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ORIGINAL ARTICLE

Labellar secretion and secretory trichomes of *Rhetinantha cerifera* (Barb. Rodr.) M.A.Blanco (Orchidaceae, Maxillariinae): micromorphology and composition

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Abstract
The structure and composition of the labellar secretory trichomes of *Rhetinantha cerifera* (Barb. Rodr.) M.A.Blanco were analyzed by scanning electron and light microscopy, as well as histochemistry and chemistry. Histological analyses revealed that the chlorophyllated and dry callus at the basal region of labellum is non-secretory. The white sticky secretion is produced by unicellular secretory trichomes (not papillae), occupying the V-shaped ridge at the apical and median regions of the labellum, and the central portion of the labellar adaxial base, behind the callus. Chemical analyses of dichloromethane extracts of the secretion detected several long carbon chain constituents common in plant waxes (*n*-alkanes, carboxylic acids, alcohols, esters) and phytosteroids, predominantly cycloartenol derivatives. Histochemical tests showed that the secretion contains terpenoids (oleoresin), free fatty acids, phenolic compounds (including flavonoids), and polysaccharides (mainly mucilage); the results were negative for alkaloids. The secretory unicellular trichomes can concomitantly activate different metabolic pathways, and the exudate should be characterized as "heterogeneous mixtures," consisting of lipophilic and hydrophilic compounds. Therefore, the labellar secretion is chemically more complex than plant waxes composition. The specific epithet *cerifera* is thus misleading, and previously reported interpretations regarding the secretion are equivocated. Based on the present results and those from the literature, it is suggested that *R. cerifera* and *R. notylioglossa* (Rchb.f.) M.A.Blanco are taxonomical entities that should be merged into a single species, as has been suggested in Flora Brasil 2020 under construction.

Keywords Free fatty acid · Phenolic compound · Plant waxes · Polysaccharide · *Rhetinantha notylioglossa* · Terpenoid

1 Introduction

Orchids have a widely diverse and complex combination of rewards, visual and olfactory appealing and tactile stimuli, all involved in the attraction of animal visitors (Knudsen et al. 2006; Fay 2010; Vereecken et al. 2011; Davies et al. 2013). Reports of investigations about the floral glands diversity of Orchidaceae have emphasized the presence of osmophores, nectaries, and elaiophores on the labellum (Davies et al. 2005, 2014; Stpiczynska and Davies 2006, 2008; Stpiczynska et al. 2007, 2015; Pansarin et al. 2009; Pacek et al. 2012; Francisco and Ascensão 2013; Nunes et al. 2013; Borba et al. 2014; Pansarin and Pansarin 2014; Kowalkowska et al. 2015). Other related structures that have been reported are nectaries on the column (Stpiczynska et al. 2003), resin trichomes on tepals (Stpiczynska and Davies 2009), as well as colleters on carpels, sepals and floral/involucral bracts (Cardoso-Gustavson et al. 2014).

Regarding species of the tribe Cymbidieae, including subtribe Maxillariinae, labellar olfactory appealing and rewards, such as nectar, oils, resin, fragrance, food-hair, and pseudopollen perform important roles, taking into account

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plant–insect interactions (Davies et al. 2000, 2006, 2013; Stpiczynska et al. 2003; Davies and Stpiczynska 2006, 2009, 2012, 2017; Aliscioni et al. 2009; Stpiczynska and Davies 2009; Pansarin and Pansarin 2010; Blanco et al. 2013; Chase et al. 2015). Among subtribes of Neotropical Orchidaceae, Maxillariinae is the most conspicuous and species richest, occurring in humid neotropical forests, from southern Florida to northern Argentina, with diversity centers in southern Central America and southeastern Brazil. This subtribe includes several genera endemic to the Neotropics (Blanco et al. 2007; Whitten et al. 2007; Blanco 2013; Szlachetko and Kolanowska 2013; Zambrano-Romero and Solano-Gómez 2016; Marcusso et al. 2018).

Dathe and Dietrich (2006) compared molecular data and pollinarium morphology from twenty-two taxa of Maxillariinae, concluding that *Maxillaria* sensu lato is paraphyletic, although the subtribe is strongly supported as a monophyletic group. Whitten et al. (2007) presented a molecular phylogeny based on combined molecular data from *Maxillaria* Ruiz & Pav. and related genera of Cymbidieae, covering ca. 354 species. Their data indicated that *Maxillaria* is grossy polyphyletic and should be split into several genera, with *M. cerifera* Barb. Rodr. (=*Rhetinantha cerifera* (Barb. Rodr.) M.A.Blanco) nested within the *M. acuminata* clade, together with *M. notylioglossa* Rchb.f. (=*R. notylioglossa* (Rchb.f.) M.A.Blanco) and other five species which were realigned by Blanco et al. (2007) in the genus *Rhetinantha* M.A.Blanco *gen. nov.* ("rhetinos"=resin, "anthos"=flower), core Maxillariinae, based on *M. acuminata* Lindl. (=*R. acuminata* (Lindl.) M.A.Blanco) as type species. Szlachetko and Smiszek (2007) recognized the genus *Rhetinantha* and created new genera in the *Maxillaria* complex. Pridgeon et al. (2009) also accepted a narrower circumscription of the genera of Maxillariinae and segregated *Rhetinantha* to an autonomous genus. A posterior classification by Szlachetko et al. (2012), based on molecular phylogeny, maintains *Rhetinantha* among the 36 genera of subtribe Maxillariinae. Using combined matK/ycf1 plastid sequences from 289 taxa, Whitten et al. (2014) determined the relationships among all subtribes of Neotropical Cymbidieae. Their results recovered the major clades supporting the generic alignment of Blanco et al. (2007). The updated classification of Chase et al. (2015) also recognized *Rhetinantha* M.A.Blanco as being related to *Maxillaria s.l.,* subtribe Maxillariinae, tribe Cymbidieae. The latter is the taxonomic circumscription adopted in the present work, although a recent and distinct interpretation is available, which accepts a broader circumscription of *Maxillaria* in Schuiteman and Chase (2015).

The labellum of flowers of *R. cerifera* has a white secretion that was initially characterized as a "white semiglutinous wax" by Porsch (1905), while Hoehne (1949) described this secretion as a soft rug ("alfombras") of wax. Singer (2002) mentioned that *Maxillaria cerifera* (=*R. cerifera*) and *M. brasiliensis* Brieger & Illg (=*Heterotaxix brasiliensis* (Brieger & Illg) F.Barros) provide pads of wax on their labella, and Singer and Koehler (2004) referred to a resin-like secretion as the flower reward in *M. discolor* (Lodd. ex. Lindl.) Rchb.f. (=*Heterotaxis discolor* (Lodd. ex. Lindl.) Ojeda & Carnevali), *M. nasuta* Rchb.f. (=*Nitidobulbon nasuta* (Rchb.f.) Ojeda & Carnevali), *M. cerifera*, *M. notylioglossa*, and *M. equitans* Schltr. (=*Heterotaxis equitans* (Schltr.) Ojeda & Carnevali). Davies et al. (2003a) also characterized the white secretion in the labellum of *M. acuminata*, *M. cerifera* and *M. notylioglossa* as a viscid wax-like material consisting of lipids and proteins. Regarding the *Maxillaria acuminata* clade, Whitten et al. (2007) claimed that flowers of the nine species of genus *Maxillaria* have labellum with linear callus, bearing a resinous/lipoidal secretion or waxy white crystals, all of them corresponding to *Rethinantha* species sensu Blanco et al. (2007), among them *M. acuminata*, *M. cerifera*, *M. friedrichsthallii* Rchb.f. (=*R. friedrichsthallii* (Rchb.f.) M.A.Blanco, and *M. notylioglossa*. Szlachetko et al. (2012) recognized the genus *Rhetinantha*, with *R. acuminata*, *R. friedrichsthallii* and *R. notylioglossa* among the species, and described a sticky resinous substance upon labellum in most species of this genus. Szlachetko and Kolanowska (2013) compared the morphology of the genera *Hoehnella* (currently *Maxillaria*), *Rhetinantha* and *Sauvetrea*, and described a new *Rhetinantha* species, but did not mention any labellar secretion. Davies and Stpiczynska (2012) described the labellar secretion of *R. notylioglossa* as an amorphous or crystalline deposits of wax upon callus and a V-shaped labellar ridge.

Flach et al. (2004) compared the composition of labellar rewards of *M. cerifera* with those of *M. brasiliensis* and *M. friedrichsthallii,* using detailed chemical analysis of ethyl acetate extracts of labellar secretion. Steroidal triterpenoids, namely cycloartenal, and several unidentified derivatives of cycloartenol were isolated. The authors noticed closer similarity between *M. cerifera* and *M. friedrichsthallii,* both species later transferred to genus *Rhetinantha* and included on the *Maxillaria acuminata* clade. A question still remains whether this labellar secretion contains or lacks typical constituents of plant waxes, i.e., fatty acids, long-chain alcohols, hydrocarbons, esters, ketones, etc. Plant wax constituents are normally extracted using chloroform or dichloromethane, which are solvents less polar than ethyl acetate, and then analyzed by gas chromatography/mass spectrometry (Tsubaki et al. 2013; Guo et al. 2017).

The present work aims to localize in situ the main classes of metabolites and determine the chemical profile of its dichloromethane extract on the labellar secretion of *R. cerifera* in order to help deepening the knowledge about the secretion composition of this orchid species.

2 Materials and methods

Plant material – Fresh flowers (Fig. 1) were obtained in several periods ranging from 2011 to 2016 from a collection of living plants maintained at the São Paulo State Orchid Collection (IBt), of the Instituto de Botânica, located in São Paulo City, state of São Paulo, Brazil. Twelve specimens growing in the Orchid Collection, originally collected in different localities of the states of São Paulo and Rio de Janeiro (Table 1), were kept under controlled conditions. Plant vouchers were deposited at herbarium SP.

Histological and histochemical methods (Table 2) – Fresh labella were sectioned by hand, using a simple blade, or using the Cryostat Leica CM 1100 at 10 µm thickness. Tangential sections were parallel to marginal portions at the apical and median region of labellum. Flowers were fixed in four different solutions: Karnovsky solution for 24 h (Karnovsky 1965, modified by Kraus and Arduin 1997); formalin–acetic acid—50% ethanol for 24 h (Johansen 1940); aqueous solution containing 3–5% formalin and 10% ferrous sulfate for 48 h (Johansen 1940), a fixative for the detection of phenolic compounds (Salatino et al. 1988), and neutral buffered formaldehyde solution for 48 h (Lillie 1965), a fixative for the detection of lipids. The samples were washed, dehydrated and stored in 70% tertiary butyl alcohol. For light microscopy (LM) observations, fixed labellum was serial sectioned using standard methods of Paraplast (Fisher Healthcare, Houston, Texas, USA) and Leica historesin (Heraeus Kulzer, Hanau, Germany) embedding media on a rotary microtome. Paraplast sections were stained with safranin and astra blue (Gerlach 1969). Some historesin sections were stained, according to Sakai (1973), with toluidine blue O, others according to procedures described by Kivimäenpää et al. (2004), during 8 min with 1% toluidine blue aqueous solution at 30 °C, rinsed with distilled water, stained again during 4 min with 1% *p*-phenylenediamine in 1:1 methanol/isopropanol solution at room temperature, rinsed twice with the 1:1 methanol/isopropanol solution, and left to dry at room temperature. The sections from both treatments were mounted in Permount (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Some fresh, Paraplast and historesin embedding sections were submitted to the histochemical tests for in situ localization of the main classes of metabolites that are present in labellum secretion, and mounted in water. Standard control procedures 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.

Scanning electron microscopy (SEM) methods – Flowers were dehydrated to 100% ethanol and rinsed along a series of different hexamethyldisilazane (HMDS) concentrations (33.3%, 50% and 66.6% in *v*/*v* 100% ethanol) and 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 in a Hummer 6.2 (Anatech, Union City, CA, USA) sputtering system and viewed with a JSM-541OLV (JEOL, Tokyo, Japan) at 10 kV. Images were treated using Adobe Photoshop version 7.0.

Extraction and analyses of constituents of the labellar secretion – Labellar secretion portions were mechanically removed from flowers of specimens IBt 6103 and IBt 14099 (Table 1). Pooled together, the secretions from the apex and base of the labellum of flowers from both specimens amounted to 25 mg. The combined material was treated in a sealed vial with dichloromethane at 40 °C for 30 min. After cooling at room temperature, the supernatant was filtered to a previously weighed glass flask. The residue was treated again with dichloromethane at 40 °C for 15 min, filtered and combined with the previous extract. The solvent was evaporated to dryness under a flow of nitrogen. The remaining residue (17 mg) was a white, wax-like material. The extract was dissolved in 200 µL of dichloromethane, and the solution fractionated by preparative thin layer chromatography (TLC), using a 500-µm-thick silica gel plate, impregnated with 0.025% sodium fluoresceine (Salatino and Silva 1988), using dichloromethane/hexane (15:85 *v*/*v*) as solvent. After development, the plates viewed under long wave UV revealed bands with RF 0.0 (F1), 0.12 (F2), 0.37 (F3), 0.56 (F4), 0.63 (F5), and 0.83 (F6). After collecting the silica band corresponding to each fraction, the impregnated substances were eluted with dichloromethane at 40 °C for 30 min. The solvent from each elution was evaporated and the residue dissolved in pyridine and treated with BSTFA for derivatization of the constituents of fractions F1–F6. The derivatized products were dissolved in hexane, and 1 µL of the solution was analyzed by gas chromatography/mass spectrometry using a chromatograph Agilent 6850, coupled with a mass spectrometer Agilent MS 5975C, equipped with a triple-axis detector. A capillary column HP-5MS (30 m × 0.3 mm × 0.25 µm thickness) and helium as carrier gas at 1 mL min^{-1} were used. The temperature of injector and detector was 300 °C, and the temperature program of the oven started at 100 °C, ramp of 5 °C min^{-1} until 320 °C and maintained at this temperature for 8 min. For MS analysis, the ionization was obtained at 70 eV with a quadrupole detector, filament current 0.2 mA, detector voltage − 0.7 kV and MS range was 40–800 *m*/*z*. The characterization of the substances corresponding to the chromatogram peaks was achieved by comparison of retention times with authentic samples of *n*-alkanes and comparison of MS with data of

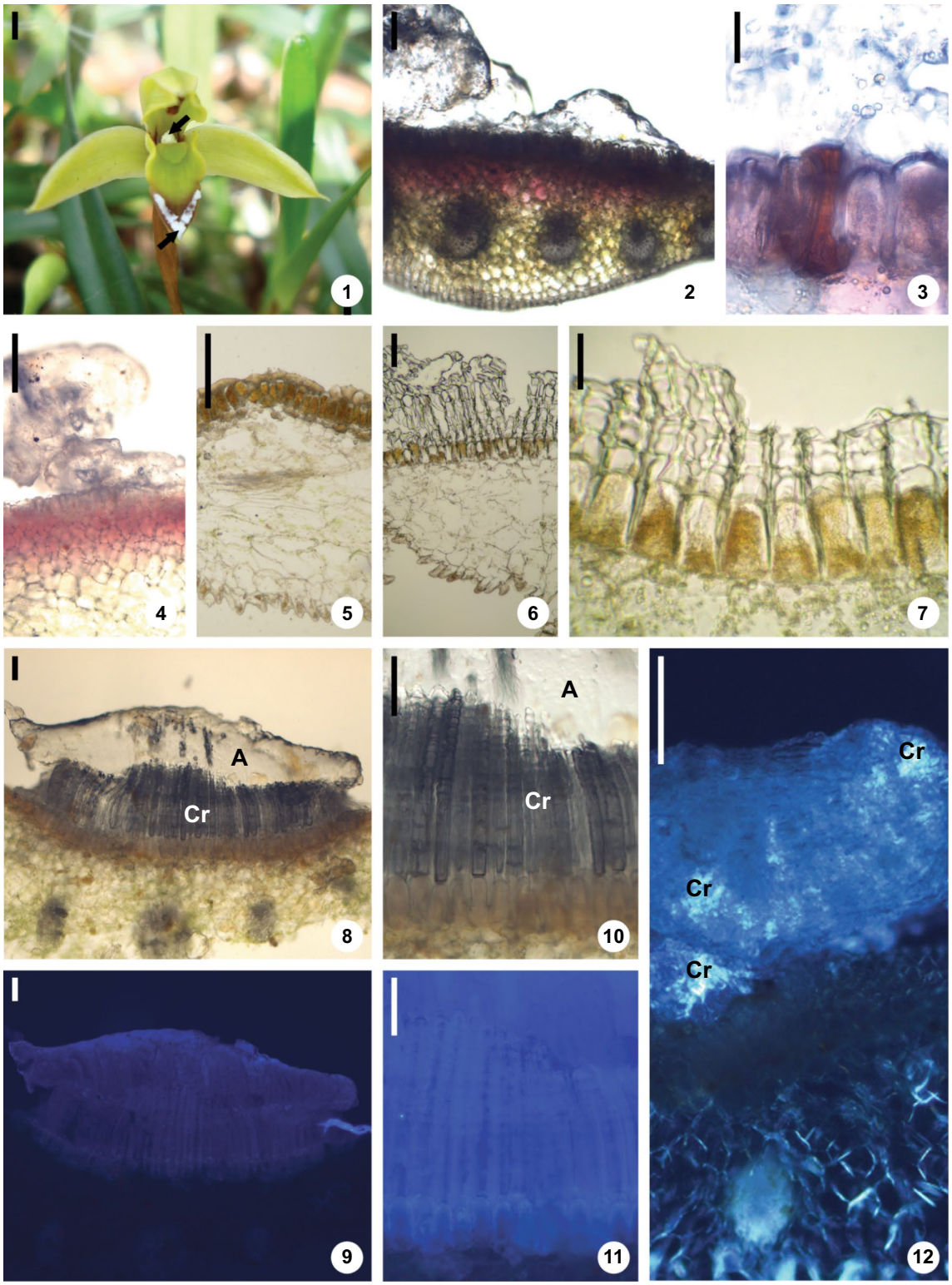

Fig. 1–12 Labellar secretion and secretory trichomes of *Rhetinantha cerifera*. **1.** Flower in frontal view, general aspect. **2**–**12** Fresh transections of labellum. **9**, **11**, **12** Secretion observed under UV light (**9**, **11**) and under polarized light (**12**). (A = amorphous material; Cr = crystalline material; arrow = white exudate). Bar = 5 mm (**1**), 200 µm (**2**, **5**, **8**, **9**, **12**), 25 µm (**3**), 100 µm (**4**, **6**, **10**, **11**), 50 µm (**7**)

Table 1 Specimens of *Rhetinantha cerifera* used to collect flowers for histological and chemical analyses

Origin locality	Date and collector	IBt collection	Flower collected dates
SP, Road Cunha to Parati: Cunha	3-VII-1963, H. J. Targa	3312 3313 3319 3324	Sep, Oct 2012 Jun, Jul, Sept 2013 Mar 2014
SP, Estação Biológica de Boracéia: Salesópolis	16-IV-1964, J. R. Mattos	4017	June 2013 Mar 2014
SP, Railroad Pindamonhangaba to Campos do Jordão, Station Eugênio Lefévre: Santo Antônio dos Pinhais	11-IX-1964, Ziro Matsui	4623 4629	Dec 2011 May, Sep, Oct 2012 July, Sep 2013
RJ, Nova Friburgo	25-VII-1966, P. Brólio	6103	Mar, Apr 2014 May 2016
SP, Reserva Biológica de Paranapiacaba: Santo André	14-I-1981, M. V. Carlucci	11.915	Dec 2011
SP, Parque Estadual da Ilha do Cardoso: Cananéia	10-11-VII-1985, F. Barros	14.099	Feb, Mar, Apr, May, June, Sep 2012 Jul 2013
SP, Serra da Bocaina, Morro das Antenas, near Pró Bocaina Base: Bananal	29-IX-1994, E. L. M. Catharino & R. T. Ninomya	16.555	Sept, Oct 2012 Sept 2013
SP, Engenheiro Marsilac: Parelheiros	w/d, Godoy 756	16.704	Sept 2012

SP, São Paulo state; RJ, Rio de Janeiro state

Table 2 Histochemical tests used to detect different classes of substances in *Rhetinantha cerifera* labellum

Target compounds	Treatments	Positive results (color/fluorescence)	Figures	References
Observed under visible light				
Total lipids	Sudan black B	Blue, black or brownish-black	31–35	Pearse (1985)
Neutral and acidic lipids	Nile blue A	Neutral lipids—pink Acidic lipids—blue	36–41	High (1984)
Terpenoids	NADI reagent (modified)	Essential oils—blue Oleoresin—violet Resiniferous acids—red	42–46	David and Carde (1964)
Free fatty acids	Copper-rubeanic acid (Holczinger reaction)	Green/dark green precipitate	47–48	High (1984)
Total phenolic compounds	Formalin-ferrous sulfate fixative	Dark brown to black	49	Salatino et al. (1988)
	Ferric chloride	Blue–green to dark brown	50–51	Gahan (1984)
Polysaccharides	PAS reaction	Magenta, purple red or pink	52–54	O'Brien and McCully (1981)
Acid mucilages	Ruthenium red	Bright red	55–56	Gregory and Baas (1989)
Mucilages	Mayer reaction	Black	57	Pizzolato (1977)
Alkaloids	Dragendorff reagent	Reddish-brown precipitate	(Negative)	Svendsen and Verpoorte (1983)
	Wagner reagent	Reddish-brown precipitate	(Negative)	Fuhr and Mahlberg (1981)
Proteins	Xylidine Ponceau	Red	58 (faint staining)	O'Brien and McCully (1981)
	Coomassie blue	Blue	(Faint staining)	Fisher (1968)
Observed under UV light				
Fluorescent compounds	Autofluorescence	Blue	59–60	Charrière-Ladreix (1976)
Flavonoids	Naturstoff reagent	Yellow to orange	61–62	Wollenweber (1982)
	aluminum chloride	Yellow	(Positive)	Charrière-Ladreix (1976)
Lipids	Neutral red	Yellow	63	Kirk (1970)

the Wiley-275 (Hewlett-Packard), Wiley/NBS and literature (Zahid et al. 2007; Sakouhi et al. 2009; Suttiarporn et al. 2015).

3 Results

The *R. cerifera* fresh labellum is greenish with some vinaceous hues (Fig. 1); it has a sticky exudate (secretion outside secretory cell) occupying the V-shaped ridge at the apical adaxial region and at the median regions of labellum, and also at the central portion of labellar adaxial base, behind the callus. The exudate is seen as white color to the naked eye (Fig. 1), and colorless by light microscopy (Figs. 2–8, 10). Under UV light and polarized light, the exudate is a mixture of amorphous and crystalline material (Figs. 9, 11–12). The dry callus, on the other hand, is chlorophyllated, devoid any kind of exudate, and is found at the basal region of the labellum (Fig. 1).

Micromorphology and distribution of the secretory trichomes (Figs. 13–30) – Under SEM, a band of secretory trichomes is visible at the V-shaped ridge occupying the apical and median regions of labellar adaxial surface, and

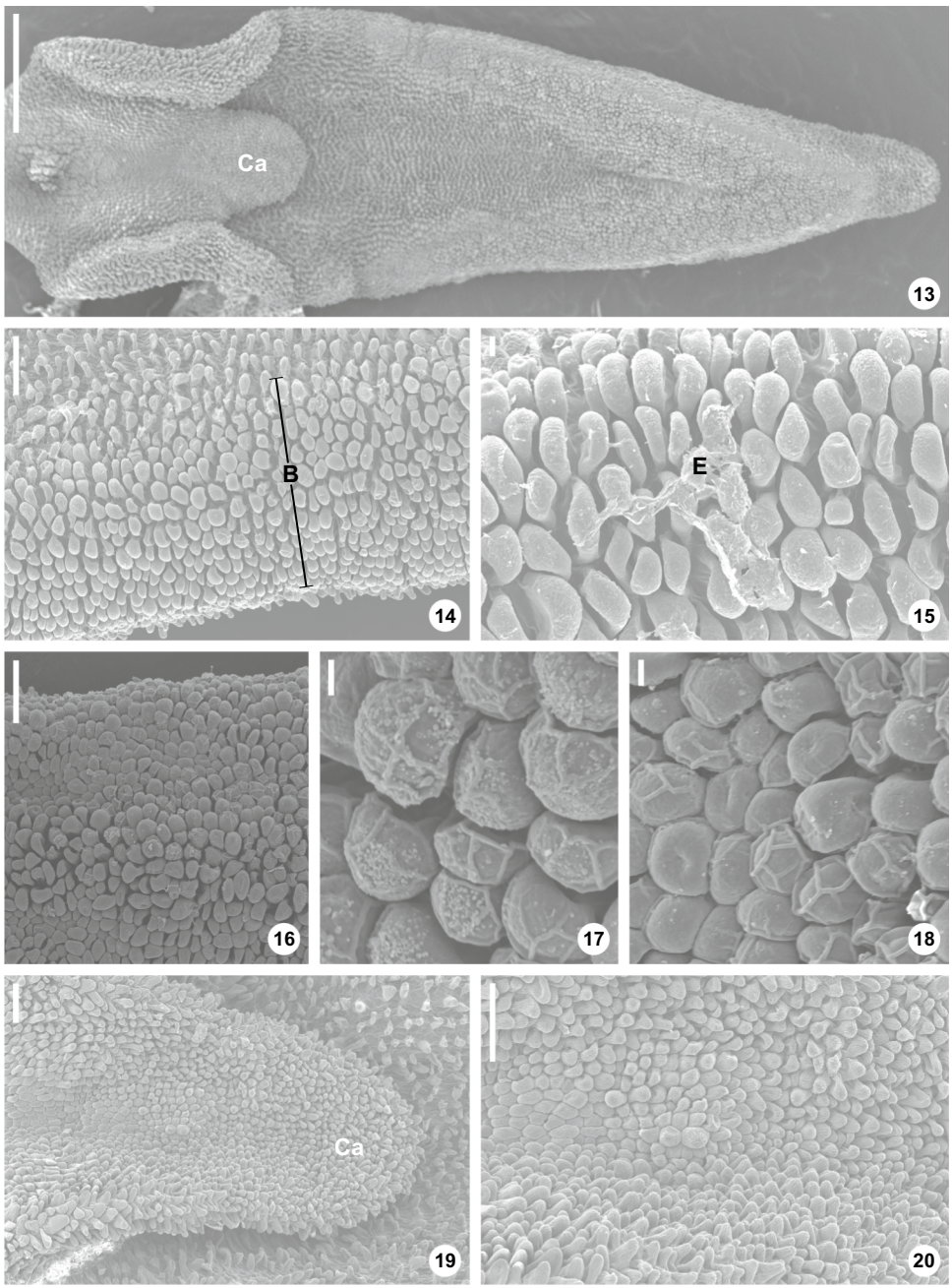

Fig. 13–20 Micromorphology of *Rhetinantha cerifera* labellum at the secretory phase. **13** General aspect of adaxial surface view. **14–18** Marginal portions at the median region of labellum; details aspects of secretory trichomes (**14**, **16**), detail of secretory trichomes after the secretory phase and exudate (**15**), and outer walls of secretory trichomes with sculpturing and small globules during the secretory phase (**17**, **18**). **19**, **20** Callus at the basal region of labellum. **20** Detail of non-secretory epidermis of callus. (B = secretory trichomes band; Ca = callus; E = exudate). Bar = 1 mm (**13**), 100 µm (**14**, **16**, **19**, **20**), 10 µm (**15**, **17**, **18**)

secretory trichomes are also found at the central portion of labellar adaxial base behind the callus (Fig. 13). The shape of the apex of the trichomes is clavate to obovate (Figs. 14–18), their outer walls present sculpturing and small globules during the secretory phase (Figs. 17–18), and the intercellular spaces that have been formed among them are clearly observed (Figs. 14–18). The callus is devoid any kind of secretion (Figs. 13, 19–20).

Secretory trichomes are distributed at the central portion of the labellum apex (Figs. 21–22), and base behind callus (Figs. 24, 27), as well as at the margins of the apical and median regions of the labellum (Figs. 23, 25–26). The identification of these structures as "unicellular secretory trichomes" is confirmed by the occurrence of intercellular spaces along tangential cell walls (Figs. 28–29). At the secretory phase, the metabolites are synthetized and accumulated inside the protoplast of the unicellular secretory trichomes (Figs. 22, 25–30). The secretory products are released together with the retraction of cytoplasm near tangential cell walls (Figs. 28–30). Cuticle detachment, cuticle rupture and pores are not observed (Figs. 27–30), and probably the secreted material may be exuded through cuticle micropores.

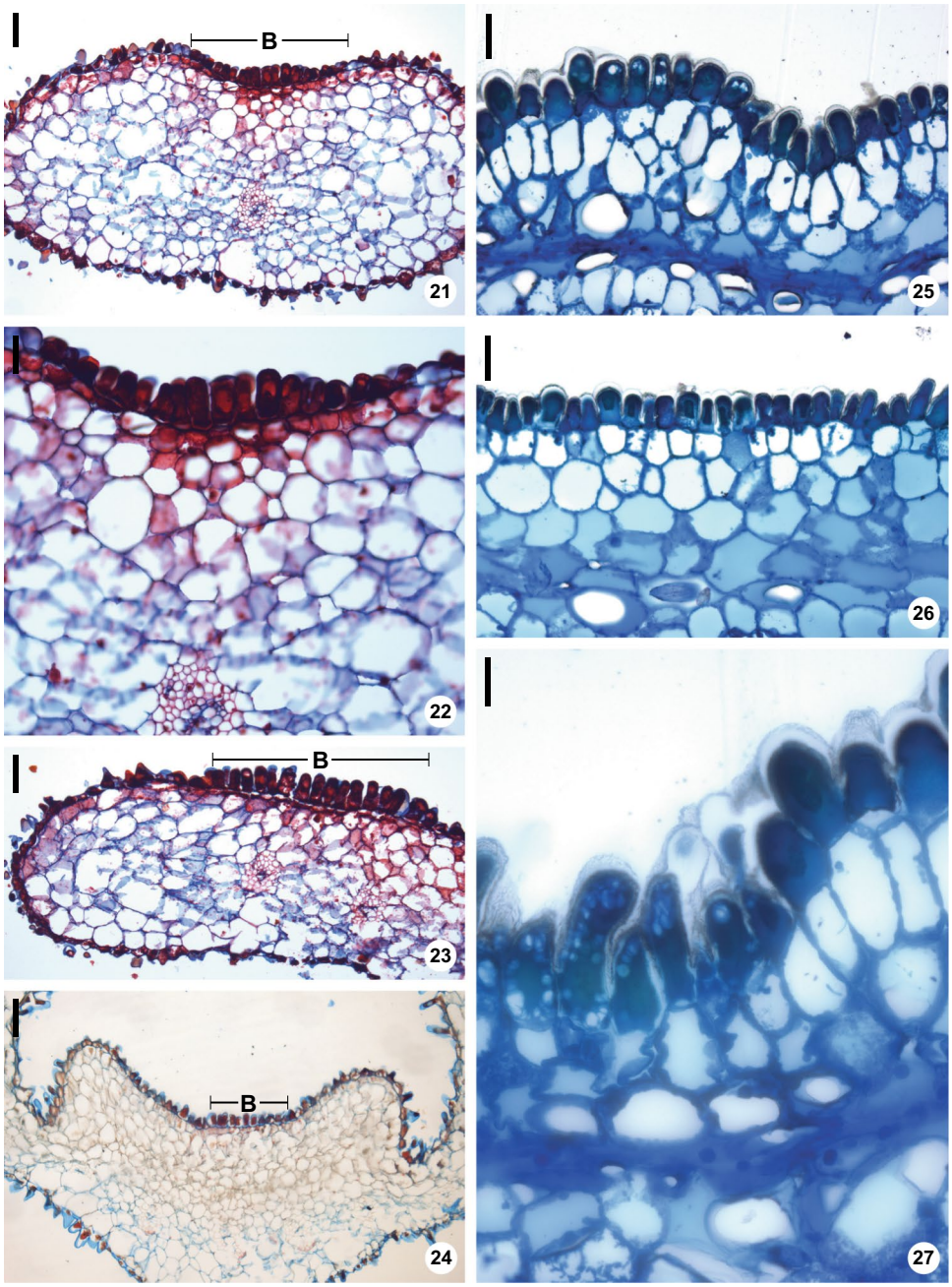

Fig. 21–27 *Rhetinantha cerifera* labellum structure and unicellular trichomes at the secretory phase; transections stained with safranin and astra blue (**21**–**24**), and tangential sections stained with toluidine blue (**25**–**27**). **21**, **22** Apical region of labellum; detail of secretory trichomes (**22**). **23**, **24** Secretory trichomes on the marginal portion at the median region of labellum (**23**) and on the central portion of labellar base behind callus (**24**). **25**–**27** Detail of secretory trichomes on the median region of labellum (**25**, **26**) and on the central portion of labellar base (**27**). (B = secretory trichomes band). Bar = 100 µm (**21**, **23**, **24**), 50 µm (**22**, **25**, **26**) 25 µm (**27**)

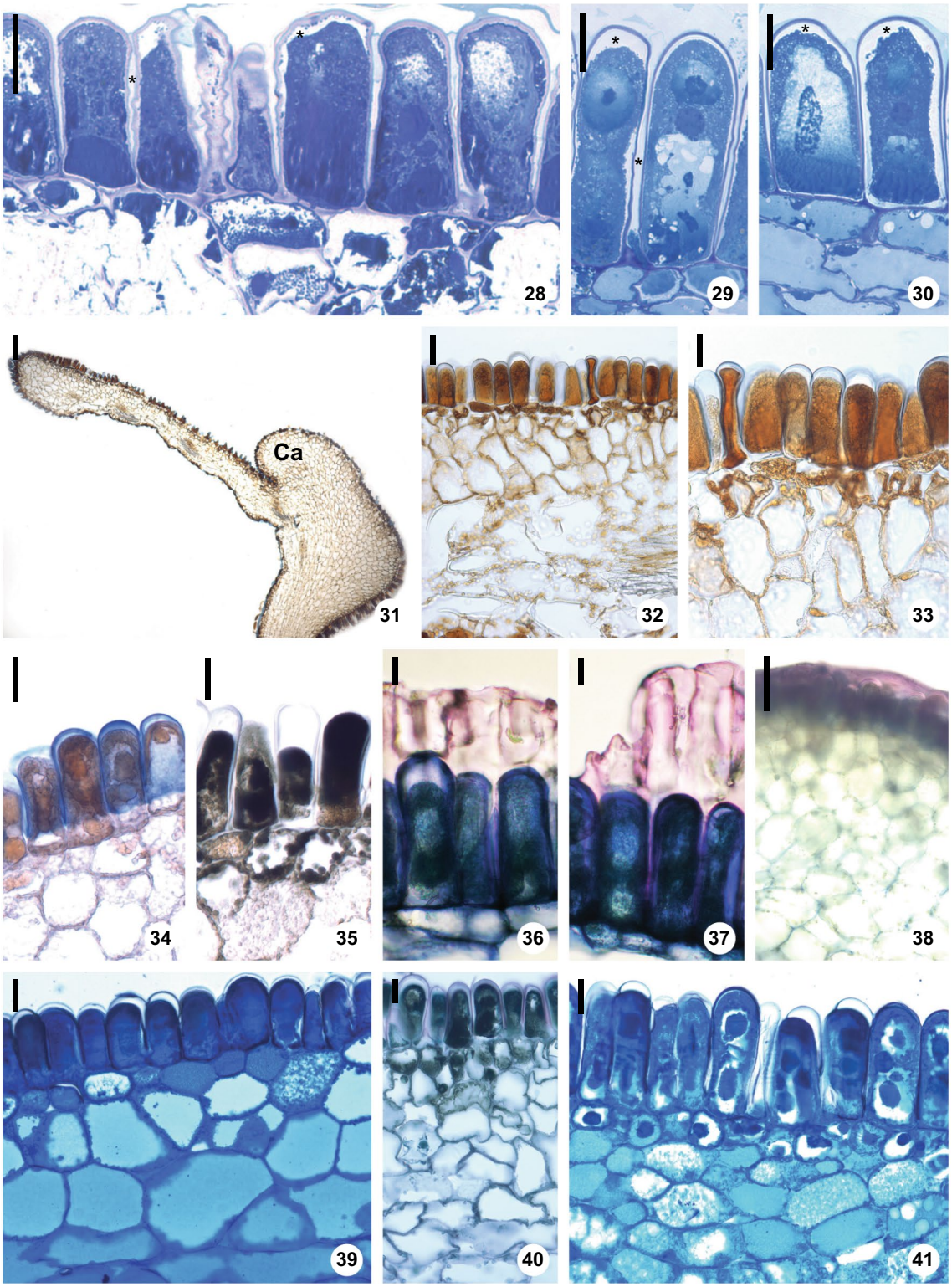

Fig. 28–41 *Rhetinantha cerifera* labellum at the secretory phase: structure and histochemical results; tangential sections of labellum embedded in historesin (**28–30**, **39–41**) or in Paraplast (**31–35**), or fresh labellum (**36–38**). **28–30** Detail of the trichomes treated with toluidine blue and *p*-phenylenediamine. **31–35** Sudan black B. **36–41** Nile blue A. (Ca = callus; * = detail of the retraction of cytoplasm). Bar = 25 µm (**28–30**, **33–37**, **39–41**), 200 µm (**31**), 50 µm (**32**, **38**)

Histochemistry (Figs. 31–63) – The exudate is observed between two unicellular secretory trichomes, occupying all the tangential space, and covering the labellar surface (Figs. 36–37, 42–46, 60–63). Table 2 shows the results of the histochemical tests that detected heterogeneous mixtures of the lipophilic and hydrophilic substances in the secreted material. Free fatty acids, terpenoids (essential oils/oleoresin), phenolic compounds, polysaccharides and proteins (faint staining) are major classes metabolites detected in the labellar secretion. Under UV light, lipids and flavonoids are detected by an induced secondary fluorescence. Procedures for alkaloid detection give negative results.

It is important to highlight that the histochemical methods applied to embedded (Paraplast or historesin) materials are as efficient as those applied to fresh materials (Figs. 31–63). The secreted material inside the trichome protoplast consists of acidic lipids (black – Fig. 35; blue – Fig. 36–37, 39–41), mono and/or sesquiterpenoids (blue – Fig. 45–46), free fatty acids (green precipitate – Fig. 48), phenolic compounds (dark-brown – Fig. 49–51), polysaccharides (red or pink – Fig. 52–56), and protein (red – Fig. 58). Under UV light, the fluorochromes for flavonoids detection induce a yellowish secondary fluorescence (Fig. 61–62).

The exudate presents neutral lipids (pink – Fig. 36–38), mono and/or sesquiterpenoids (blue – Fig. 42–44), oleoresin (violet – Fig. 45–46), free fatty acids (green/dark green precipitate – Fig. 47) and is autofluorescent (blue – Fig. 59–60). Under UV light, lipids are detected (yellow secondary fluorescence – Fig. 63).

Chemical analysis of the labellar secretion – The GC/MS analysis of the labellar secretion detected and characterized 46 constituents (Table 3). Several other substances were detected, but no characterization was possible. Several constituents are long carbon chain, common constituents of plant waxes. Among them, compounds **8**, **10**, **11**, **13**, **14**, **17**, and **18** are alkanes; **1**, **12**, **19**, **25** and **36** are ketones; **3**, **6**, **7**, **23**, and **43** are alcohols or their trimethylsilyl derivatives; **2**, **4**, **5**, and **9** are esters of fatty acids and simple alcohols; **30**, **39**, and **44** are esters of a fatty acid (hexadecanoic) and long carbon chain alcohols. All other characterized constituent are phytosteroids derived either from cholesterol, lanosterol or cycloartenol, with predominance of the latter. Compounds **15** and **16** are steroidal hydrocarbons and **20**–**22**, **32** and **41** are steroidal ketones. Compound **28** is an acetyl ester and **29** a carboxylic acid of a cycloartenol derivative. All other compounds (**24**, **26**, **27**, **31**, **33**–**35**, **37**, **38**, **40**, **42**, and **45**) are steroids with alcoholic function.

4 Discussion

The structure and ultrastructure of the labellar epidermis have been studied in some species of Cymbidiinae and Maxillariinae subtribes, whose secretory trichomes are described either as unicellular trichomes or secretory papillae (Davies and Winters 1998; Davies and Turner 2004; Davies et al. 2006; Davies and Stpiczynska 2012), or conical papillae with ornaments, such as fine longitudinal sculpturing of wall, on the labellum of *M. cerifera* (=*R. cerifera*) (Davies et al. 2003a). The present results about the labellar secretory trichomes of *R. cerifera* demonstrate that each unicellular trichome cell is totally individualized, from the summit to the base, a reason why we choose the designation "unicellular secretory trichomes," in agreement with the description and classification of Theobald et al. (1979). Such trichomes are absent upon the callus of *R. cerifera*, contrary to claims of Whitten et al. (2007) regarding *M. acuminata* clade, and are distinct from the description of *R. notylioglossa* by Davies and Stpiczynska (2012). The outer cell wall may have visible sculpturing, according to Davies et al. (2003a), and small globules, so far unreported for *R. cerifera*. The cuticle remains without modifications and lacked pores during the secretory activity, in agreement with results of Davies et al. (2003a) regarding the same species, and of Davies and Stpiczynska (2012) regarding *R. notylioglossa*.

Davies et al. (2003a, b) also described the labellar secretion of *Maxillaria violaceopunctata* Rchb.f. (=*Heterotaxis violaceopunctata* (Rchb,f.) F.Barros) and *M. villosa* (Barb. Rodr.) Cogn. (=*Heterotaxis villosa* (Bar.Rodr.) F.Barros) as a "viscid" secretion, that stained positively with histochemical tests for aromatic amino acids and lipids, resembling the "wax-like" labellar secretion of members of the *M. acuminata* alliance, comprising *M. acuminata* Lindl. (=*R. acuminata* (Lindl.) M.A.Blanco), *M. cerifera* (=*R. cerifera*) and *M. notylioglossa* (=*R. notylioglossa*). Davies and Turner (2004) described the labellar secretion of *M. cerifera* as a "viscid wax-like material" rich in lipids and protein. Stpiczynska and Davies (2009) examined the labellar trichomes of *Maxillaria dichroma* Rolfe using LM, SEM, TEM (transmission electron microscopy) and described them as "resin-secreting trichomes."

Based on histochemical tests, Davies and Stpiczynska (2012) claimed that the labellum of *R. notylioglossa* secretes "wax," not resin. However, the same authors stated earlier that *Rhetinantha* secretes a "resin-like substance" (hence not wax), when they proposed to compare floral elaiophores histology in *Rudolfiella picta* (Schltr.) Hoehne (Maxillariinae sensu lato) and *Oncidium ornithorhynchum* H.B.K. (Oncidiinae sensu lato) with those tissues that are involved with secretion in *Rhetinantha*

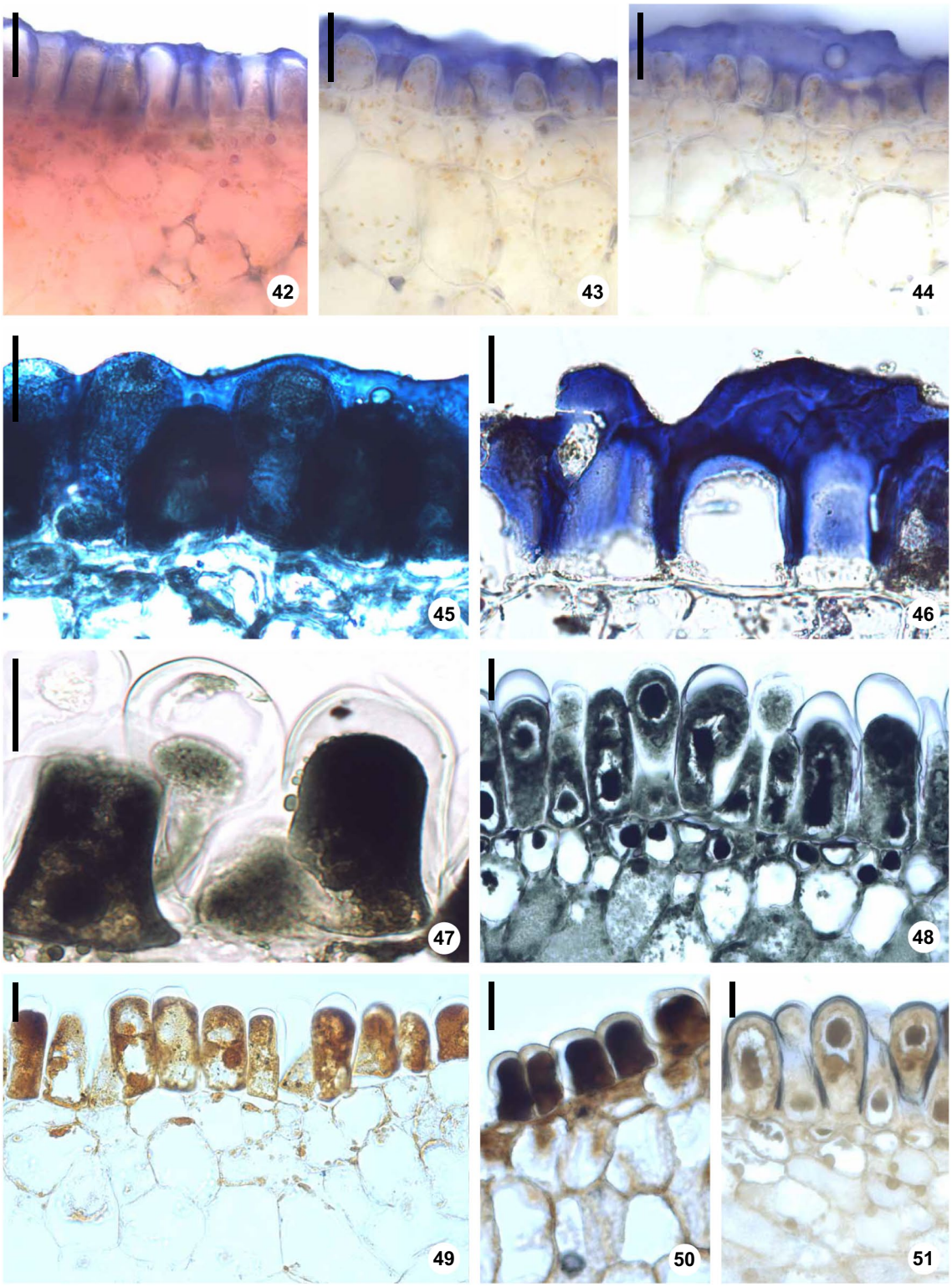

Fig. 42–51 Histochemical results of *Rhetinantha cerifera* secretion; fresh tangential sections of labellum (**42**–**47**) or labellum embedded in historesin (**48**, **49**, **51**) and in Paraplast (**50**). **42**–**46** Nadi reagent. **47**, **48** Holczinger reaction. **49** Formalin-ferrous sulfate fixative. **50**, **51** Ferric chloride. Bar = 25 µm (**42**–**51**)

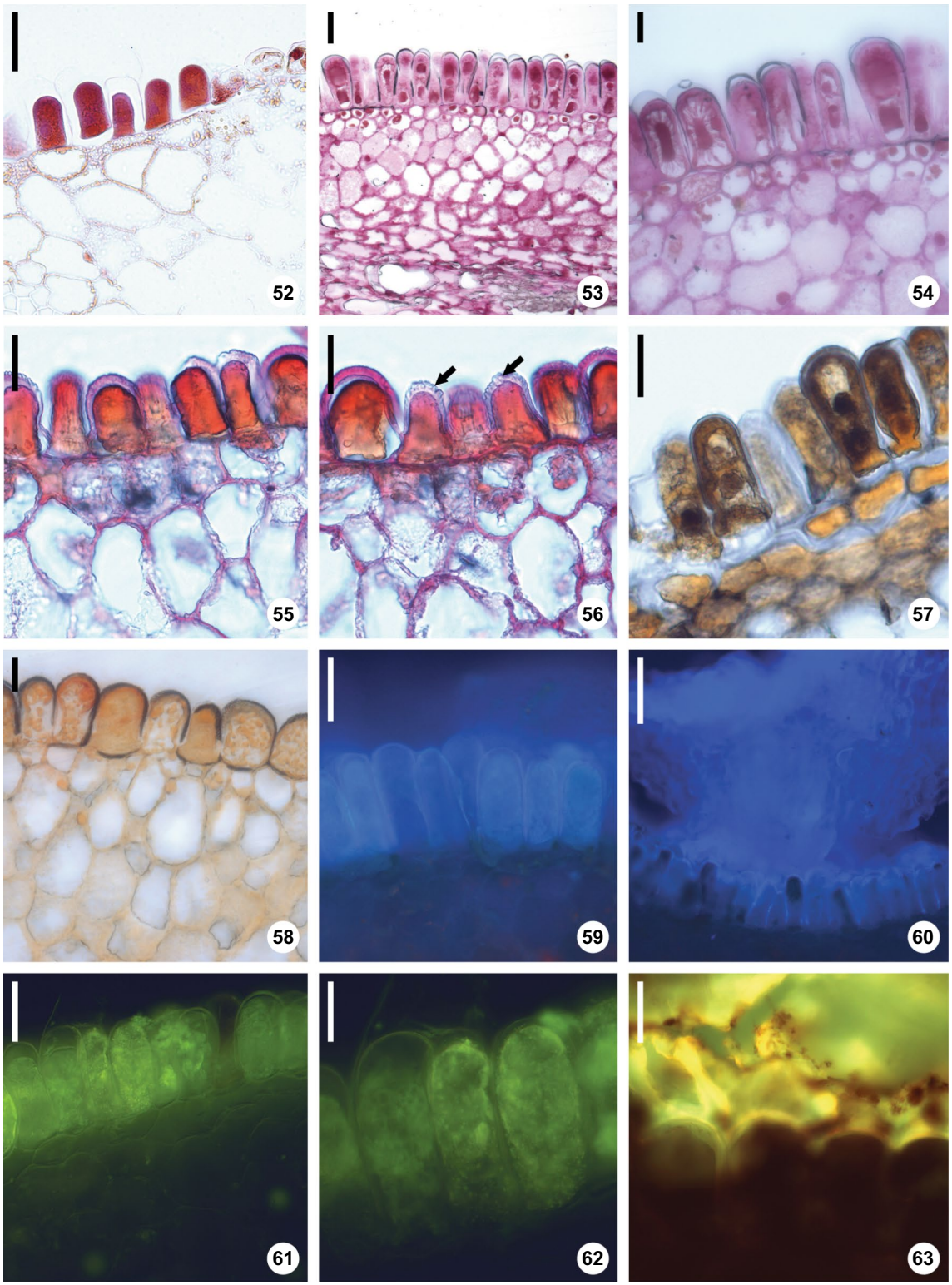

Fig. 52–63 Histochemical results of *Rhetinantha cerifera* secretion; tangential sections of labellum embedded in Paraplast (**52**, **55**, **56**) or in historesin (**53**, **54**, **57**, **58**), or fresh labellum (**59**–**63**). **52**–**54** PAS reaction. **55**, **56** Ruthenium red. **57** Mayer reaction. **58** Xylidine Ponceau. **59**, **60** Autofluorescence of secretion under UV light. **61**, **62** Naturstoff reagent under UV light. **63** Neutral red under UV light. (arrow = outer wall of secretory trichome with sculpturing). Bar = 50 µm (**52**, **53**, **59**, **61**), 25 µm (**54**–**58**, **62**, **63**), 100 µm (**60**)

Table 3 Retention times (Rt), mass spectra data and characterization of chemical constituents (Const.) of labellar secretion of *Rhetinantha cerifera*

Const.	Rt	Molecular ions and fragments (*m/z*, %)	Characterization
1—F_5	8.56	212 ($C_{14}H_{28}O$) (30), 154 (30), 127 (40), 126 (100),	Tetradecanone
2—F_6	14.82	270 ($C_{17}H_{34}O_2$) (20), 227 (40), 143 (50), 85 (80), 74 (100)	Hexadecanoic acid methyl ester
3—F_3	17.93	242 ($C_{16}H_{34}O$) (1), 224 (20), 126 (40), 111 (60), 95 (80), 83 (100)	Hexadecanol
4—F_5	18.76	298 ($C_{18}H_{38}O_2$) (20), 255 (20), 199 (20), 143 (40), 87 (80), 74 (100)	Octadecanoic acid methyl ester
5—F_5	20.97	282 ($C_{18}H_{34}O_2$) (10), 239 (100), 116 (50), 98 (100)	Hexadecanoic acid vinyl ester
6—F_2	21.59	270 ($C_{19}H_{38}O$) (1), 252 (20), 111 (60), 95 (80), 83 (100)	Octadecanol
7—F_2	24.94	298 ($C_{20}H_{42}O$) (1), 125 (40), 113 (60), 95 (80), 83 (100)	Eicosanol
8—F_6	25.10	352 ($C_{25}H_{52}$) (1), 125 (20), 113 (20), 99 (40), 85 (70), 71 (90)	Pentacosane
9—F_5	26.45	328 ($C_{19}H_{40}O_2Si$) (5), 313 (60), 145 (50), 117 (100), 73 (100)	Hexadecanoic acid trimethylsilyl ester
10—F_6	26.64	366 ($C_{26}H_{54}$), 113 (20), 99 (40), 85 (70), 71 (90)	Hexacosane
11—F_6	28.12	380 ($C_{27}H_{56}$) (1), 113 (20), 99 (40), 85 (70), 71 (90)	Heptacosane
12—F_4	29.07	338 ($C_{23}H_{46}O$) (5), 239 (100), 259 (70), 154 (50)	Tricosanone
13—F_6	29.55	394 ($C_{28}H_{58}$) (1), 113 (20), 99 (40), 85 (70), 71 (90)	Octacosane
14—F_6	30.93	408 ($C_{29}H_{60}$) (1), 155 (20), 141 (30), 127 (40), 113 (50), 99 (60), 85 (70), 71 (90)	Nonacosane
15—F_4	31.35	370 ($C_{27}H_{46}$) (40), 313 (50), 232 (40), 175 (60), 95 (100)	5-Cholestene
16—F_3	31.54	372 ($C_{27}H_{48}$) (10), 357 (40), 354 (30), 339 (50), 311 (35), 232 (40), 203 (35), 175 (60), 95 (100)	Cholestane
17—F_6	32.26	436 ($C_{31}H_{64}$) (1), 113 (20), 99 (50), 85 (70), 71 (90)	Hentriacontane
18—F_6	33.56	464 ($C_{33}H_{68}$) (1), 113 (20), 99 (60), 85 (70), 71 (90)	Tritriacontane
19—F_4	34.4	310 ($C_{21}H_{42}O$) (50), 309 (60), 295 (70), 154 (50), 111 (40), 57 (100)	Heneicosanone
20—F_3	34.76	412 ($C_{29}H_{48}O$) (50), 398 (20), 370 (20), 329 (50), 289 (40), 229 (70), 124 (100)	Stigmast-4-en-3-one
21—F_3	34.87	382 ($C_{27}H_{42}O$) (50), 174 (100)	Cholesta-3,5-dien-7-one
22—F_3	34.90	382 ($C_{27}H_{42}O$) (40), 340 (45), 246 (50), 174 (80), 121 (100)	Cholesta-4,6-dien-3-one
23—F_5	34.95	398 ($C_{25}H_{54}OSi$) (1), 383 (100), 283 (50), 255 (60), 75 (100)	Docosanol-trimethylsilyl ether
24—F_3	35.03	412 ($C_{29}H_{48}O$) (10), 379 (50), 272 (30), 239 (30), 175 (60), 95 (100)	Stigmasta-7,24(28)-dien-3-ol-7-avenasterol
25—F_3	35.86	450 ($C_{31}H_{62}O$) (5), 254 (50), 239 (100)	Hentriacontanone
26—F_3	36.13	412 ($C_{29}H_{48}O$) (10), 397 (20), 394 (20) 379 (50), 272 (40), 175 (70), 95 (100)	Stigmasta-5,7-dien-3-ol
27—F_3	36.38	416 ($C_{29}H_{52}O$) (50), 401 (30), 233 (80), 215 (100), 107 (50)	Stigmastanol
28—F_5	36.48	398 ($C_{36}H_{38}O_3$) (20), 383 (30), 313 (40), 260 (40), 175 (80), 95 (100)	Artocarpuate A
29—F_3	36.59	454 ($C_{30}H_{46}O_3$), 408 (20), 383 (20), 379 (30), 367 (50), 260 (40), 175 (70), 95 (100)	3-Oxo-9,19-Cyclolanost-24-en-26-oic acid
30—F_5	36.83	480 ($C_{32}H_{64}O_2$) (1), 340 (40), 257 (100)	Hexadecanoic acid hexadecyl ester
31—F_3	37.20	426 ($C_{30}H_{50}O$) (10), 393 (50), 286 (40), 159 (60), 107 (60), 95 (100)	Lanosta-8,24-dien-3-ol
32—F_3	37.38	410 ($C_{29}H_{46}O$) (50), 174 (100)	Stigmasta-3,5-dien-7-one
33—F_3	37.42	412 ($C_{29}H_{48}O$) (20), 397 (30), 313 (50), 175 (80), 95 (100)	14-Methyl-9,19-cycloergost-25-en-3-ol
34—F_3	37.59	428 ($C_{30}H_{52}O$) (30), 410 (20), 381 (50), 274 (40), 175 (90), 95 (100)	Cycloartan-29-ol
35—F_3	37.85	440 ($C_{31}H_{52}O$) (5), 425 (30), 422 (30), 407 (50), 353 (30), 300 (20), 175 (60), 95 (100)	24-Methylenecycloartan-3-ol
36—F_3	38.24	478 ($C_{33}H_{66}O$) (1), 283 (40), 268 (60), 254 (50), 239 (100)	Tritriacontanone
37—F_2	38.75	426 ($C_{30}H_{50}O$) (20), 411 (20), 313 (50), 175 (60), 95 (100)	Cycloarten-3-ol
38—F_2	38.92	428 ($C_{30}H_{52}O$), 412 (20), 395 (50), 175 (80), 95 (100)	Cycloartan-3-ol
39—F_5	39.13	508 ($C_{34}H_{68}O_2$) (20), 368 (30), 257 (100)	Hexadecanoic acid octadecyl ester
40—F_2	39.28	440 ($C_{29}H_{46}O_2$) (20), 354 (30), 313 (50), 175 (80), 95 (100)	Cyclosadol
41—F_2	39.44	426 ($C_{29}H_{46}O_2$) (20), 409 (50), 302 (30), 175 (80), 95 (100)	3-Hydroxy-27-norcycloart-23-en-25-one
42—F_2	39.65	426 ($C_{30}H_{50}O$) (20), 393 (50), 286 (50), 187 (60), 145 (70), 95 (100)	24-Methyl-28-norcycloart-25-en-3-ol
43—F_3	40.53	454 ($C_{29}H_{62}OSi$) (1), 439 (100), 175 (50), 75 (100)	Hexacosanol trimethylsilyl ether
44—F_3	41.32	536 ($C_{36}H_{72}O_2$) (10), 257 (100)	Hexadecanoic acid eicosanyl ester
45—F_2	41.26	440 ($C_{31}H_{52}O$), 407 (50), 300 (40), 175 (60), 107 (80), 95 (100)	24-Methyl cycloartenol

F_1–F_6, fractions providing constituents from preparative thin layer chromatography

(Maxillariinae sensu stricto) (Davies and Stpiczynska 2009). Although these papers characterized the labellar secretion by different genera of Maxillariinae as lipophyllic (either waxy or resin-like material), our observations and the available descriptions of species, have led us to conclude that only the species of *R. cerifera* and *R. notylioglossa* in their subtribe have a similar sticky white secretion occupying the V-shaped ridge at the apical and median regions of the labellum, as well as at the labellar adaxial base behind the callus.

Given the distinct terminology proposed to designate the labellar secretion of *R. cerifera*, detailed chemical analyses are needed to attain a consistent basis about its composition. The data of the GC/MS analysis of the labellar secretion (Table 3) agrees and expands the results reported by Flach et al. (2004), corresponding to the isolation of cycloartenol itself and its derivatives. Given the distinct terminology proposed to designate the labellar secretion of *R. cerifera*, detailed chemical analyses are needed to attain a consistent basis about its composition. The data of the GC/MS analysis of the labellar secretion (Table 3) corroborates and expands the results reported by Flach et al. (2004), regarding cycloartenol. In the present study, cycloartenol itself (**37**) and its derivatives (**28**, **34**, **35**, **38**, **40**–**42**) were characterized, as well as steroids with other carbon skeletons (e.g., **15**, **27**, **31** and **32**; Table 3). In addition, other classes of lipophilic substances not previously reported for *R. cerifera* have been characterized (Table 3). Pentacyclic terpenoids and lipophilic long-chain alkanes (e.g., **9**), carboxylic acids (e.g., **14**), alcohols (e.g., **43**) and esters (e.g., **44**) are common constituents of plant cuticular waxes (Fernández et al. 2016). Pentacyclic triterpenoids may be major constituents of plant cuticular wax, as observed on the skin of apple fruits (Belding et al. 1998) and leaves of many plant species (Oliveira et al. 2003; Buschhaus and Jetter 2010). This result is not in disagreement with former characterization of the labellar secretion as "wax" (Hoehne 1949) and "wax-like" (Davies et al. 2003a; Davies and Turner 2004). Tetracyclic triterpenoids (phytosteroids) such as cycloartenol are not common wax constituents. However, in some groups, such as Solanaceae, steroids may be abundant (Halinski et al. 2012). Cholesterol is a relevant constituent of cuticular waxes of potato leaves (*Solanum tuberosum* L.) (Szafranek and Synak 2006). A question arises whether cycloartenol and its derivatives (e.g., **34**, **37**, **45**) might be a characteristic also of the cuticular wax of Orchidaceae or of groups within the family.

Although the list of substances of Table 3 could characterize the studied secretion as a plant wax, the histochemical tests of the present study reveal that the labellar secretion of *R. cerifera* is more complex than just wax. The labellar secretion is really a heterogeneous mixture, with amorphous and crystalline material, containing mainly lipophilic substances (in agreement with Davies et al. 2003a and Flach et al. 2004), free fatty acids, phenolic compounds and polysaccharides (mucilage) at the first time. These results are in accordance with Davies and Turner (2004), who reported a material rich in lipids, although they did not detect a high protein content, suggesting that the proteins detected are structural or enzymatic (Table 2). The results of the present work also indicate that the labellar secretory unicellular trichomes of *R. cerifera* can concomitantly activate different metabolic pathways.

Based on the present histochemical and chemical analyses, the exudate of the labellum of *R. cerifera* should not be characterized by the terms "waxy" or "resinous." It is true that the results obtained revealed that the secretion contains oleoresin (terpenoids), and this complies with the word "*rhetinos.*" The secretion contains also wax constituents, and this is in line with the specific epithet "*cerifera.*" Since, however, the composition of the exudate is highly complex, the authors of the present work propose to characterize the studied labellar secretion as a "heterogeneous mixture," until a better terminology is found to designate the exudate of the labellar unicellular trichomes, taking into account its overall chemical composition.

The results demonstrate that the chlorophyllated and dry callus of *R. cerifera* labellum is non-secretory. By contrast, Davies and Stpiczynska (2012) stated that a labellar secretion is produced by *R. notylioglossa* both on the callus and on V-shaped ridge. We have never observed secretion on the callus of this species, but only behind the callus as described on *R. cerifera* labellum. Orchidaceae species belonging to tribe Maxillarieae often present a secretory callus or protuberance upon adaxial surface, specially elaiophores in species of Oncidiinae subtribe. Examples are *Cyrtochilum meirax* (Rchb.f.) Dalström, *Gomesia bifolia* (Sims) M.W.Chase & N.H. Williams, *Lockhartia verrucosa* Lindl. ex Rchb.f., *Oncidium truliferum* Lindl., *Ornithophora radicans* (Rchb.f.) Garay & Pabst, *Phymatidium falcifolium* Lindl., *Zygostates grandiflora* (Lindl.) Mansf., *Z. lunata* Lindl. (Stpiczynska and Davies 2008; Aliscioni et al. 2009; Pacek et al. 2012; Blanco et al. 2013; Davies et al. 2014). Nectary is known from species of subtribe Maxillariinae, as examples *Maxillaria anceps* Ames & C.Schweinf. (= *Maxillariella anceps* (Ames & C.Schweinf.) M.A.Blanco & Carnevali), *Maxillaria coccinea* (Jacq.) L.O.Williams ex Hodge (= *Ornithidium coccineum* (Jacq.) Salisb. ex R.Br.), *Ornithidium sophronitis* Rchb.f. (Stpiczynska et al. 2003, 2009; Davies et al. 2005). In addition, Stpiczynska and Davies (2009) described callus cells containing dark polyphenols on *Maxillaria dichroma* Rolfe. Pansarin and Pansarin (2010) emphasized that although the central callus on *Trichocentrum pumilum* (Lindl.) M.W.Chase & N.H.Williams (tribe Maxillarieae) resembles the elaiophore of some others species,

only the finger-like unicellular trichomes scattered on the surface of the lateral lobes of the labellum are responsible for lipid secretion; thus, the callus of this species is also non-secretory.

In summary, the characters described in the present report concerning the labellum structure and secretion of *R. cerifera* highlights several points, as follows: (a) the white sticky exudate occupies the V-shaped ridge at the apical and median regions of the labellum, where unicellular secretory trichomes are distributed; (b) the white sticky exudate is also seen at the central portion of labellum base, behind callus, where unicellular secretory trichomes are present; (c) a non-secretory callus occurs at the basal region of the labellum; (d) as analyzed by GC/MS, the secretion contains substances with long carbon chain, commonly found in plant waxes, in addition to phytosteroids, such as cycloartenol and its derivatives; (e) as seen by in situ histochemical analyses, the main classes of metabolites, are terpenoids (oleoresin), free fatty acids, polysaccharides (mainly mucilage) and phenolic compounds (including flavonoids); (f) the secretion is a heterogeneous mixture, with hydrophilic and lipophilic constituents.

The occurrence of *Rhetinantha* species is confirmed from the North to the South of Brazil as reported by Flora do Brasil 2020 under construction (2018). *Rhetinantha friedrichsthallii* is restricted to the Northern region, but *R. cerifera* and *R. notylioglossa* are commonly quoted in floristic inventories throughout Brazil. Based on our observations, only *R.cerifera* and *R. notylioglossa* have non-secretory callus and a white exudate from labellar secretory cells occupying the contours of the labellar apex and the adaxial base behind the callus. Our own observations and those from the literature show the high similarity between these two species. Even the chemical composition of the labellar exudate of these two species is described as similar by Davies et al. (2003a). This set of information reinforces that both constitute a single taxonomic entity. In this case, the accepted binomium must be *Rhetinantha notylioglossa*, respecting the nomenclature priority rule, corroborating the synonymy suggested by Barros et al. (2015) and the site Flora do Brasil 2020 under construction (2018).

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Author's contributions SCMV, PCG, and MMC were involved histological and histochemistry contributions; AS, MLS, and GN were involved biochemical contributions; FB was involved taxonomy contributions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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