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Anatomy of the floral nectary of ornithophilous *Elleanthus brasiliensis* (Orchidaceae: Sobralieae)

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Orchidaceae show enormous floral diversity. However, anatomical studies of nectary tissues relative to nectar composition and pollinators are scarce. This work aims to present a detailed anatomical study of the labellar nectary of *Elleanthus brasiliensis*, analyse the chemical composition of its nectar and relate these findings to pollination biology. Basally, the labellum bears a pair of fleshy, whitish, ovoid calli on its adaxial surface. Nectariferous callus tissue consists of a papillate epidermis and enlarged subepidermal parenchyma cells with thin walls, large nuclei and dense cytoplasm which stained positively for hydrophilic substances, interpreted as pre-nectar. The paired calli lack vascular tissues, but at the point of callus insertion, the diameters of vascular bundles supplying the lip are larger. Nectar is secreted as droplets on the adaxial callus surface. It is produced in small quantities, *c*. 4 μL per flower. Callus cell contents tested negative for polysaccharides, lipids and phenolic compounds. The nectar is sucrose-dominant, as in other hummingbird-pollinated species. It is suggested that other ornithophilous species of Sobralieae have anatomically similar nectaries. *Botanical Journal of the Linnean Society*, 2013, **171**, 764–772.

ADDITIONAL KEYWORDS: Atlantic Rainforest – callus – glands – histochemistry – labellum – lip – nectar – orchid – pollination – secretion.

INTRODUCTION

Most flowering plants offer floral rewards, such as nectar and pollen, to their pollinators (Proctor, Yeo & Lack, 1996). However, nectar is considered to be particularly important, given its wide distribution as a floral reward (Smets, 1986). Two-thirds of orchid species offer floral rewards to animal visitors. The remaining species employ deceit strategies and are pollinated mainly by insects (Schiestl, 2005). Orchid flowers may offer oil, resin, fragrance or pseudopollen. However, nectar is the most common reward. All these rewards are essential for the attraction of pollinators and are consequently linked to the reproductive success of the plant (van der Pijl & Dodson, 1969; Dressler, 1993; Ackerman, Rodríguez-Robles & Meléndez, 1994; Neiland & Wilcock, 1998; Pedron *et al*., 2012).

Over the last 20 years, a series of studies has investigated the anatomy of floral glands in orchids and their relevance to pollination. Among the glands described to date are: elaiophores and osmophores (Stpiczyńska, Davies & Gregg, 2007; Aliscioni *et al*., 2009; Davies & Stpiczyńska, 2009; Pansarin, Castro & Sazima, 2009), secretory trichomes (Davies & Stpiczyńska, 2006), spur-shaped nectaries (Figueiredo & Pais, 1992; Galetto, Bernardello & Rivera, 1997; Stpiczyńska, Davies & Gregg, 2005), nectariferous flower surfaces (Stpiczyńska, Davies & Gregg, 2003) and other lip structures (Galetto *et al*., 1997; Davies,

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Stpiczyńska & Gregg, 2005). Studies describing nectariferous glands in ornithophilous orchids are rare and the diversity of these nectaries indicates that they may have originated from a number of floral structures. For example, in *Ornithidium coccineum* (Jacq.) Salisb. ex R.Br. and *O. sophronitis* Rchb.f., a faucet and sink mechanism operates, in which the nectary is represented by a protuberance (the 'faucet') on the ventral surface of the column (Stpiczyńska *et al*., 2003; Stpiczyńska, Davies & Gregg, 2009) and droplets of nectar produced here collect in a 'sink' formed from the bases of the column and tepals. By contrast, the nectary of *Scaphyglottis imbricata* (Lindl.) Dressler [as *Hexisea imbricata* (Lindl.) Rchb.f.] takes the form of a spur formed by the fusion of the column basis and part of the lip (Stpiczyńska *et al*., 2005), whereas in *Oncidium strictum* (Cogn.) M.W.Chase & N.H.Williams [as *Symphyglossum sanguineum* (Rchb.f.) Schltr.], nectar is secreted by the labellar callus (Stpiczyńska & Davies, 2006). Recently, Mondragón-Palomino & Theißen (2008, 2009) proposed how genes may regulate differentiation of the orchid labellum. They subsequently (Mondragón-Palomino & Theißen, 2011) modified their model and concluded that tissue differentiation occurs by the differential expression of two pairs, including genes from two clades of 'DEF-like' genes. The specific differentiation of the lip (rather than the whole of the inner whorl) is determined by the hormonal influence of the anthers present on the opposite side of the flower column (Mondragón-Palomino & Theißen, 2009).

The few studies that address nectar production and composition in ornithophilous orchids show that the nectar volume can vary from 3 to 50 μL, with sugar concentrations varying from 10 to 50% (mass per total mass) (Johnson, 1996; Galetto *et al*., 1997; Singer & Sazima, 2000; Johnson & Brown, 2004; Micheneau, Fournel & Pailler, 2006). The major solutes present in the nectar of these plants are sucrose (disaccharide), glucose and fructose (hexoses), but sucrose may dominate (Johnson & Brown, 2004) or all three sugars may be present in more or less equal quantities, as in *Sacoila lanceolata* (Aubl.) Garay (Galetto *et al*., 1997).

Elleanthus brasiliensis (Lindl.) Rchb.f. is found throughout the humid forests of Brazil, occurring in different physiognomies of the Atlantic Forest of the Parque Estadual da Serra do Mar, São Paulo. Several species of this genus are considered ornithophilous (Dressler, 1993; Dressler, 2006). Hummingbirds are probably the main pollinators of *E. brasiliensis*. If so, one would expect the flowers to display adaptations to this type of pollination system, such as modification of floral structures to fit the morphology of the bill and foraging behaviour of the bird. It is also expected that the reward offered would fit the nutritional requirements of the pollinator (Baker & Baker, 1983a, 1990; Schiestl & Schlüter, 2009).

To date, no investigations into the composition of the floral nectar or the nectary anatomy of tribe Sobralieae (*sensu* Pridgeon *et al*., 2006) have been undertaken and hummingbirds have not been confirmed to be the main pollinators of species of *Elleanthus* C.Presl. Nevertheless, *Phaethornis pretrei* (Trochilidae: Phaethornithinae) has been observed visiting *E. brasiliensis* in south-eastern Brazil (Singer & Sazima, 2000; Singer, 2003) and other hummingbirds have been observed visiting Andean *Elleanthus* spp. (van der Pijl & Dodson, 1969). Therefore, the purpose of this study is to describe the structure of the nectary of *E. brasiliensis* and the chemical composition of its nectar relative to pollination biology.

MATERIAL AND METHODS

Data were collected from individuals of *E. brasiliensis*, occurring as epiphytes at the margins of a stream in the Atlantic Rainforest (Rio da Fazenda 23°20′22.13″S and 44°50′14.54″W), Núcleo Picinguaba, municipality of Ubatuba in the Parque Estadual da Serra do Mar, State of São Paulo. The climate is tropical-humid ('Af.'; *sensu* Köppen, 1948), with a maximum annual rainfall of 2600 mm, a relative air humidity of > 80% and an average annual temperature of 22 °C. The wet season occurs from October to April, with a mean precipitation of 285 mm per month (data source: Instituto Agronômico de Campinas, Campinas, Brazil). The flowering period (January–March) of *E. brasiliensis* coincides with the wet season. A voucher specimen (*12/02/2010, C.E.P. Nunes 01*) was deposited in the herbarium of UEC.

For general histology, tissue samples of fully open flowers were fixed in formalin–acetic acid–alcohol (FAA) for 24 h (Johansen, 1940) or in neutral buffered formaldehyde solution (NBF) for 48 h (Lillie, 1965) and subjected to reduced pressure to allow adequate penetration of the fixative. They were subsequently stored in 70% (v/v) ethanol. The material was dehydrated through a tertiary butanol series (Johansen, 1940), embedded in Paraplast® and sectioned with a rotary microtome (Microm International GmbH – HM340 E). Longitudinal and transverse serial sections were cut at a thickness of 12 μm and stained with Safranin O and Astra blue (Gerlach, 1969). The serial sections were examined microscopically (Olympus BX51) under polarized light to verify the occurrence of starch grains, crystals and lignified cell walls.

Some histochemical procedures were carried out to detect the main classes of chemical compounds produced by the callus cells. The following tests were applied to samples fixed in FAA: periodic acid–Schiff

reagent (PAS reaction) for total polysaccharides (McManus, 1948) and ruthenium red for cellular acid mucilages in the cell contents (Gregory & Baas, 1989). The following tests were applied to samples fixed in NBF: Sudan black B for total lipids (Pearse, 1985), ferric chloride for phenolic compounds (Johansen, 1940) and copper acetate–rubeanic acid for fatty acids (Ganter & Jolles, 1969). For all the applied histochemical tests, standard controls were carried out simultaneously.

Images of inflorescence and lip details were made in the field with a digital camera (Canon EOS20D) and in the laboratory with a camera (DFC295) coupled to a Leica M80 stereoscopic microscope. Digital images (600 d.p.i.) of the histological sections were obtained with a DP71 camera coupled to an Olympus BX51 microscope, electronically processed using the software Olympus DP Controller and edited with the software GNU Image Manipulation Program (GIMP, version 2.6.6).

Nectar volume (μL, $n = 72$ flowers) and sugar concentration in sucrose equivalents (%, mass per total mass, $n = 66$ flowers) were measured in the field using microlitre syringes and a manual refractometer, respectively. Nectar appeared as droplets on the pair of calli of each flower and samples of accumulated nectar were removed for sugar composition analysis from three flowers in full anthesis, in turn derived from three individual plants, using the procedure of Galetto & Bernardello (2005). Nectar droplets were placed on Whatman #1 chromatography paper (Maidstone, UK) and dried quickly. In the laboratory, nectar was redissolved in distilled water and sugar separation and quantification was accomplished by means of gas-liquid chromatography. Nectar was lyophilized and silylated according to Sweeley *et al*. (1963). The derivatives were then injected into a Konik KNK 3000-HRGS gas chromatograph equipped with a Spectra-Physics SP 4290 data integrator, a flame ionization detector and a capillary column OV 101 3% (2 m length) over a film 100–120 Cromosorb G/AW-DMCS. Nitrogen was used as the carrier gas (30 mL per min) and the following temperature regime was followed: 208 °C for 1 min, 1 °C rise per minute to 215 °C and 10 °C rise per minute to 280 °C (maintained for 5 min). Carbohydrate standards (Sigma) were prepared using the same approach. The proportion of sugars (r) and of hexoses (hr) was calculated following Baker & Baker (1983b):

$$r = \text{sucrose}/(\text{glucose} + \text{fructose})$$

$$hr = \text{glucose}/\text{fructose}$$

The quantity of sugars (mg) available in the analysed samples was obtained using the following equation:

$$y = 0.00226 + (0.00937x) + (0.0000585x^2)$$

where x is the concentration reading on the refractometer (Galetto & Bernardello, 2005).

RESULTS

The tubular (8–10 mm long) flowers of *E. brasiliensis* occur in dense inflorescences. The sepals are purplish-red, the petals are white and the lip is white with two purplish-red spots on the adaxial surface (Fig. 1A). A combination of resupination and a pendulous inflorescence results in the lip pointing upwards (Fig. 1A). Basally, the lip bears a pair of fleshy, whitish, ovoid calli 1.8 mm long and 1.5 mm wide (Fig. 1B). Nectar is present as droplets on the callus surface. It accumulates at the flower base in a chamber enclosed by the lip and the column, together with the column appendage (Fig. 2A).

The paired calli consist of secretory (nectariferous) and non-secretory cells (Figs 2–4). The nectariferous

Figure 1. Inflorescence and lip of *Elleanthus brasiliensis*. A, pendulous inflorescence and resupinate flowers, with upwardly pointing labella (arrows). B, a labellum (preserved) with basal paired calli (arrows). Scale bars: A, B, 1 cm.

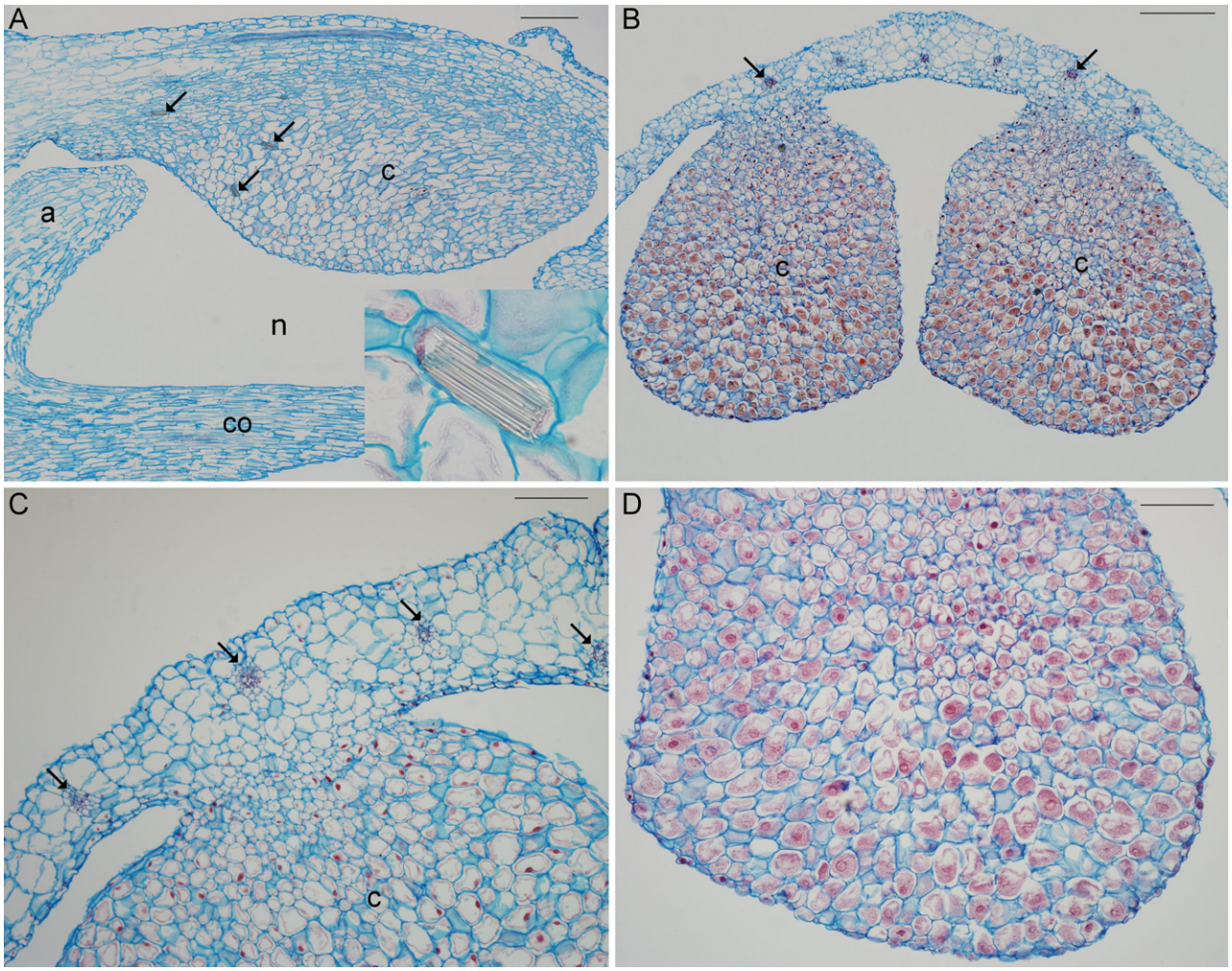

Figure 2. Labellar nectary of *Elleanthus brasiliensis* at the secretory phase stained with safranin O and Astra blue. A, longitudinal section of the flower through the calli (c) and column (co) with appendage (a); note the nectar chamber (n) and raphide cells (arrows and inset). B, the pair of calli (c) and the vascular bundles (arrows) of the lip. C, position of the vascular bundles (arrows) supplying the labellar callus (c). D, nectariferous parenchyma of the callus; note that the cytoplasm stains pink with safranin O. B–D, transverse sections. Scale bars: A, B, 200 μm; C, D, 100 μm.

cells have thin walls that stained with Astra blue and relatively large nuclei and dense cytoplasm. The cytoplasm stained positively for hydrophilic substances (light pink with safranin O) and was well preserved in samples fixed in FAA (Figs 2B, D, 3A–C). In the secretory phase, the callus cell content gave negative results for polysaccharides (including mucilage), lipids and phenolic compounds (Fig. 4).

The nectariferous epidermis is composed of papillate cells, the short papilla arising from the central region of the outer periclinal wall (Fig. 3A, C, arrows), and is covered by a thin cuticle (Fig. 4D, inset). No stomata or detachment or rupture of the cuticle were observed. Large, almost isodiametric, nectariferous parenchyma cells occupy the central and distal regions of the calli and these contain dense cytoplasm (Figs 2B, D, 3A, B). Basally, however, the parenchyma cells are smaller, non-secretory and more compactly packed (Figs 2C, 3D).

Ruthenium red revealed strands of narrow cells with thick walls rich in cellulose and pectic substances (Fig. 4A, B). The absence of lignin was checked under polarized light. These cells differ in form and content from neighbouring cells. Raphide cells are found scattered throughout the ground tissue (Fig. 2A, arrows and inset).

The paired calli have no special vascularization (Figs 2A, C, 3D), although several vascular bundles (*c.* 13) supply the lip, and larger bundles were observed close to the points of insertion of the calli (Fig. 2B, C arrows). Analysis of serial sections under polarized light revealed the absence of starch grains

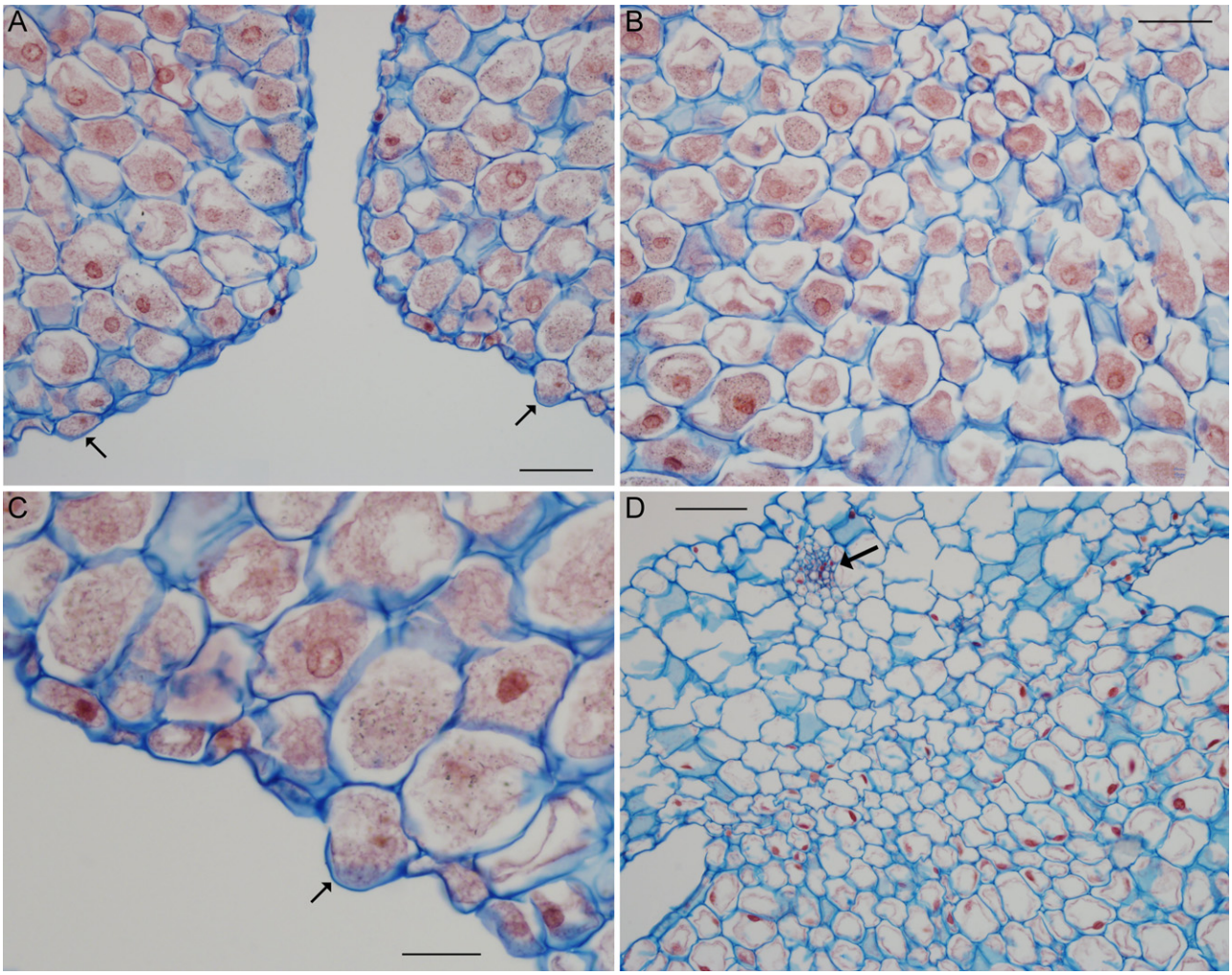

Figure 3. Details of the labellar callus (in transverse section) of *Elleanthus brasiliensis* at the secretory phase and stained with safranin O and Astra blue; note that the cell contents are stained light pink with safranin O. A, C, nectariferous, papillate epidermis of callus; note the short papillae (arrows). B, nectariferous parenchyma. D, vascular bundle (arrow) supplying the labellum. Scale bars: A, B, D, 50 μm; C, 20 μm.

from parenchyma and epidermal secretory cells of the calli.

Nectar is produced in small quantities, 4.15 ± 2.93 μL, has a total concentration of 21.11 ± 5.27% sugars, and is composed of 96.55 ± 5.28% sucrose, 2.00 ± 2.25% fructose and 1.46 ± 3.03% glucose.

DISCUSSION

The morphological characteristics of *E. brasiliensis* flowers and their lack of human-perceived fragrance agree with the field observations of C. E. P. Nunes & M. Sazima (unpubl. data) that hummingbirds are the main pollinators of this species. An unusual feature of *E. brasiliensis* is that owing to resupination and a pendulous inflorescence, the labellum points upwards. Thus, instead of a landing platform, the lip is an inverted half tube, such that bees like *Trigona* experience difficulties in accessing the flower. Besides flower shape, colour and lack of odour can also be related to pollination by hummingbirds (van der Pijl & Dodson, 1969).

The floral nectary of this species is represented by a pair of calli borne basally on the labellum. Although such nectaries are known in orchids, few records are known for ornithophilous species (e.g. *Oncidium strictum*; Stpiczyńska & Davies, 2006). Paired calli also occur in other *Elleanthus* spp. (Pridgeon *et al.*, 2006). That the labellar callus is often related to pollinator attraction in orchids is evident from the fact that, in many species, it secretes nectar (Davies *et al.*, 2005), oil (Stpiczyńska *et al.*, 2007) or fragrance (Dodson, 1962); in others, it has been interpreted as a rewardless gland that deceives pollinators by imitating a

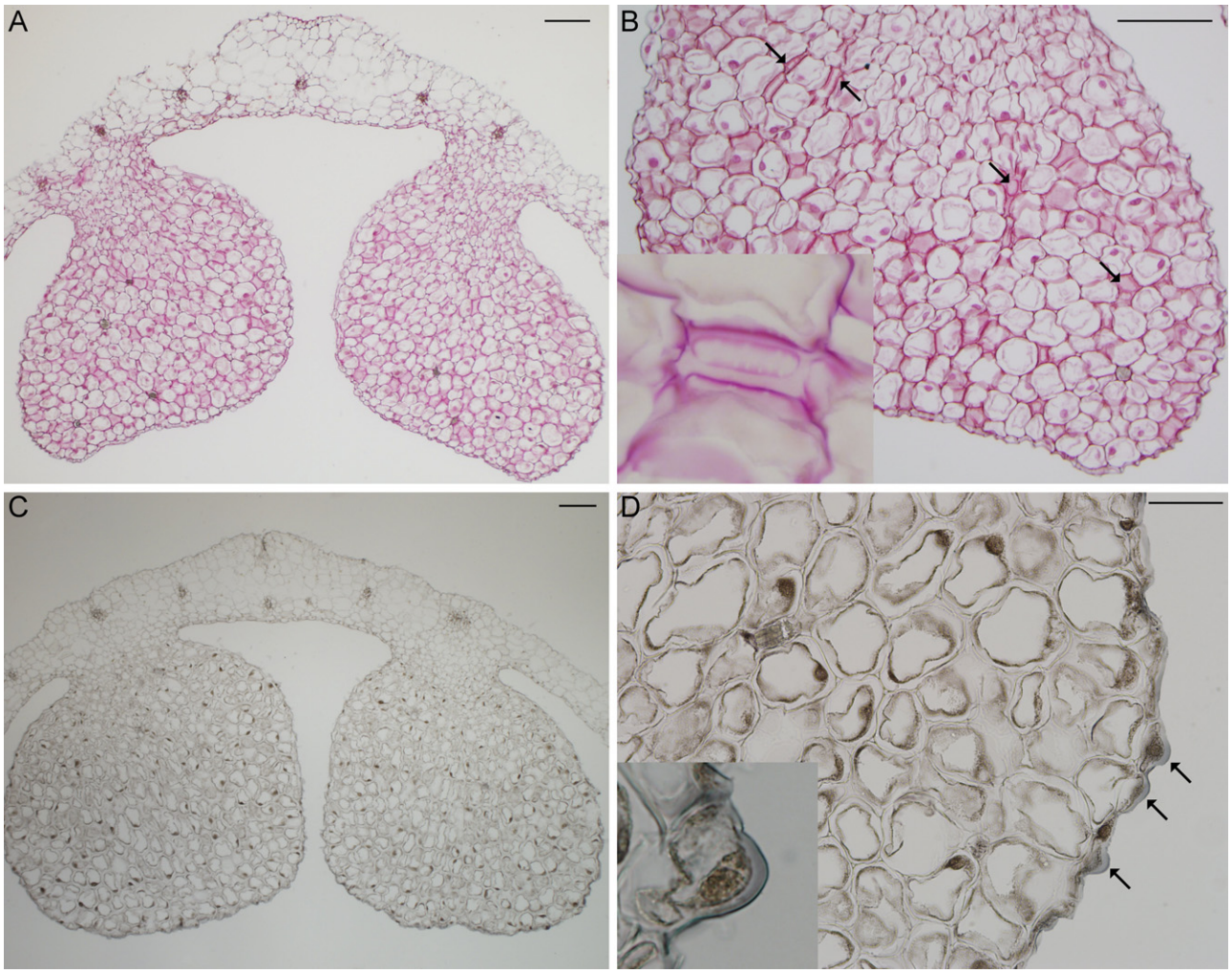

Figure 4. Histochemical features of the paired labellar calli (transverse sections) of *Elleanthus brasiliensis*. A, B, cell walls stain intensely with ruthenium red showing distribution of pectins. B, detail of collenchymatous cells (arrows and inset). C, D, nectariferous cells stain weakly for lipids with Sudan black B. D, the thin cuticle stains navy-blue (arrows and inset) with Sudan black B, indicating lipid content. Scale bars: A, C, 200 μm; B, 50 μm; D, 20 μm.

pollen source (Cheng *et al*., 2009). However, despite this morphological diversity and the large number of species that have floral glands, few anatomical and morphological studies of this kind have been carried out in Orchidaceae. Therefore, such studies are important because they provide greater insight into the diversity of floral glands in the family and how they have evolved to interact with different groups of pollinators, considering that this interaction is one of the main evolutionary forces driving floral diversification in orchids (Gravendeel *et al*., 2004).

Analysis of serial sections enables the localization of nectariferous cells in the papillate epidermis and parenchyma of the paired calli. Characteristics of the cytoplasmic content of these cells, such as positive reactions for hydrophilic substances and negative reactions for lipophilic compounds, are typical of pre-nectar (Fahn, 1979, 2000). All these features allow the classification of the paired calli of *E. brasiliensis* as structural nectaries, as they are anatomically differentiated and can be recognized macroscopically (Zimmermann, 1932, *apud* Fahn, 1979).

Owing to the high concentration of sucrose, the nectar of *E. brasiliensis* is considered to be 'sucrose-dominant', according to the classification of Baker & Baker (1983b). Plants pollinated by hummingbirds generally offer nectar containing high concentrations (> 60%) of sucrose (Baker & Baker, 1983b, 1990). Thus, the nectar composition of *E. brasiliensis* supports the observations of Singer (2003) and C. E. P. Nunes & M. Sazima (unpubl. data) that this species is pollinated by hummingbirds, as previously suggested by van der Pijl & Dodson (1969) for several other *Elleanthus* spp. Gas chromatography coupled

with histochemical procedures on either the droplets of nectar or secretory cell contents enabled the identification of metabolites secreted by the labellar calli of *E. brasiliensis*. Histochemical tests confirmed that neither polysaccharides (including mucilage), nor lipids or phenolic compounds are present in the cell contents of the calli, interpreted here as pre-nectar; analyses showed that they are also absent from the nectar. According to Heil (2011), if non-carbohydrate substances are constituents of the nectar, they should also be present at the pre-nectar stage. Moreover, Sawidis (1998), who reported on the occurrence of cells containing oil and mucilage in the sub-nectary parenchyma of *Hibiscus rosa-sinensis* L., proposed that mucilage cells offer a water regulatory mechanism during nectar secretion and protect nectary tissue from damage caused by water-stress. However, in the habitat of *E. brasiliensis*, atmospheric humidity is high and such mechanisms for protection against water loss are unlikely to be necessary.

Unlike the majority of nectaries, which are composed of small, densely packed cells (Fahn, 1979; Durkee, 1983), those of *E. brasiliensis* consist of enlarged, almost isodiametric, nectariferous parenchyma cells, interspersed by collenchymatous cells. The latter cells are also found in nectaries of ornithophilous orchids of the genera *Ascocentrum* Schltr. (Stpiczyńska, Davies & Kaminska, 2011), *Ornithidium* Salisb. ex R.Br. (Stpiczyńska *et al*., 2003, 2009), *Oncidium* Sw. (Stpiczyńska & Davies, 2006) and *Scaphyglottis* Poepp. & Endl. (Stpiczyńska *et al*., 2005). Such cells would provide mechanical support to the paired calli of *E. brasiliensis*, by reinforcing the parenchymatous areas.

The presence of raphide cells in the labellar calli of *E. brasiliensis* was expected, as these cells are commonly found in flower parts of Orchidaceae (Galetto *et al*., 1997; Stpiczyńska *et al*., 2003, among others). The probable composition of the raphide crystal is calcium oxalate, as found in other orchid nectaries (Stpiczyńska *et al*., 2003), those of other flowers and in extrafloral nectaries (Nepi, 2007). According to Nepi (2007), calcium ions (Ca^{2+}) inhibit the ATPase protein involved in the transport of sucrose across membranes. Calcium ions are immobilized with the formation of calcium oxalate crystals, thereby allowing sugar transport in nectariferous parenchyma. It is also possible that crystals represent excretory products or discourage herbivory by insects and other invertebrates (Davies, 1999; Davies, Winters & Turner, 2000; Nepi, 2007).

The absence of starch or any significant amount of other polysaccharides in the cell content of the secretory tissue might indicate that nectar sugars are transported directly from the phloem sap, a suggestion made by Fahn (2000) and corroborated here by the presence of larger vascular bundles near the secretory calli. According to Vassilyev (2010), 'pre-nectar sugars are transported from the phloem into nectary secretory cells in the symplasm (in the cytoplasm and through plasmodesmata) by diffusion'. Thus, the direct origin of nectar-sugar in *E. brasiliensis* differs from that of many other plant families (reviewed by Pacini, Nepi & Vesprini, 2003) and from other orchid species, e.g. *Limodorum abortivum* (L.) Sw. (Figueiredo & Pais, 1992), *Maxillariella anceps* (Ames & C.Schweinf.) M.A.Blanco & Carnevali (as *Maxillaria anceps* Ames & C.Schweinf.; Davies *et al*., 2005) and *Ascocentrum* spp. (Stpiczyńska *et al*., 2011).

The nectariferous papillate epidermis, as observed in *E. brasiliensis*, is a characteristic of floral nectaries of other orchid species (Galetto *et al*., 1997; Aliscioni *et al*., 2009; Davies & Stpiczyńska, 2009). There have been reports of floral nectar being secreted via modified stomata or through cuticular pores for a number of orchid species (Stpiczyńska *et al*., 2003, 2005, 2011; Davies *et al*., 2005). However, as neither secretory stomata nor cuticle detachment and rupture were observed for the paired calli of *E. brasiliensis*, it is speculated that nectar droplets in this species might be exuded through micro-pores in the cuticle. Confirmation, however, awaits transmission electron microscopy investigations.

The lack of special vascularization of the paired calli of *E. brasiliensis* might also explain the low nectar production observed. Furthermore, the position of the nectar chamber at the flower base, coupled with the high air humidity of the Atlantic Rainforest, probably reduces the transpiration and evaporation, thus enabling the production and accumulation of a low nectar volume (1–7 μL). However, such a small amount of nectar is still sufficient to attract hummingbirds, as has been shown for *Comparettia falcata* Poepp. & Endl. (Rodríguez-Robles, Meléndez & Ackerman, 1992; Ackerman *et al*., 1994). These values are considered low relative to other ornithophilous plants, including orchids (Galetto *et al*., 1997; Buzato, Sazima & Sazima, 2000), even though the nectar volume of hummingbird-pollinated plants varies widely from 0.5 to 56.0 μL per flower (Opler, 1983). Low nectar volume promotes brief visits by hummingbirds and consequently influences the reproductive biology of *E. brasiliensis*, as these animals have to visit several flowers from different individual plants in sequence along their foraging route, thereby promoting cross pollination (C. E. P. Nunes & M. Sazima, unpubl. data).

The anatomical features of the paired calli of other species of Sobralieae remain unknown, but it is likely that nectaries of other ornithophilous species of this

tribe have similar structure as a synapomorphy. The presence of collenchymatous cells in the secretory parenchyma of ornithophilous orchid species may be due to evolutionary convergence, as proposed by Stpiczyńska *et al*. (2011). Detailed studies of the anatomy, morphology and pollination of Sobralieae and the phylogenetic relationships of the genera are now necessary if we are to gain a better understanding of the mechanisms by which flowers have evolved in this tribe. For now, when it comes to detailed, floral anatomical studies of Orchidaceae, we have barely scraped the surface.

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REFERENCES

Ackerman JD, Rodríguez-Robles JA, Meléndez EJ. 1994. A meager nectar offering by an epiphytic orchid is better than nothing. *Biotropica* **26:** 44–49.

Aliscioni SS, Torretta JP, Bello ME, Galati BG. 2009. Elaiophores in *Gomesa bifolia* (Sims) M.W. Chase, N.H. Williams (Oncidiinae: Cymbidieae: Orchidaceae): structure and oil secretion. *Annals of Botany* **104:** 1141–1149.

Baker HG, Baker I. 1983a. A brief historical review of the chemistry of floral nectar. In: Bentley B, Elias TS, eds. *The biology of nectaries*. New York: Columbia University Press, 126–152.

Baker HG, Baker I. 1983b. Floral nectar sugar constituents in relation to pollinator type. In: Jones CE, Little RJ, eds. *Handbook of experimental pollination biology*. New York: Van Nostrand Reinhold Co., 117–141.

Baker HG, Baker I. 1990. The predictive value of nectar chemistry to the recognition of pollinator type. *Israel Journal of Botany* **39:** 157–166.

Buzato S, Sazima M, Sazima I. 2000. Hummingbird-pollinated floras at three Atlantic forest sites. *Biotropica* **32:** 824–841.

Cheng J, Shi J, Shangguan F-Z, Dafni A, Deng Z-H, Luo Y-B. 2009. The pollination of a self-incompatible, food-mimic orchid, *Coelogyne fimbriata* (Orchidaceae), by female *Vespula* wasps. *Annals of Botany* **104:** 565–571.

Davies KL. 1999. A preliminary survey of foliar anatomy in *Maxillaria*. *Lindleyana* **14:** 126–135.

Davies KL, Stpiczyńska M. 2006. Labellar micromorphology of Bifrenariinae Dressler (Orchidaceae). *Annals of Botany* **98:** 1215–1231.

Davies KL, Stpiczyńska M. 2009. Comparative histology of floral elaiophores in the orchids *Rudolfiella picta* (Schltr.) Hoehne (Maxillariinae *sensu lato*) and *Oncidium ornithorhynchum* H.B.K. (Oncidiinae *sensu lato*). *Annals of Botany* **104:** 221–234.

Davies KL, Stpiczyńska M, Gregg A. 2005. Nectar-secreting floral stomata in *Maxillaria anceps* Ames & C.Schweinf. (Orchidaceae). *Annals of Botany* **96:** 217–227.

Davies KL, Winters C, Turner MP. 2000. Pseudopollen: its structure and development in *Maxillaria* (Orchidaceae). *Annals of Botany* **85:** 887–895.

Dodson CH. 1962. Pollination and variation in the subtribe Catasetinae (Orchidaceae). *Annals of the Missouri Botanical Garden* **49:** 35–56.

Dressler RL. 1993. *Phylogeny and classification of the orchid family*. Portland, OR: Diocorides Press.

Dressler RL. 2006. *Elleanthus*. In: Pridgeon AM, Cribb P, Chase MW, Rasmussen FN, eds. *Genera Orchidacearum Volume 4: Epidendroideae (part 1)*. Oxford: Oxford University Press, 598.

Durkee LT. 1983. The ultrastructure of floral and extrafloral nectaries. In: Bentley B, Elias T, eds. *The biology of nectaries*. New York: Columbia University Press, 1–29.

Fahn A. 1979. *Secretory tissues in plants*. London: Academic Press.

Fahn A. 2000. Structure and function of secretory cells. *Advances in Botanical Research* **31:** 37–75.

Figueiredo ACS, Pais MS. 1992. Ultrastructural aspects of the nectary spur of *Limodorum abortivum* (L) Sw. (Orchidaceae). *Annals of Botany* **70:** 325–331.

Galetto L, Bernardello G. 2005. Rewards in flowers – nectar. In: Dafni A, Kevan PG, Husband BC, eds. *Practical pollination biology*. Cambridge: Enviroquest, 261–313.

Galetto L, Bernardello G, Rivera GL. 1997. Nectar, nectaries, flower visitors, and breeding system in five terrestrial Orchidaceae from Central Argentina. *Journal of Plant Research* **110:** 393–403.

Ganter P, Jolles G. 1969. *Histochemie normale et pathologique*. Paris: Gauthier-Villars.

Gerlach G. 1969. *Botanische Mikrotechnik: eine Einführung*. Stuttgart: Georg Thieme.

Gravendeel B, Smithson A, Slik FJW, Schuiteman A. 2004. Epiphytism and pollinator specialization: drivers for

orchid diversity? *Philosophical Transactions of the Royal Society B: Biological Sciences* **359:** 1523–1535.

Gregory M, Baas P. 1989. A survey of mucilage cells in vegetative organs of the dicotyledons. *Israel Journal of Botany* **38:** 125–174.

Heil M. 2011. Nectar: generation, regulation and ecological functions. *Trends in Plant Science* **16:** 191–200.

Johansen DA. 1940. *Plant microtechnique*. New York: McGraw-Hill Book Co.

Johnson SD. 1996. Bird pollination in South African species of *Satyrium* (Orchidaceae). *Plant Systematics and Evolution* **203:** 91–98.

Johnson SD, Brown M. 2004. Transfer of pollinaria on bird's feet: a new pollination system in orchids. *Plant Systematics and Evolution* **244:** 181–188.

Köppen W. 1948. *Climatologia: con un estudio de los climas de la Tierra*. México DF: Fondo de Cultura Econômica.

Lillie RD. 1965. *Histopathologic technic and practical histochemistry, 3rd edn*. New York: McGraw-Hill Book Co.

McManus JFA. 1948. Histological and histochemical uses of periodic acid. *Biotechnic & Histochemistry* **23:** 99–108.

Micheneau C, Fournel J, Pailler T. 2006. Bird pollination in an angraecoid orchid on Reunion Island (Mascarene Archipelago, Indian Ocean). *Annals of Botany* **97:** 965–974.

Mondragón-Palomino M, Theißen G. 2008. MADS about the evolution of orchid flowers. *Trends in Plant Science* **13:** 51–59.

Mondragón-Palomino M, Theißen G. 2009. Why are orchid flowers so diverse? Reduction of evolutionary constraints by paralogues of class B floral homeotic genes. *Annals of Botany* **104:** 583–594.

Mondragón-Palomino M, Theißen G. 2011. Conserved differential expression of paralogous DEFICIENS- and GLOBOSA-like MADS-box genes in the flowers of Orchidaceae: refining the 'orchid code'. *The Plant Journal* **66:** 1008–1019.

Neiland MRM, Wilcock CC. 1998. Fruit set, nectar reward, and rarity in Orchidaceae. *American Journal of Botany* **85:** 1657–1671.

Nepi M. 2007. Nectary structure and ultrastructure. In: Nicolson SW, Nepi M, Pacini E, eds. *Nectaries and nectar*. Dordrecht: Springer, 129–166.

Opler PA. 1983. Nectar production in a tropical ecosystem. In: Bentley B, Elias T, eds. *The biology of nectaries*. New York: Columbia University Press, 30–79.

Pacini E, Nepi M, Vesprini JL. 2003. Nectary biodiversity: a short review. *Plant Systematics and Evolution* **238:** 7–21.

Pansarin LM, Castro MM, Sazima M. 2009. Osmophore and elaiophores of *Grobya amherstiae* (Catasetinae, Orchidaceae) and their relation to pollination. *Botanical Journal of the Linnean Society* **159:** 408–415.

Pearse AGE. 1985. *Histochemistry: theoretical and applied, 4th edn*. Edinburgh: C. Livingstone.

Pedron M, Buzatto CR, Singer RB, Batista JAN, Moser A. 2012. Pollination biology of four sympatric species of *Habenaria* (Orchidaceae: Orchidinae) from southern Brazil. *Botanical Journal of the Linnean Society* **170:** 141–156.

van der Pijl L, Dodson CH. 1969. *Orchid flowers: their pollination and evolution*. Coral Gables, FL: University of Miami Press.

Pridgeon AM, Cribb P, Chase MW, Rasmussen FN, eds. 2006. *Genera Orchidacearum Vol. 4: Epidendroideae (part 1)*. Oxford: Oxford University Press.

Proctor M, Yeo P, Lack A. 1996. *The natural history of pollination*. Portland, OR: Timber Press (and London: Collins).

Rodríguez-Robles J, Meléndez EJ, Ackerman JD. 1992. Effects of display size, flowering phenology, and nectar availability on effective visitation frequency in *Comparettia falcata* (Orchidaceae). *American Journal of Botany* **79:** 1009–1017.

Sawidis T. 1998. The subglandular tissue of *Hibiscus rosa-sinensis* nectaries. *Flora* **193:** 327–335.

Schiestl FP. 2005. On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* **92:** 255–264.

Schiestl FP, Schlüter PM. 2009. Floral isolation, specialized pollination, and pollinator behavior in orchids. *Annual Review of Entomology* **54:** 425–446.

Singer RB. 2003. Orchid pollination: recent developments from Brazil. *Lankesteriana* **7:** 111–114.

Singer RB, Sazima M. 2000. The pollination of *Stenorrhynchus lanceolatus* (Aublet) L. C. Rich (Orchidaceae: Spiranthinae) by hummingbirds in south-eastern Brazil. *Plant Systematics and Evolution* **223:** 221–227.

Smets E. 1986. Localization and systematic importance of floral nectaries in the Magnoliatae (dicotyledons). *Bulletin du Jardin Botanique National de Belgique* **56:** 51–76.

Stpiczyńska M, Davies KL. 2006. Nectary structure in *Symphyglossum sanguineum* (Rchb.f.) Schltr. (Orchidaceae). *Acta Agrobotanica* **59:** 7–16.

Stpiczyńska M, Davies KL, Gregg A. 2003. Nectary structure and nectar secretion in *Maxillaria coccinea* (Jacq.) L.O. Williams ex Hodge (Orchidaceae). *Annals of Botany* **93:** 87–95.

Stpiczyńska M, Davies KL, Gregg A. 2005. Comparative account of nectary structure in *Hexisea imbricata* (Lindl.) Rchb.f. (Orchidaceae). *Annals of Botany* **95:** 749–756.

Stpiczyńska M, Davies KL, Gregg A. 2007. Elaiophore diversity in three contrasting members of Oncidiinae (Orchidaceae). *Botanical Journal of the Linnean Society* **155:** 135–148.

Stpiczyńska M, Davies KL, Gregg A. 2009. Nectary structures of *Ornithidium sophronitis* Rchb.f. (Orchidaceae: Maxillariinae). *Acta Agrobotanica* **62:** 3–12.

Stpiczyńska M, Davies KL, Kaminska M. 2011. Comparative anatomy of the nectary spur in selected species of Aeridinae (Orchidaceae). *Annals of Botany* **107:** 327–345.

Sweeley EC, Bentley R, Makita M, Wells WW. 1963. Gas liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *Journal of the American Chemical Society* **85:** 2497–2507.

Vassilyev AE. 2010. On the mechanisms of nectar secretion: revisited. *Annals of Botany* **105:** 349–354.