al.* 2013). Thus, we used LYCH to assess nectar reabsorption and NR to test if that stain could diffuse internally without interruption or if these glands specifically select which substance can penetrate to the subjacent nectary tissues.

Application of LYCH to floral and extrafloral nectaries

We followed the procedures detailed in Cardoso-Gustavson *et al.* (2013). A 1% solution of LYCH dilithium salt (Sigma-Aldrich, St. Louis, MO, USA) in distilled water (pH 6.9) was prepared in the dark at room temperature. According to the species, different volumes of LYCH solution were applied to standing nectar droplets using an insulin syringe (1 ml capacity). There was no direct injection of the solution into the nectary tissues; instead, the solution was added to naturally accumulated nectar. It was not possible to directly access nectar on the floral nectaries *in situ*, such that LYCH solution was applied to the nectar chamber through the same route that a pollinator takes to consume it.

We applied 180 μl LYCH solution to each of the five floral nectaries in *H. sinensis* and 30 μl into the opening of the nectar spur of *C. ruber*, both in non-pollinated flowers on the first day of anthesis. Following those single applications, no further LYCH solution was reapplied later during flowering. Sepals of *H. sinensis* were harvested 3 and 5 days post-anthesis. Flowers of *C. ruber* were investigated 3 days after anthesis, an interval leading to a moderate natural decline in nectar volume within the spur of this species (Mack 2013).

For the extrafloral nectaries on the midvein near the base of the abaxial leaf surface of *G. hirsutum* and those on the stipules of *V. faba*, a drop of LYCH (*ca.* 10 μl) was applied onto the accumulated nectar droplet in the early morning (07:00 h), for 5 consecutive days. Thus, each extrafloral nectary received a total of 50 μl LYCH solution. LYCH was applied to six leaves from the first to third visible nodes of two branches of *G. hirsutum*, and to six stipules from the first and second nodes from three specimens of *V. faba*.

Following each LYCH application, flowers and leaves were covered with aluminium foil. Control experiments were carried

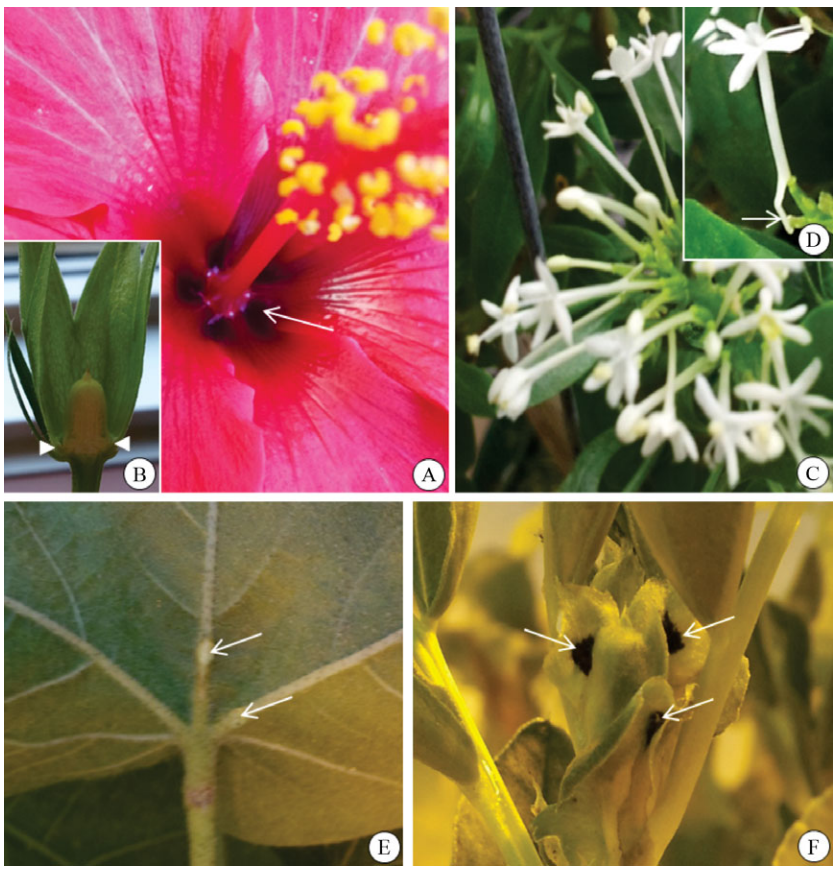

Fig. 1. Morphological aspects of the floral (A–D) and extrafloral (E–F) nectaries investigated. (A–B) *Hibiscus sinensis* at anthesis (A) and 3 days later (B). (A) Apertures in the base of the corolla through which flower visitors access nectar (arrow). (B) Longitudinal section of ovary, after abscission of petals and reproductive whorls. Arrowheads indicate the region where nectaries are found. (C–D) *Centranthus ruber* inflorescence (C) with flowers at several stages of development; note spur (arrow) of a single flower (D). (E) Abaxial leaf surface of *Gossypium hirsutum.* Extrafloral nectaries situated in midrib plus along a major branch vein, secreting a profuse exudate (arrows); for standardisation purposes, LYCH was applied only to midrib glands. (F) Stipular extrafloral nectaries of *Vicia faba*; secretory trichomes are present on abaxial surface exactly where the dark spots are evident (arrows).

out using the same conditions (volume, number of applications, covering), but with distilled water alone.

Application of NR to floral and extrafloral nectaries

Neutral red was applied to floral nectaries of *H. sinensis* and extrafloral glands of *G. hirsutum* based on the use of this dye as a confocal probe (described in Dubrovsky *et al.* 2006). Solutions of 0.3% NR in 0.1% sodium phosphate buffer were prepared at three pH levels (5.3, 6.8, 8.0). Each solution was applied to two different flowers or extrafloral nectaries following the same pattern of volume of dye, number of applications and covering carried out with the LYCH salt analysis.

Fixation and microscopy analysis

After LYCH treatment, single nectaries were isolated, washed several times with distilled water and immediately fixed with Karnovsky's glutaraldehyde solution (modified by Kraus & Arduin 1997) for 24 h. LYCH does not spread to tissues during fixation (Stewart 1981). This fixation step should reduce the tissue tendency to produce autofluorescence, although it was found to be insufficient to remove autofluorescence completely. After fixation, material was washed several times in distilled water, free-hand sectioned and slides mounted in glycerine:distilled water (1:1, v:v). Since some water-soluble dyes can spread within plant tissues during fixation (Canny & McCully 1986), glands treated with NR were not fixed, but immediately washed, sectioned and mounted.

To visualise any lipophilic compounds impregnating the thick anticlinal walls of the stalk cells of secretory trichomes, some sections from control samples were submitted to 0.03% Sudan black B dye (in 70% ethanol), a marker for aliphatic compounds (Pearse 1985), for 30 min at room temperature. Since suberin and/or lignin autofluoresce (Rost 1995; Roshchina 2008) and can constitute these impregnations, some control samples were examined fresh (*i.e.*, without fixation) to observe any autofluorescence within the tissues.

Fluorescence (UV light) and bright-field observations were performed with a Zeiss microscope using Fujifilm Superia 400 ASA film. Magnifications were obtained through the projection of the micrometer scale in the same optical conditions used for the illustrations.

Slides containing a fresh drop of 1% LYCH solution, 0.3% NR solutions at different pH, and 0.1% sucrose or 0.1% of glucose (BDH Laboratory reagent) were prepared to determine the emission spectra of these standards under our experimental conditions. An inverted Zeiss LSM 510-Meta confocal laser scanning microscope was used to image the results with LYCH and NR using a Zeiss water immersion objective (25×).

We also acquired lambda stack images (a series of images of the same microscopic region each taken with different wavelengths in 10- to 11-nm increments) of all treatments to visualise the specific emission from different cells or intracellular structures (including autofluorescence) for the various fluorescent probes used. The 405 nm line of a diode laser was used to excite the LYCH-treated samples plus the LYCH, sucrose and glucose standards. NR standards were excited with a 543 nm

line of a He/Ne laser. Observations were taken of the emission bands from 449 to 695 nm (diode laser) and 556 to 748 nm (He/Ne laser), with emission filters BP 530–600 and BP 560–615, respectively.

Terminology

We followed the simplified terminology of Nepi (2007) to describe the general anatomy of nectaries comprised of trichomes.

RESULTS

Morphology of floral and extrafloral nectaries investigated

The floral nectaries of *H. sinensis* are located within conspicuous solitary flowers in which the insertion of the five petals formed five apertures through which the nectar is accessible to pollinators (Fig. 1A), occurring on the lower inner surface of the fused sepals (Fig. 1B). On the third day of anthesis, flowers started to wilt and the petals and reproductive whorl fell, leaving only the superior ovary and the deeply toothed pentamerous calyx (Fig. 1A). *Centranthus ruber* had tiny flowers (about 2-cm length) at different developmental stages per inflorescence (Fig. 1C). Each flower possessed a single nectar spur at the lower abaxial side of the tubular corolla (Fig. 1D).

Each extrafloral nectary of *G. hirsutum* appeared as a pyriform depression on the abaxial surface of the leaf midrib, *ca.* 1 cm from the lamina base (Fig. 1E). Although an additional nectary could occur near the base of a large branch vein (Butler *et al.* 1972) of the same leaf (Fig. 1E right), for standardisation purposes, reabsorption experiments were limited to the midrib gland. Nectar secretion by these glands was profuse throughout the day. Each extrafloral nectary of *V. faba* was situated in a dark purple region on the stipule abaxial surface (Fig. 1F). Largest accumulation of nectar from these glands occurred during the morning (around 07:00 h).

Nectar reabsorption phenomena in floral and extrafloral nectaries

Floral nectaries – Hibiscus sinensis

In this species, each floral nectary consisted of numerous densely packed multicellular trichomes at the base of each sepal. All trichomes examined comprised a terminal secretory cell (which effectively exudes the nectar during the secretory phase), intermediate cells (of variable number), stalk cells (where the anticlinal wall impregnations occur) and a basal cell at the same level as the non-specialised epidermal cells (Fig. 2A).

Histochemical tests with Sudan black B highlighted the lipophilic nature of the cuticle and the impregnations of the

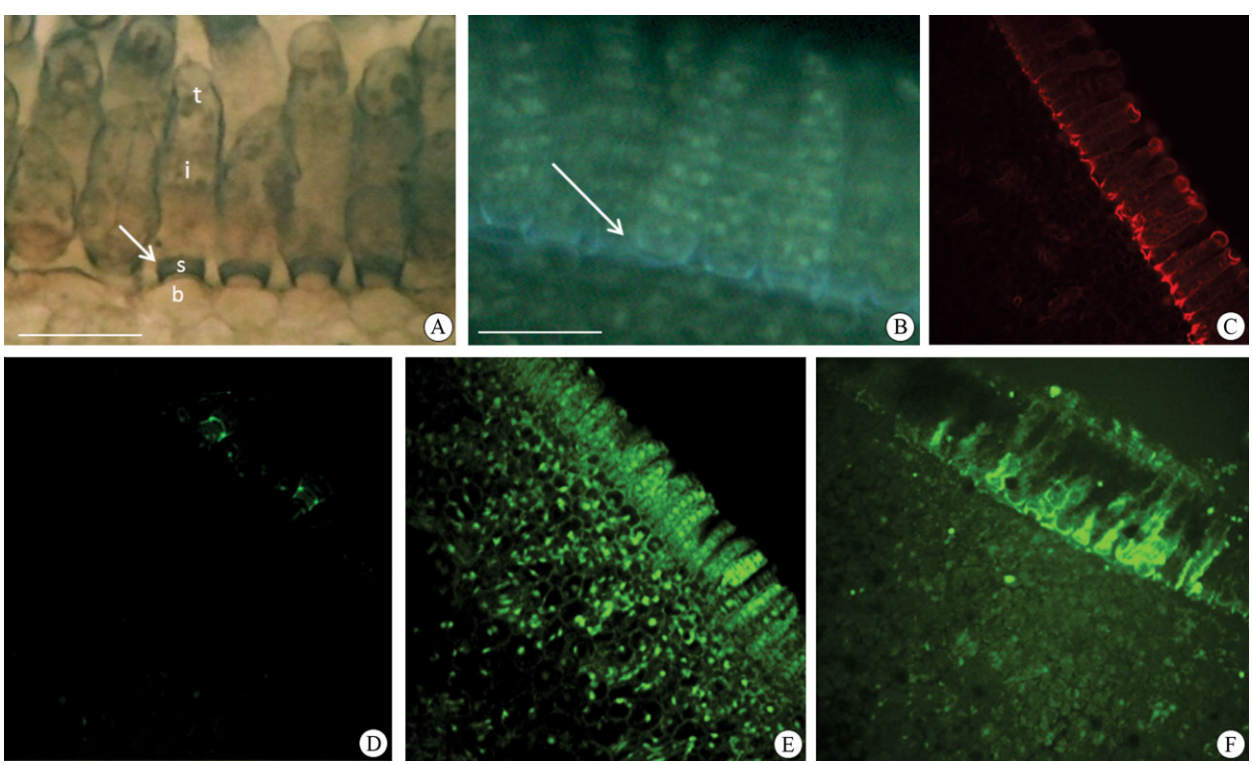

Fig. 2. Reabsorption process in the floral nectary of *Hibiscus sinensis*. (A) Multicellular trichomes are composed of tip (t), intermediate (i), stalk (s) and basal (b) cells. Lipophilic compounds stained with Sudan black B constitute impregnations in anticlinal walls of the stalk cells (arrow). (B) Autofluorescence (UV light) of a fresh control sample (without fixation); note fluorescence from anticlinal walls (arrow). (C) Nectary 3 days post-anthesis, following treatment with neutral red (pH 6.8) on day of anthesis. Note strong emission from stalk cells. (D) Only anticlinal walls fluoresce when control samples were exposed to UV light. (E–F) Absorption of LYCH through secretory trichomes and nectary parenchyma 3 (E) and 5 days (F) after anthesis. Note the plasmolysed trichomes (F). Scale bars: 75 μm.

anticlinal walls of the stalk cells (Fig. 2A); these impregnations also exhibited strong autofluorescence in fresh (*i.e.,* non-fixed) control samples (Fig. 2B).

The emission spectra of NR excited at 543 nm peaked between 550–650 nm (Dubrovsky *et al.* 2006). All samples yielded the same result of incorporation of NR as well as the emission pattern, regardless of pH. After treatment with NR, multicellular trichomes of each nectary showed a strong emission, mainly in the anticlinal walls of the stalk cells (Fig. 2C), due to the lipophilic nature of these inclusions.

The LYCH solution itself had a strong emission between 520–585 nm (see images in lambda stack mode) with a peak observed around 550 nm when excited at 405 nm (Fig. S1). In control samples, fluorescence was verified only from the anticlinal walls of stalk cells (Fig. 2D). However, glands whose standing nectar received an application of LYCH solution presented an intense emission of this salt along the entire length of the multicellular trichomes, as well as the walls and protoplasts of the underlying nectary parenchyma cells (Fig. 2E). Five days after anthesis, tissues exhibited a similar pattern of LYCH emission as observed on day 3 post-anthesis, although the trichomes were now plasmolysed (Fig. 2F).

Detailed analyses of the emission spectra from samples submitted to LYCH are shown in Fig. 3. Nectaries from control flowers presented a pronounced peak corresponding to chlorophyll emission (Fig. S2). On day 3 after anthesis, a strong emission between 450–500 nm – related to phenolic compounds (Rost 1995; Vermerris & Nicholson 2006; Roshchina 2008) – was detected in nectary trichomes and parenchyma, as well as an emission corresponding to the LYCH signal peak, indicating that the salt was indeed incorporated by these tissues (Fig. 3A). Five days after anthesis, the LYCH signal was more intense in nectary trichomes (although plasmolysed) and parenchyma, whereas the signal related to phenolic compounds had decreased at this time (Fig. 3B).

To infer whether entrance of the LYCH salt occurred passively (*i.e.,* without governance from the trichomes), we examined the floral nectaries after addition of NR to the nectar. On day 3 of anthesis, the NR signal was not detected in the nectary parenchyma, being confined to the full length of the active nectary trichomes themselves (Figs 2C and 4A). However, when the floral nectaries were examined on day 5 post-anthesis following NR application, the NR emission signal was detected throughout each nectary, including within plasmolysed cells

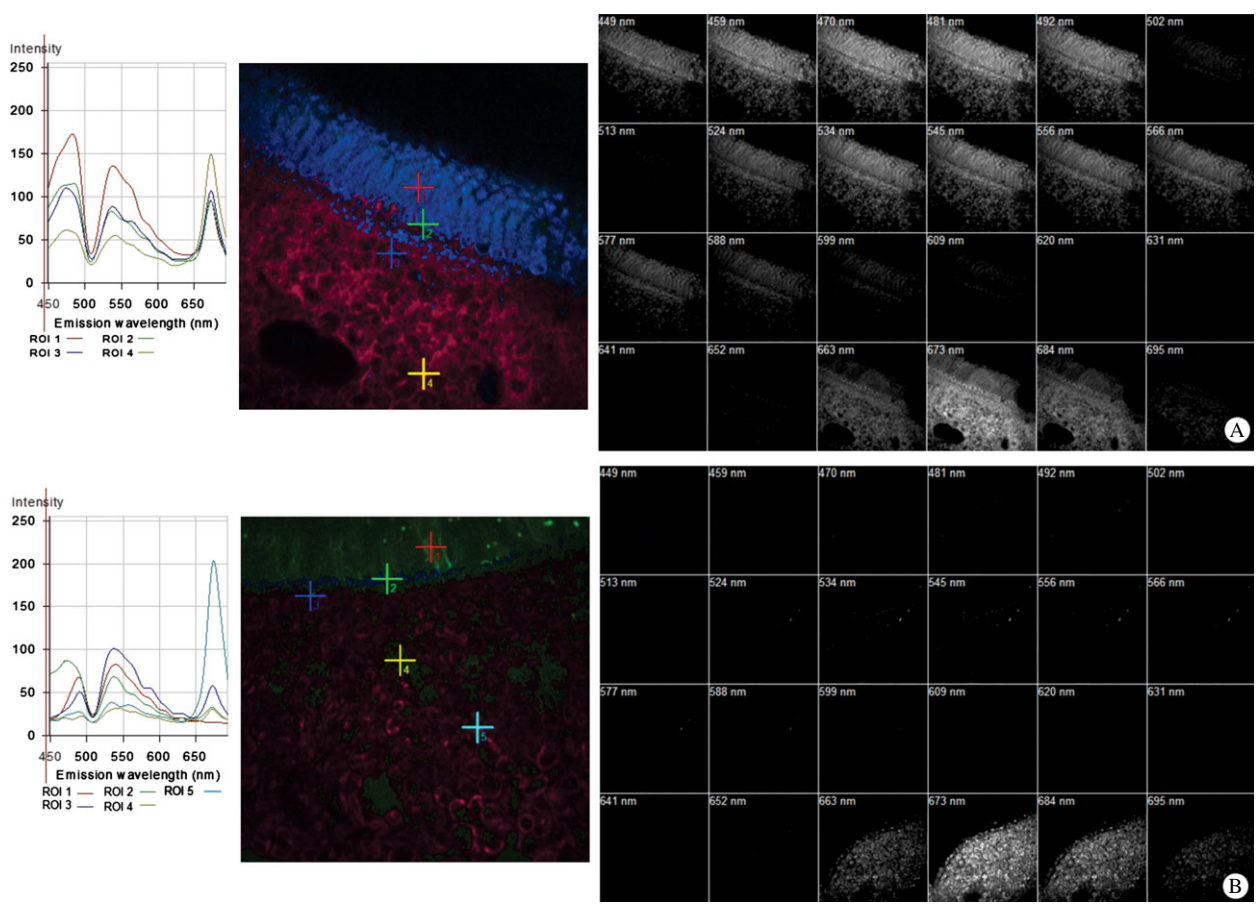

Fig. 3. Emission spectra from floral nectary tissues of *Hibiscus sinensis* treated with LYCH solution on the day of anthesis. Nectaries observed 3 (A) and 5 (B) days after anthesis. The peak between 450–500 nm refers to phenolic compounds, 500–600 nm to LYCH, and above 600 nm to chlorophyll emissions. A lambda stack of data from 24 channels (spectrum 449–695 nm) allows visualisation of luminescence intensity from different regions of the nectary. Note that emission intensity is higher on the third day (A) of analysis, wherein the peak related to phenolic compounds is more intense than LYCH; this pattern is reversed in samples from the fifth day of anthesis (B).

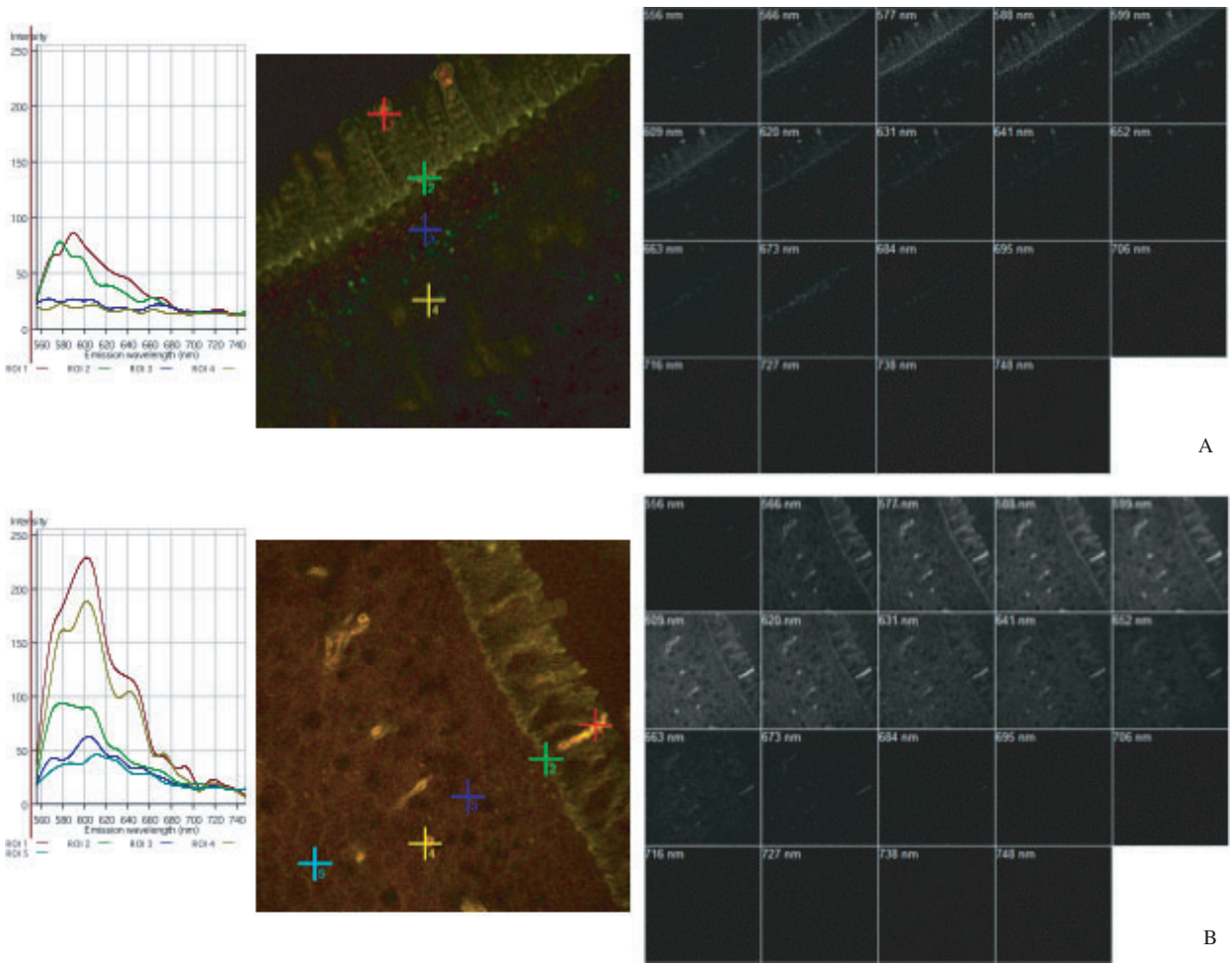

Fig. 4. Emission spectra from floral nectary tissues of *Hibiscus sinensis* treated with neutral red (NR), pH 6.8, on day of anthesis. Nectaries observed 3 (A) and 5 (B) days after anthesis. The peak related to NR emission is observed between 560–640 nm. Luminescence intensity of NR is higher on fifth day of anthesis (B). Emission of NR is confined to nectary trichomes at day 3 of anthesis (A) but at day 5 (B) is exhibited from all nectary tissues, including vascular endings.

and also in the subjacent nectary tissues, including the ends of the vasculature nearest the trichomes (Fig. 4B).

Floral nectaries – Centranthus ruber

In this species, the floral nectary comprises a file of unicellular capitate secretory trichomes confined to one side that runs opposite the vascular bundle (Fig. 6) along the entire length of the inner surface of the spur. The cuticle stained intensely with Sudan black B, as did the trichome thickened basal region of wall and cuticle before it contacted neighbouring, non-specialised epidermal cells (Fig. 5A). In addition to these glandular trichomes, many more non-secretory trichomes – each with a thin cuticle and narrow protoplast that stained weakly with Sudan black B (Fig. 5B) – lined the remaining circumference of the inner spur surface (Fig. 6). Nectary trichome content was colourless under bright field (Fig. 5C), and their autofluorescence was not fully removed after fixation with glutaraldehyde (Fig. 5D).

Non-secretory trichomes exhibited an emission peak related to phenolic compounds, while the secretory trichomes and other nectary tissues had a peak due to chlorophyll (Fig. S3). LYCH samples exhibited strong emission peaks in cell walls and protoplasts of the nectary secretory trichomes, parenchyma (Fig. 5E) and vasculature (Fig. 5F). When compared to control samples (Fig. S3), LYCH luminescence activity was strong and also an increase in the emission of phenolic compounds was observed (Fig. 6). LYCH emission was not evident inside non-secretory trichomes (Fig. 5E), although autofluorescence of these hairs due to phenolic content was high (Fig. S3).

Extrafloral nectaries – Gossypium hirsutum

Each extrafloral nectary of this species was composed of many multicellular secretory trichomes confined in a depression of the midrib. The cuticle, as well as the anticlinal walls of the stalk cells, stained with Sudan black B (Fig. 7A). In sections from fresh control samples, the anticlinal walls of these stalk cells also showed a strong autofluorescence (Fig. 7B).

The emissions of phenolic compounds and chlorophyll presented a weak intensity in control samples (Fig. S4). However, when LYCH and NR were applied separately to the extrafloral nectar of this species, an intense emission of both LYCH and NR in the nectary secretory trichomes and anticlinal walls of their stalk cells was visualised after five consecutive days of reagent application (Fig. 7C and D). There was reabsorption of

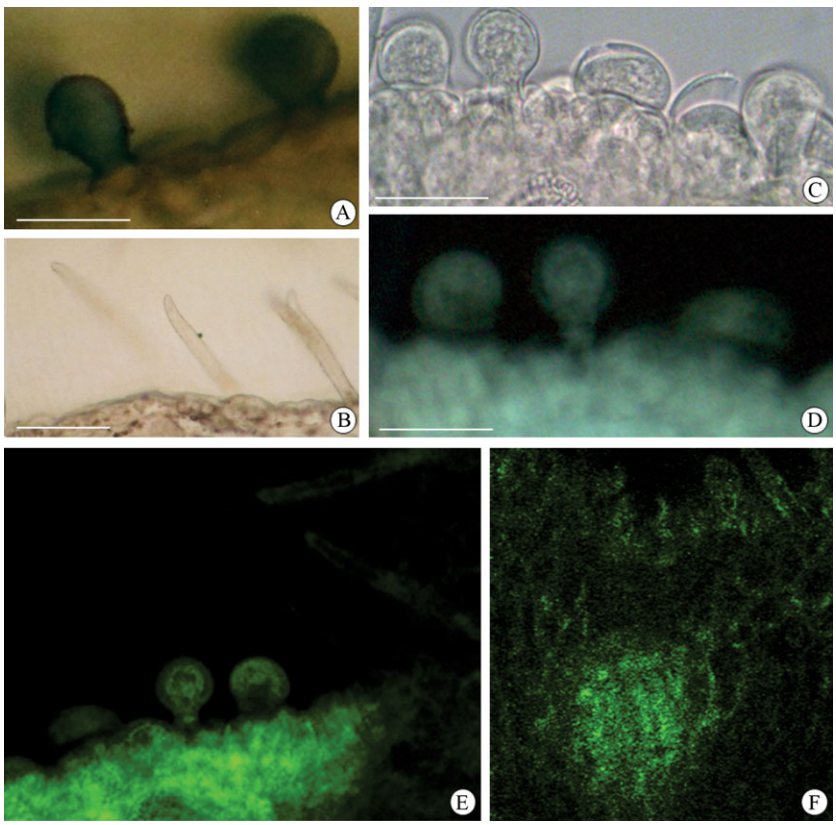

Fig. 5. Reabsorption process in floral nectary of *Centranthus ruber* 3 days after anthesis. (A–B) Lipophilic compounds in cuticle and base of unicellular secretory (A) and non-secretory (B) trichomes after staining with Sudan black B. (C–D) Control sample (after fixation) visualised under bright field (C) and UV light (D). (E–F) LYCH emission from nectary trichomes, parenchyma and vasculature. Scale bars: 75 μm.

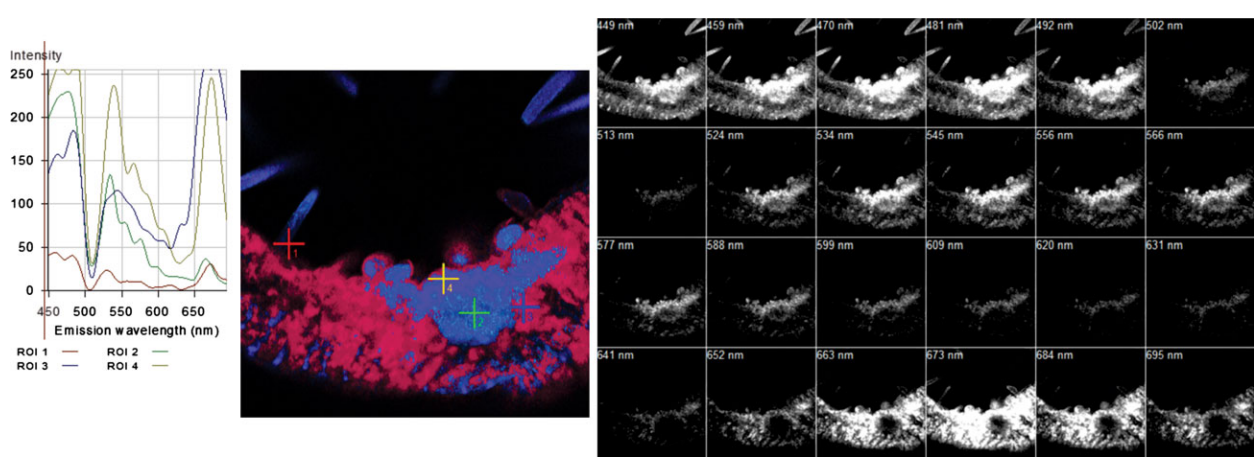

Fig. 6. Emission spectrum from floral nectary tissues of *Centranthus ruber* 3 days after anthesis, following treatment with LYCH on day of anthesis. Emission peaks between 450–500 nm, 500–600 nm and above 600 nm refer to phenolic compounds, LYCH absorption and chlorophyll, respectively. Nectary tissues present a strong emission related to phenolic compounds and LYCH; the low emissions are related to non-secretory trichomes.

LYCH solution from the multicellular trichomes to the parenchyma, but barely reaching the nectary innermost tissues, such as subnectary parenchyma or vascular tissue (Fig. 8A). The uptake of NR was even more restrictive, in that this dye did not reach the nectary parenchyma, being confined to the secretory trichomes (Fig. 8B).

Extrafloral nectaries – Vicia faba

Extrafloral nectaries of *V. faba* consisted of hundreds of multicellular secretory trichomes located in a slight depression easily recognised from the occurrence of anthocyanin inside the secretory trichomes and other nectary tissues (Fig. 7E). The cuticle of both secretory and non-secretory trichomes was intensely stained with Sudan black B, interfering with the visualisation of the anticlinal walls of the stalk cells (Fig. 7F); however, this impregnation possessed a strong blue autofluorescence in both secretory and non-secretory trichomes caused by UV light (Fig. 7G). This blue colour is indicative of lignin (Roshchina 2008), as demonstrated histochemically (Davis *et al.* 1988). Considering the occurrence of polyphenols in the

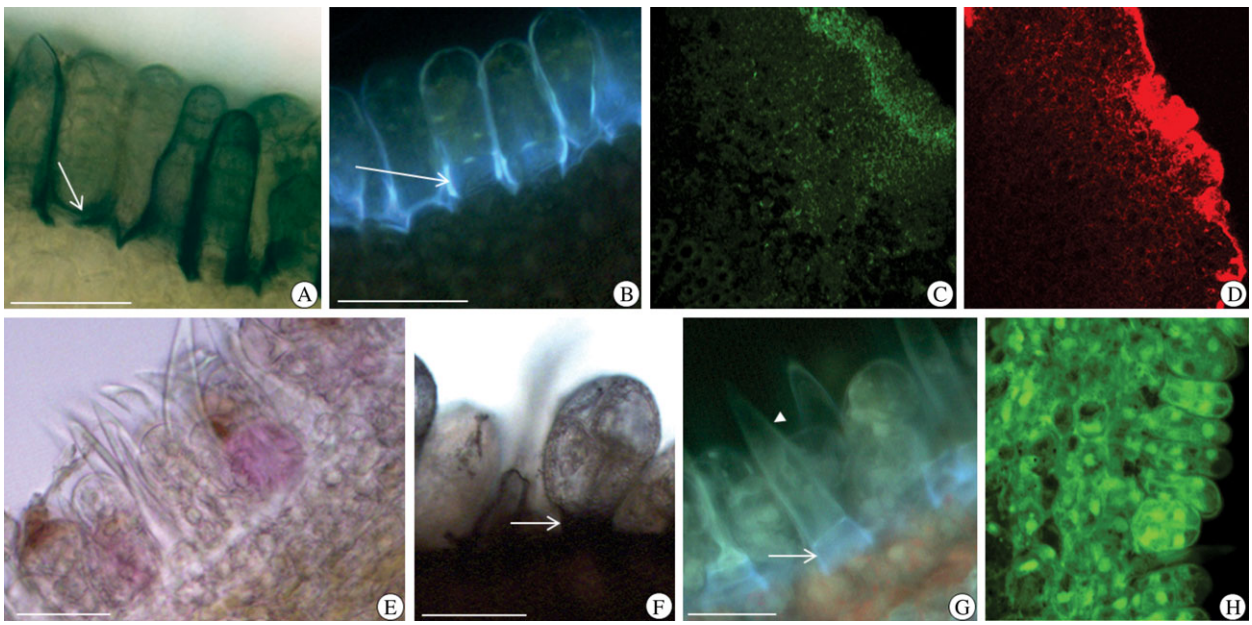

Fig. 7. Reabsorption process in the extrafloral nectaries of *Gossypium hirsutum* (A–D) and *Vicia faba* (E–H). (A, F) Cuticle and impregnations of anticlinal walls possess a lipophilic nature as evidenced by Sudan black B (arrows). (B, E, G) Fresh control samples visualised under bright field (E) and UV light (B, G). Anticlinal walls of stalk cells from secretory (long arrow, B) and non-secretory trichomes (short arrow, G) present strong autofluorescence; note thick cuticle (arrowhead, G) of non-secretory trichome. (E) Presence of anthocyanin in nectary and non-secretory tissue. (C, H) Emission from all nectary tissues after LYCH applications for five consecutive days. Strong LYCH emission following LYCH absorption by all nectary tissues, except non-secretory trichomes (bottom right, H). (D) Emission of neutral red from nectary trichomes after daily applications for the same period. Scale bars: 75 μm.

cuticle and their green fluorescence under UV light (Roshchina 2008), it appears that the cuticle of *V. faba* non-secretory hairs is thicker than that within the spur of *C. ruber*.

Control samples presented an intense emission of phenolic compounds from non-secretory trichomes, while only chlorophyll was detected from the nectary internal tissues (Fig. S5). The application of LYCH solution for five consecutive days resulted in an intense incorporation of this salt into the nectary secretory trichomes and subnectary tissues (Figs 7H and 9). Similar to *C. ruber*, *V. faba* non-secretory trichomes did not incorporate LYCH (Fig. 7H, bottom right), but exhibited a higher emission of secondary metabolites (Fig. 9) compared to the non-secretory trichomes within the *C. ruber* spur.

DISCUSSION

When LYCH solution was applied to standing nectar droplets (Cardoso-Gustavson *et al.* 2013), it resulted in successful visualisation of nectar reabsorption that emulated the results obtained when radioactively labelled sugars were applied to the floral nectary of *Cucurbita pepo* (Nepi & Stpiczyńska 2007). Thus, use of this fluorochrome together with proper controls, coupled with confocal laser scanning microscopy, has provided an ideal methodology to investigate nectar reabsorption in trichome-possessing nectaries of the four studied species.

Confocal laser scanning microscopy ideally is applied to tissues exhibiting minimal autofluorescence and nonspecific background fluorescence (Viegas *et al.* 2007). Despite the use of Karnovsky's glutaraldehyde solution, this fixative did not eliminate interfering autofluorescence of all constitutive substances within the nectaries. Accordingly, acquisition of lambda stack images plus direct observation of emissions from specific, arbitrary pinpoints within the nectaries (Dickinson *et al.* 2001) allowed spectra of the LYCH probe to be distinguished from tissue autofluorescence of control samples (*e.g.*, Fig. 2D–F). Although characterisation of all fluorescent compounds comprising nectary tissues was not our primary purpose, awareness of such compounds that constitute the tissue background emissions is important for correct interpretation of LYCH uptake from nectaries, even when examining fresh material.

Some physical aspects of luminescence intensity

The observed fluorescence of intact cells is the sum of several emission patterns from different groups of substances (Roshchina 2008). The luminescence intensity of some compounds can be enhanced or decreased according to the interaction with other fluorophores, such as LYCH.

The floral and extrafloral nectars and nectaries may fluoresce, whereas sugar itself does not. These emissions may be due to fluorescent secondary metabolites such as alkaloids, terpenes or phenolic compounds dissolved in nectar (Roshchina 2008). We observed three peaks in all nectaries examined, related to chlorophyll, phenolic compounds, and – in the experimental samples – to LYCH. Chlorophyll represents a large characteristic maximum of 680–685 nm in the red region of the spectrum (Roshchina 2008), while the source of blue-green fluorescence may be related to different classes of phenolic compounds, such as flavonoids (Rost 1995; Hutzler *et al.* 1998; Vermerris & Nicholson 2006; Roshchina 2008).

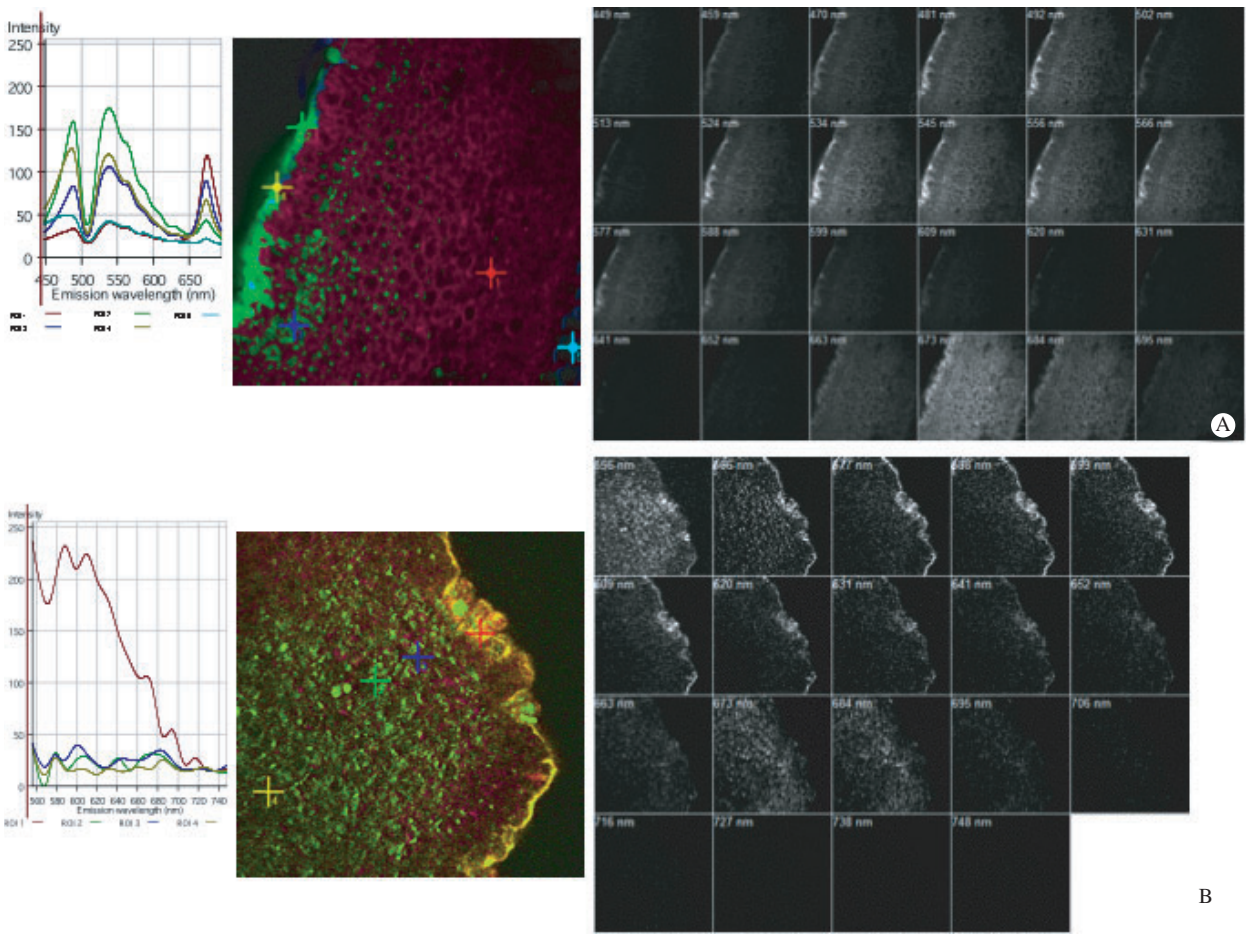

Fig. 8. Emission spectra from extrafloral nectary tissues of *Gossypium hirsutum* after application of LYCH (A) or neutral red (B) for five consecutive days. (A) Emission peaks between 450–500 nm, 500–600 nm and above 600 nm pertain to phenolic compounds, LYCH absorption and chlorophyll, respectively. Emission of LYCH is observed only in nectary trichomes and parenchyma, not reaching innermost tissues where chlorophyll emission is conspicuous (detailed in lambda stack mode). (B) The peak related to neutral red emission is observed between 560–640 nm. A strong emission of this stain is evident from nectary trichomes.

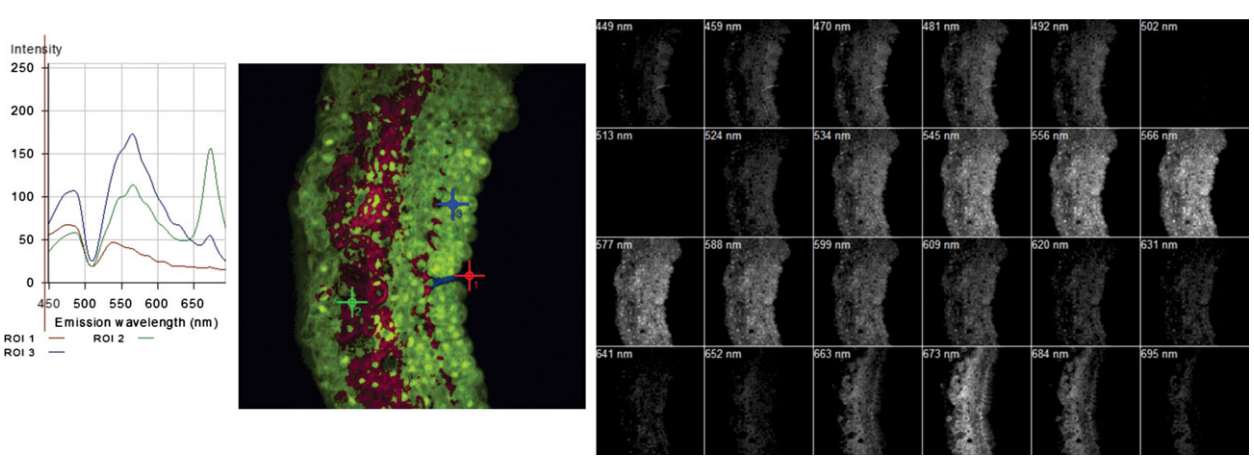

Fig. 9. Emission spectrum from extrafloral nectary tissues of *Vicia faba* after application of LYCH for five consecutive days. Emission peaks between 450–500 nm, 500–600 nm and above 600 nm refer to phenolic compounds, LYCH reabsorption and chlorophyll, respectively. LYCH emission is observed from all nectary tissues except non-secretory trichomes, which emit only in the range of phenolic compounds.

Comparing emission patterns of controls and those nectaries treated with LYCH, some compounds had their luminescence intensity increased while others (sometimes the same one) decreased. The peak related to phenolic compounds from *C. ruber*, *G. hirsutum* and *V. faba* increased in emission intensity when samples were submitted to LYCH, whereas in

H. sinensis (regardless of the period of analysis) there was a decrease in this pattern. Meanwhile, chlorophyll emission was enhanced in all nectaries treated with LYCH.

At least two factors may explain the observed alterations in luminescence activity: (i) LYCH or Karnovsky fixative (or both) can interact with the sample and somehow increase or reduce the other emissions; or (ii) LYCH possesses a very small emission peak between 490–520 nm (Fig. S1), possibly causing an overlap with phenolic/terpenoid emission, thereby resulting in an increase in emission intensity. Indeed, other factors can affect the luminescence intensity of even known compounds: molecule size; occurrence of any differential element in the structure of the nectary that allows electron transition that emits in the region of the analysed spectra; occurrence of a change in the crystalline field over the optical centre whenever a new component is introduced (such as LYCH or Karnovsky fixative); and formation of new substances when all these compounds are combined (Azaroff & Brophy 1963; Ralls *et al.* 1976; Cao & Wang 2011). As the exact chemical composition of the phenolic compounds and nectary tissues are unknown, it is impossible, in our analysis, to precisely indicate the reason for the observed specific differences in enhancement/reduction caused by the presence of LYCH in nectar. These important aspects await further investigation.

Nectar reabsorption by floral and extrafloral nectaries

These experiments with LYCH application to standing nectar droplets demonstrated that the route of nectar sugars through these secretory trichomes is actually bidirectional. Furthermore, reabsorption of accumulated nectar can occur through apoplastic and symplastic routes, since LYCH was visualised in both cell walls and protoplasts of the secretory trichomes and underlying nectary tissue, regardless of the nature of the nectary (*i.e.,* floral or extrafloral). Maintenance of contact between accumulated nectar and the floral nectary itself is a prerequisite for nectar reabsorption (Burquez & Corbet 1993), and this condition was met not only for the floral nectaries of *C. ruber* and *H. sinensis*, but also for the extrafloral nectaries of *G. hirsutum* and *V. faba.*

Despite the qualitative variation in cuticle thickness of the nectary trichomes, nectar reabsorption occurred in floral and extrafloral nectaries of all four species examined. At least concerning water passage through the cuticle, permeability is not correlated with cuticle thickness nor with wax coverage (Riederer & Schreiber 2001); in fact, thin cuticles tend to be more efficient barriers than thick cuticles, since both diffusion and partition coefficients are elevated with increasing cuticle thickness (Becker *et al.* 1986). Thus, for the species examined here, it is not features of the cuticle that preclude nectar reabsorption; instead, this event is probably under strict metabolic control (Christensen *et al.* 2009).

The reabsorption process is linked with the ability of tissues for uptake, which can be inferred by the absence of LYCH within the nectary non-secretory trichomes of *C. ruber* and *V. faba*. Thus, regions where reabsorption starts, such as at the tips of secretory trichomes, are probably where 'sugar sensing' structures – the existence of receptors within flowers to allow regulation of nectar levels, *i.e.,* to control nectar properties such as volume, concentration and viscosity that change dynamically over time – occur (Castellanos *et al.* 2002; Rolland *et al.* 2006; Nepi & Stpiczyńska 2008).

The floral nectaries readily absorbed LYCH. *Hibiscus* nectaries were highly permeable to LYCH after 3 days of flowering. This uptake of LYCH across the trichome cuticle and inwardly beyond the cutinised stalk cell at the trichome base occurred while the flowers were open and the glandular trichomes still in their secretory phase. After 5 days of anthesis, *H. sinensis* trichomes were plasmolysed and LYCH influx occurred freely through the trichomes and into the innermost nectary tissues up to the vascular bundles. Similarly, an intense uptake of LYCH by the floral spur of *C. ruber* was observed from the nectary unicellular trichomes inward to the spur vascular bundle, supporting the onset of net nectar reabsorption at day 3 of flowering in *C. ruber* (Mack 2013). These glands, which resemble the capitate unicellular trichomes of the closely related *Lonicera japonica* (Fahn 1979), showed a pattern of nectar reabsorption similar to the unicellular papillae within the nectar spur of *Platanthera chlorantha* (Orchidaceae; Stpiczyńska 2003a,b; Nepi & Stpiczyńska 2007). However, LYCH reabsorption by the unicellular capitate secretory trichomes of *Centranthus* occurred despite possession of a basal region impregnated with a cutin-like material (Mack 2013), reportedly lacking in the spur papillae of *P. chlorantha* (Stpiczyńska 2003a). Thus, possession of lipidic impregnations at the trichome base did not prevent passage by LYCH to the underlying nectary tissues and vascular bundle.

Thus, the floral secretory trichomes in *Centranthus* and *Hibiscus* are engaged in both nectar secretion and reabsorption, even when covered by a cuticle lacking pores or cracks (Mack 2013), as in *Platanthera* (Stpiczyńska 2003a,b). In the latter, labelled sugar predominated in cell walls (an indication of an apoplastic route) but also inside cells (symplastic route) (Stpiczyńska 2003a,b; Nepi & Stpiczyńska 2007). From a technical standpoint, the difficulty in observing small grey dots representing radiolabelled compounds *via* autoradiography – even in unstained nectary tissues – contrasts with the facile tracking of the prominent emissions of LYCH. LYCH reached the vascular bundles of floral nectaries, indicating its potential reallocation to various floral organs. Due to fluorescence microscopy's high contrast and the increased sensitivity of confocal microscopy, even compounds at low concentrations, such as sucrose being dispersed to nectary parenchyma and innermost tissues, were visualised with confidence.

In the extrafloral nectaries of *Gossypium* and *Vicia*, reabsorption was detected after five consecutive days of LYCH application to accumulated nectar, but the emission of LYCH fluorescence only reached the nectary parenchyma. Since sugar concentration of nectar from extrafloral nectaries varies less because ants are main consumers of extrafloral nectar (Pacini & Nicolson 2007), LYCH uptake may simply indicate a diffusion of sugar back to the nectary once nectar remained uncollected by any visitor. The occurrence of reabsorbed nectar only into the nectary parenchyma may indicate that, in contrast to floral nectaries (Nepi & Stpiczyńska 2008; Cardoso-Gustavson *et al.* 2013), extrafloral nectar is generally not reallocated to distant regions of the plant, at least for the two species and periods of analysis examined here. Also, sugars can act by affecting osmotic potentials as well as by functioning as signalling molecules (Gibson 2005). It is hypothesised that reabsorption could be a response to modifications of cell turgor, which in turn responds rapidly to changes in osmolality (Castellanos *et al.* 2002). This possibility could also explain the non-

occurrence of LYCH in innermost tissues of the extrafloral nectaries of *G. hirsutum* and *V. faba*, because changes in sugar concentration gradient could favour nectar influx only until the nectary parenchyma is reached.

Noteworthy is that LYCH, taken up preferentially in the presence of sucrose (Botha *et al.* 2000; Exteberria *et al.* 2005), was reabsorbed from nectar droplets of variable carbohydrate composition. In floral nectar of *C. ruber*, sucrose predominates over glucose and fructose (Percival 1961; Mack 2013), whereas the floral nectar of *H. sinensis* is 54.6% glucose, 45.0% fructose and just 0.4% sucrose (Sawidis 1988). Also, those monosaccharides prevail in extrafloral nectar of both *G. hirsutum* (Butler *et al.* 1972) and *V. faba* (Davis *et al.* 1988).

Neutral red: evidence that the control of dye influx ceases in secretory trichomes at the post-secretory phase

The exact protocol used for the LYCH analysis was applied to floral and extrafloral nectaries of the Malvaceae, namely *H. sinensis* and *G. hirsutum*, respectively. NR, a lipid fluorochrome possessing affinity to suberin and lignin (Kirk 1975), is useful as a confocal probe (Dubrovsky *et al.* 2006). Having applied this stain to nectar, we had to consider that strong emission of this stain in the stalk cells may occur due to its binding to the lipophilic impregnations, similar to when this stain is applied during typical anatomical analysis. However, the only tissues into which NR diffused were glandular trichomes of both floral and extrafloral nectaries, during their secretory phase. In both species, NR influx was impeded, such that NR did not access nectary parenchyma. The diffusion through stalk cells to the innermost nectary tissues was observed only in floral nectaries of *H. hirsutum* at the post-secretory phase. At that phase, the tip and intermediate cells of secretory trichomes had plasmolysed and their permeability increased, allowing entrance of solutes as well as NR. The occurrence of a passive diffusion of NR through multicellular secretory trichomes at the post-secretory phase is evidence that a controlled process occurs during the secretory phase itself, probably to regulate nectar concentration and that, after the secretion period has ceased, nectar not collected by visitors can simply diffuse back to nectary tissues.

Samples subjected to NR treatment yielded several smaller peaks constituting the emission peak itself (see Figs 4 and 8B). This effect is attributed to the occurrence of multiple optical transitions, in which the more intense is the most likely transition at the local electric field of NR. The higher peaks (inside nectary trichomes) were related to NR emissions. The other peaks of low luminescence intensity were associated with chlorophyll emission. As the NR samples were not fixed prior to examination, the *in vivo* metabolism was maintained, and the multiple optical transitions may be associated with metabolic processes of the nectary cells occurring at the moment of analyses.

Autofluorescence and the lipophilic nature of stalk cell wall impregnations

The mechanism of nectar reabsorption through multicellular trichomes resembles that of water uptake *via* secretory or non-secretory trichomes, in which water is able to cross the stalk cells despite the occurrence of the lipophilic impregnations (Pridgeon 1981; Limm *et al.* 2009 and references therein). The formation of anticlinal impregnations in stalk cells of water absorbing (Pridgeon 1981) and nectary trichomes (Gunning & Hughes 1976; Eleftheriou & Hall 1983a) is similar to development of the Casparian strip (CS) in *Pinus* needles (Wu *et al.* 2005). Indeed, the occurrence of CS in stalk cells of glandular trichomes has already been suggested (Lűttge 1971; Fahn 1979, 2000; Paiva 2009). The impregnations of stalk cells contain lignin in *V. faba* (Davis *et al.* 1988) and a cutin-like compound in *H. sinensis* (Sawidis *et al.* 1987, 1989; Sawidis 1991) and *G. hirsutum* (Eleftheriou & Hall 1983a,b). The strong autofluorescence emitted by the impregnations from all multicellular trichomes examined here, as well as their lipophilic nature exposed by Sudan black B, can be related to the occurrence of lignin and suberin, since both substances have phenolic compounds constituting their structure (Rost 1995; Roshchina 2008; Ranathunge & Schreiber 2011).

The primary function attributed to these lipophilic anticlinal impregnations in the stalk cells of trichomes is to block apoplastic passage of solutes (Lűttge 1971; Fahn 2000): solutes can cross these cells either symplastically (after uptake into trichome tips) or directly across the stalk cells through polar localised influx and efflux carriers (transcellular transport) (Geldner 2013). Ecological roles have also been attributed to these impregnations, such as barriers against pathogen entry and/or microorganism colonisation (Meyer & Peterson 2011). Considering that nectar can provide a nutritious medium for microorganism development – although deterred by the presence of secondary metabolites (González-Teuber & Heil 2009) – the uptake of nectar by multicellular trichomes has an additional advantage over other nectary epidermis, because stalk cells can impede pathogen penetration.

The lipophilic impregnations of stalk cells are also considered responsible for preventing any secretion reflux (Fahn 2000; Paiva 2009). Although the strong autofluorescence from the impregnations did not allow visualisation of the sugar route back to nectary tissues through stalk cells, our experiments with LYCH clearly demonstrate that nectar uptake occurs. Similarly, LYCH has already been applied by injection to study solute movement through nectary multicellular trichomes of *Abutilon*, in which this bidirectional passage through stalk cells was also noted (Zellnig *et al.* 1991).

In control samples, non-secretory trichomes of *C. ruber* and *V. faba* exhibited two emission peaks: one of relatively high intensity (450–500 nm) and a second peak between 520–600 nm (Figs S3 and S5). Both are related to different classes of phenolic compounds (Vermerris & Nicholson 2006; Roshchina 2008). The luminescence intensity of these compounds is higher in *V. faba*, especially for the former peak. Non-secretory trichomes presented the same emission pattern as in samples submitted to LYCH treatment (Figs 6 and 9), and themselves lack LYCH staining (Figs 5E and 7H), indicating that unlike their neighbouring glandular hairs, these non-secretory trichomes did not take up nectar. This differential uptake was demonstrated using fluorescent probes and can be related to the differential permeability that impregnations like Casparian strips exhibit (Wu *et al.* 2005). These differences in permeability could explain why some trichomes allow bidirectional movement while others prevent backflow.

We conclude that the wall impregnations at the base of the secretory trichomes of the four nectary systems (floral – *C. ruber*, *H. sinensis*; extrafloral – *G. hirsutum*, *V. faba*) investi-

gated do not prevent sugar re-entry to the subjacent nectary tissues when LYCH is supplied externally to standing droplets of nectar. These studies have elucidated that a sugar sensing mechanism appears to be present in the stalk cells themselves, highlighting the relevance of stalk cells not only as presenting barriers to reduce an apoplastic route of outflow, but also controlling inflow from the extracellular environment into actively secreting nectary tissues. Therefore, even relatively complex secretory trichomes of nectaries are readily capable of nectar reabsorption, similar to previous studies of LYCH reabsorption by floral nectaries that possess surface stomata (Cardoso-Gustavson *et al.* 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Confocal analysis of the 1% LYCH solution applied in these experiments. Visualisation of spectra emission in the range of 449–695 nm with sample excited in a 405 nm line of the diode. The LYCH spectrum presents two peaks: between 534–566 nm (maximum emission) and 490–520 nm, also visualised in lambda stack mode.

Figure S2. Emission spectra from floral nectary of *Hibiscus sinensis* treated with water (control). Sample excited with a 405 nm line of a diode laser. The obvious peak (above 650 nm) at right is related to chlorophyll emission in nectary and subnectary parenchyma.

Figure S3. Emission spectra from floral nectary of *Centranthus ruber* treated with water (control). Non-secretory trichomes exhibit a peak related to phenolic compounds (450–500 nm), whereas nectary tissues present a high peak relative to chlorophyll emission (above 650 nm).

Figure S4. Emission spectra from extrafloral nectary of *Gossypium hirsutum* treated with water (control). Note smooth peak related to phenolic compound emission (450–500 nm).

Figure S5. Emission spectra of extrafloral nectary of *Vicia faba* treated with water (control). Non-secretory trichomes exhibit a peak related to phenolic compounds (450–500 nm), whereas nectary tissues present a smooth peak relative to chlorophyll emission (above 650 nm).

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