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EVIDENCE FOR THE DUAL ROLE OF FLORAL SECRETORY CELLS IN *BULBOPHYLLUM*

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Floral epidermal cells of most species of *Bulbophyllum* Thouars studied to date produce both lipid-rich food-rewards and fragrance. Since fragrances largely consist of terpenoids and have an affinity for lipophilic stains, the simultaneous presence of lipid-rich food-rewards frustrates identification of fragrance-secreting cells by conventional histochemistry. Furthermore, since both lipid-rich food-rewards and fragrances are probably synthesized by a similar complement of organelles, interpretation of TEM images can prove difficult. All members of section *Racemosae* Benth. & Hook. f. investigated to date, however, are unusual in their secretion of a predominantly proteinaceous food-reward, and lipids are seemingly absent. This might enable their use as models for the identification and characterization of fragrance-secreting tissues and organelles.
Three members of sect. *Racemosae* were chosen, namely *Bulbophyllum dissitiflorum* Seidenf., *B. lilacinum* Ridl. and *B. tricorne* Seidenf. & Smitinand. All produced food-rewards. Of these, one (*B. dissitiflorum*) lacked fragrance and was used as a control, whereas the remaining two species produced fragrance. Having established that the food-reward was mainly proteinaceous in each case, and did not test positively for lipid, we undertook further histochemical investigations, as well as light microscopy, SEM and TEM. Specialized palisade-like epidermal cells of all species contained protein bodies and rough endoplasmic reticulum consistent with the production and secretion of a protein-rich food-reward. Cuticular pores were also present. In fragrant species, these cells also contained abundant smooth endoplasmic reticulum, oil droplets and many, well-developed, spherical plastids with numerous plastoglobuli, similar to those found in the osmophores (fragrance-producing structures) of other orchids. Indeterminate, osmiophilic cytoplasmic inclusions were also present. By contrast, the non-fragrant species lacked oil droplets and other osmiophilic inclusions and the plastids were scant, poorly developed, often elongate or irregular in shape and contained few plastoglobuli. Smooth endoplasmic reticulum was also less frequent. Since food-rewards tested negatively for lipid, it is probable that any oil droplets present were involved in fragrance production, especially since they were absent from the non-fragrant species. Thus, the unusual absence of lipids from the food-rewards of sect. *Racemosae* provided a rare opportunity, permitting, for the first time, the unraveling of these two secretory processes (food-reward and fragrance) in *Bulbophyllum* and clearly demonstrating the plasticity of these cells and their dual role in secretion.

Key words: Anatomy, *Bulbophyllum*, fragrance, histochemistry, micromorphology, oil, protein, secretion, ultrastructure.

INTRODUCTION

Plants of the genus *Bulbophyllum* Thouars have some of the most intricate flowers and complex pollination mechanisms to be found amongst the Orchidaceae (van der Pijl and Dodson, 1969; van der Cingel, 2001). For an up-to-date, overall account of the genus, readers are referred to Pridgeon et al. (2014). Many of its members are pollinated by dipterans (van der Pijl and Dodson, 1969; van der Cingel, 2001) attracted by food-rewards and fragrance. However, to date, these food-rewards have been investigated for relatively few species, mostly Neotropical and African taxa, and have generally been found to be lipid-rich and produced by palisade-like epidermal cells of the

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labellum (de Pádua Teixeira et al., 2004; Nunes et al., 2014, 2015; Stpiczyńska et al., 2015). Other food-rewards are heterogeneous and contain both lipids and sugars (e.g., Pohl, 1935; Stpiczyńska et al., 2015).

The fragrance-producing structures of *Bulbophyllum* are not always obvious and are often indistinguishable from typical epidermal cells based on micromorphological characters alone. Since in many species lipid-rich food-rewards thickly coat the labellar epidermis and, like the terpenoid compounds that occur in fragrances, stain strongly with lipophilic stains, their presence frustrates any attempts to identify fragrance-secreting tissues by conventional histochemistry. Furthermore, since both lipid-rich food-rewards and fragrances are likely to be synthesized by a similar complement of organelles, interpretation of TEM images can prove difficult. Hitherto, the labellar anatomy and secretion of food-rewards have been described for four species of the Asian section *Racemosae* Benth. & Hook. f., namely, *B. careyanum* (Hook.) Spreng., *B. morphologorum* Kraenzl., *B. orientale* Seidenf. and *B. wangkaense* Seidenf. All these taxa were unusual in that their food-rewards comprised protein-laden mucilage (Davies and Stpiczyńska, 2014), but seemingly no lipid. Members of this section, in that they lack lipid-rich food-rewards, might provide useful models for the identification of fragrance-secreting cells.

Section *Racemosae* contains approximately 38 provisionally accepted species distributed throughout India, Nepal, Bhutan, Myanmar, China, Vietnam, Laos, Thailand, Peninsular Malaysia and the Philippines (pers. comm., J.J. Vermeulen, 2014). Moreover, Ong and Tan (2012), who investigated three of its species (including *B. lilacinum* Ridl. and *B. peninsulare* Seidenf.), reported that these plants are pollinated by fruit flies (*Drosophila ananassae*) that remove pollinia while feeding on labellar secretions.

Here, we establish the presence of predominantly proteinaceous floral food-rewards for further three species of sect. *Racemosae*, namely, *B. dissitiflorum* Seidenf., *B. lilacinum* Ridl. and *B. tricorne* Seidenf. & Smitinand, and subsequently use these taxa as models for identifying and characterizing floral scent-producing tissues, unimpeded by the presence of surface lipid-rich food-rewards.

MATERIALS AND METHODS

Plants used in this study include *Bulbophyllum dissitiflorum* Seidenf. (accession number KLD 201305), *B. lilacinum* Ridl. (accession number KLD 201306) and *B. tricorne* Seidenf. & Smitinand (accession number KLD 201315) obtained from one of the authors' collection. Spirit-preserved, voucher material of each of these species was deposited at the herbarium of the Royal Botanic Gardens, Kew, UK under the accession numbers Davies 2015-1 (*B. dissitiflorum*), Davies 2015-2 (*B. lilacinum*) and Davies 2015-3 (*B. tricorne*). Their identities were confirmed by J.J. Vermeulen (pers. comm., 2013–2014). Abbreviations for authors of plant names follow Brummitt and Powell (1992) throughout.

Since the presence of lipid-rich food-rewards on the labellum surface would obscure the selective staining of fragrance-producing tissues by lipophilic stains, histochemical analyses of food-rewards were firstly undertaken in order to establish their chemical composition before using appropriate histochemical methods to detect tissues involved in fragrance production.

Localization of putative lipid-secreting floral tissues was performed on intact flowers by immersing them for 30 min in a saturated ethanolic solution of Sudan III. The presence of putative mucilage-secreting tissues was detected by immersing labella for 10 min in 0.05% (w/v) aqueous ruthenium red solution, whereas the presence of proteins was verified by immersing whole flowers for 15 min in solutions of Coomasie Brilliant Blue R 250 (CBB) and Ponceau 2R (Fisher, 1968; Ruzin, 1999). Those parts of the flower that showed clear evidence of secretion (namely, parts of the labellum) were subsequently examined using light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM), as follows:

Representative pieces of secretory tissues (approx. 1 mm^3) were excised and fixed in 2.5% (v/v) glutaraldehyde / 4% (v/v) formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C, transferred and washed three times in 0.1M sodium cacodylate buffer pH 6.8 and post-fixed in 1.5% (w/v) osmium tetroxide solution for 1.5 h at 0°C. The fixed material was then dehydrated using a graded ethanol series, and infiltrated and embedded in Spurr resin (Spurr Low-Viscosity resin, Sigma). Following polymerization at 60°C, sections were cut at 70 nm for transmission electron microscopy using a Leica EM UC7 ultramicrotome and a diamond knife, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined using a JEM 1400 (JEOL Co., Japan, 2008) transmission electron microscope, at an accelerating voltage of 80 kV.

Semi-thin sections (0.9–1.0 μm thick) were prepared for light microscopy and stained for general histology using a 1:1aqueous solution of 1% (w/v) methylene blue: 1% (w/v) azure II (MB/AII) for 5–7 min. The periodic acid-Schiff (PAS) reaction was applied to detect the presence of insoluble

polysaccharides (Jensen, 1962). Ponceau 2R and Sudan III or Sudan Black B (SBB) were used to test for the presence of proteins and lipids, respectively (Ruzin, 1999).

The presence of lipids, starch, mucilage and proteins in hand-cut sections of labellar tissue was detected by treating the latter with a saturated ethanolic solution of Sudan III and aqueous solutions of IKI (iodine-potassium iodide), ruthenium red solution and Ponceau 2R, respectively. Furthermore, CBB was used to test sections for total protein (Fisher, 1968; Ruzin, 1999).

Micrometry and photomicrography were accomplished using a Nikon Eclipse E200 (NIS-Elements AR software) and a high resolution digital camera (CCD MORADA, SiS-Olympus, Germany) for LM and TEM images, respectively.

Entire labella were dehydrated for SEM with acetone and subjected to critical-point drying using liquid CO_2.They were then sputter-coated with gold and examined using a Tescan Vega II LS or LEO 1430VP (Zeiss) scanning electron microscope, at an accelerating voltage of 30 kV.

RESULTS

Since the boundaries of many *Bulbophyllum* species are poorly defined and may eventually be subject to realignment, the floral habit and gross morphology of the labellum of each investigated species are shown for future reference (Fig. 1). Characteristics of labellar secretory tissues are summarized in Table 1.

TABLE 1. Summary of characteristics of labellar secretory tissues.

Character	*Bulbophyllum dissitiflorum* Seidenf.	*Bulbophyllum lilacinum* Ridl.	*Bulbophyllum tricorne* Seidenf. & Smitinand
Fragrance	-	+	+
Median longitudinal labellar groove	+	+	+
Surface secretion	+ (copious)	+ (copious)	+ (copious)
Sweet taste (sugars?)	-	-	-
Stains for proteins	+	+	+
Stains for lipids	-	-	-
Stains for mucilage	-	-	+ (minute quantities)
Type of secretion (TEM)	heterogeneous	heterogeneous	heterogeneous
Secretory tissue (LM)	palisade-like cells	palisade-like cells	palisade-like cells
Proteins	+	+	+
Lipids	-	+	-
Starch	+ (few, small grains)	+ (few, small grains)	+ (few, small grains)
Mucilage	+ (associated with cell wall)	+ (associated with cell wall)	+ (associated with cell wall)
Cuticle			
Striate (SEM)	+	+	+
Cuticular blisters (SEM)	+	+	+
Cracks or pores (SEM)	+	+	+
Micro-channels (TEM)	-	-	+
Organelle complement (TEM)			
Endoplasmic reticulum	mainly RER	RER and SER	RER and SER
Plastids	leucoplasts with few plastoglobuli	presumed chromoplasts with plastoglobuli	chromoplasts with plastoglobuli
Oil droplets	-	+	+
Protein bodies	+	+	+
Mitochondria	+	+	+
Dictyosomes	+	+	+
Vesicles	+	+	+ (also multivesiculate bodies)
Sub-epidermal parenchyma	amyloplasts	amyloplasts	amyloplasts
Type of ground parenchyma	compact storage parenchyma	compact storage parenchyma	compact storage parenchyma
Vascular bundles	collateral	collateral	collateral
Idioblasts with raphides	+	+	+

LM = light microscopy; RER = rough endoplasmic reticulum; SEM = scanning electron microscopy; SER = smooth endoplasmic reticulum; TEM = transmission electron microscopy

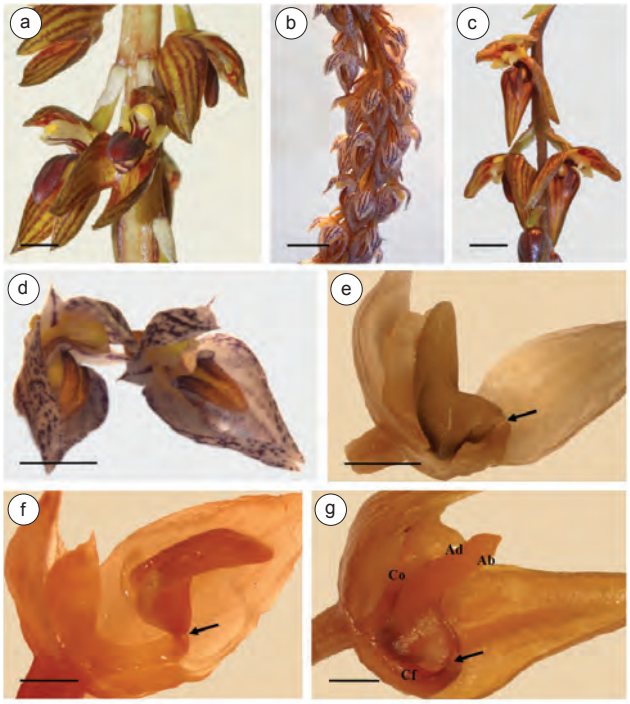

Fig. 1. Inflorescences and flowers of investigated species of *Bulbophyllum* sect. *Racemosae* (**a–c**). Pendulous racemose inflorescences of *Bulbophyllum dissitiflorum* (**a**), *Bulbophyllum lilacinum* (**b**) and *Bulbophyllum tricorne* (**c**). Flowers of *B. lilacinum* with orange-yellow labellum (**d**). Flowers of *B. dissitiflorum* (**e**), *B. lilacinum* (**f**) and *B. tricorne* (**g**) showing articulation of labellum to column-foot (arrows), and flower of *B. dissitiflorum* (**e**) with labellum adpressed to column. Scale bars = 2mm, 4 mm, 5 mm, 2 mm, 2 mm, 1 mm, 1 mm, respectively. Ab = abaxial surface of labellum; Ad = adaxial surface of labellum; Cf = column-foot; Co = column.

BULBOPHYLLUM DISSITIFLORUM

The inflorescence is pendulous, lax and racemose, with widely spaced flowers (Fig. 1a) that lack distinctive fragrance. The sepals are yellow, striped dark-red, the petals yellow, spotted with red, the labellum dark-red and the column white, heavily marked ventrally with dark-red. The stelidia are white, forwardly pointing, often with hooked tips. The linguiform labellum is mobile, has lateral lobes and, adaxially, a median longitudinal groove or sulcus. Secreted material can be found associated with this groove (Fig. 2a). The labellum is articulated with the base of the column-foot and, when in the upright position, is adpressed to the column (Fig. 1e).

The labellar groove is lined with palisade-like epidermal cells (Fig. 2e, f, h) of mean dimensions 52.3 x 12.5 µm. These cells contain dense cytoplasm and a centrally located nucleus. Their thin, outermost cellulosic cell walls have a thick, and often blistered cuticle (Figs. 2e, f; 3a, d). Beneath the labellar epidermis are located smaller subepidermal parenchyma cells. These isodiametric cells have a mean diameter of 24.7 µm, and each contains a relatively large nucleus and cytoplasm that stains intensely with MB/AII (Fig. 3a). Collateral vascular bundles are present in the ground parenchyma (Fig. 2e), and numerous idioblasts containing raphides occur predominantly in the subepidermal parenchyma (Fig. 2e–f).

The secretion present on the labellar surface stained for proteins with CBB (Fig. 2a) and

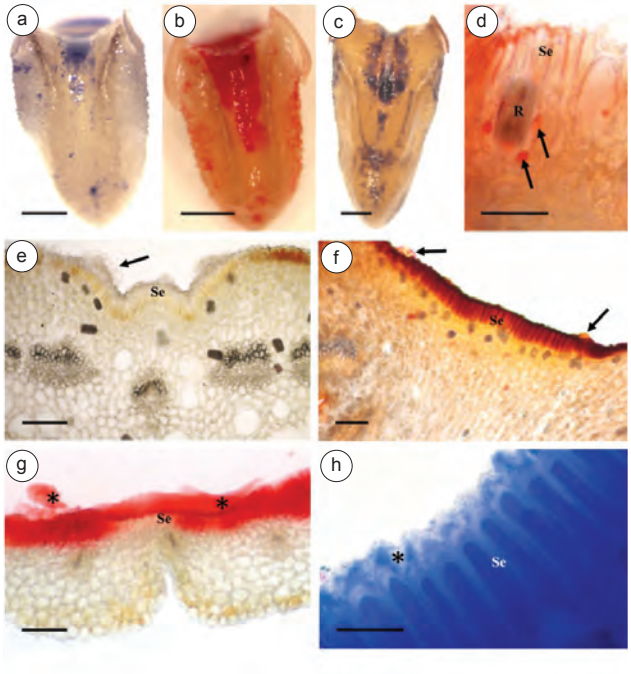

Fig. 2. Labella of investigated species of *Bulbophyllum* – macro-photography images and hand-cut transverse sections. (**a**) Adaxial view of labellum of *B. dissitiflorum* stained with CBB. Note that the secretion on the proximal part of the median longitudinal groove stained selectively for protein with this reagent. (**b**) Proteinaceous secretion on labellum of *B. lilacinum* stained with Ponceau 2R. (**c**) Labellum of *B. tricorne* stained for proteins with CBB. (**d**) Cuticle and lipid droplets (arrows) in subepidermal idioblast of *B. lilacinum* stained with Sudan III. (**e**) Cuticular blisters (arrow) on the surface of palisade-like epidermis of *B. dissitiflorum*. Testing with IKI reveals only a small amount of starch in subepidermal parenchyma cells. (**f**) Section of labellum of *B. dissitiflorum*. Note that only palisade-like epidermal cells, the thick cuticle and cuticular blisters (arrows) stained strongly with Sudan III. (**g**) Surface secretion (asterisks) on labellum of *B. lilacinum* stained with Ponceau 2R for proteins. (**h**) Palisade-like epidermal cells of *B. dissitiflorum* and granular, secreted surface material (asterisk) stained for proteins with CBB. Scale bars = 0.5 mm, 0.5 mm, 0.5 mm, 40 µm, 100 µm, 100 µm, 100 µm, 20 µm, respectively. Se = palisade-like secretory epidermis; R = raphides.

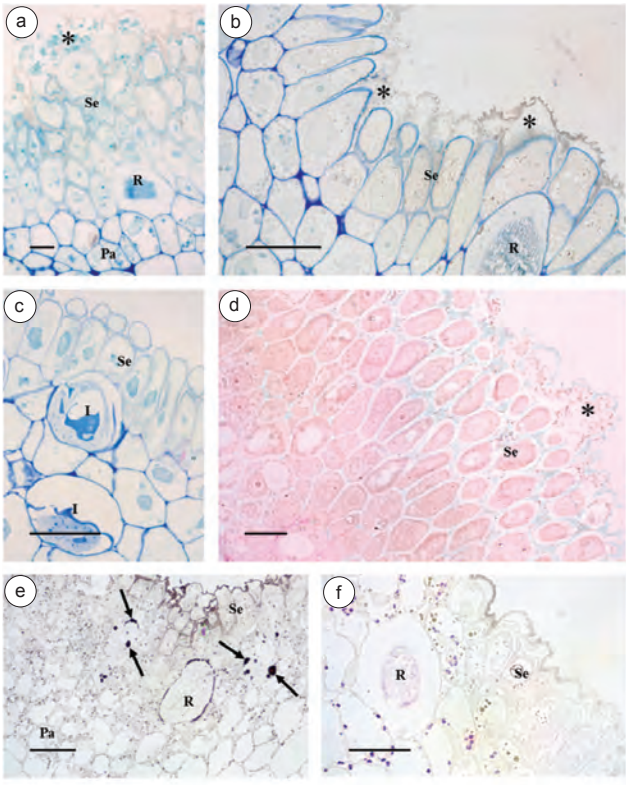

Fig. 3. Palisade-like epidermis and subepidermal parenchyma, LM. (**a**) Slightly oblique section of palisade-like epidermis and parenchyma of *B. dissitiflorum* stained with MB/AII. Note granular material accumulated beneath cuticular blister (asterisk). (**b**) Distended cuticle overlying the labellar epidermis of *B. lilacinum*. Note that secreted material has accumulated beneath the cuticle (asterisk). (**c**) Large idioblasts in subepidermal parenchyma of *B. tricorne* containing material that has stained strongly with MB/AII. (**d**) Epidermal cells of *B. dissitiflorum* with granular secreted material (asterisk) that has stained for protein with Ponceau 2R. (**e**) Section of labellum with small, individual oil droplets in epidermal and parenchymatous cells of *B. lilacinum* stained with SBB. These may coalesce to form larger globules (arrows) or a peripheral layer within idioblasts. (**f**) Starch in subepidermal cells of *B. lilacinum* following the PAS reaction. Scale bars = 10 μm, 25 μm, 30 μm, 25 μm, 25 μm, 25 μm, respectively. Se = palisade-like secretory epidermis; I = idioblast; Pa = parenchyma; R = raphides.

Ponceau 2R (Fig. 3d), but did not stain for mucilage with ruthenium red, nor for lipids with Sudan III. Moreover, in sectioned material, globules of secretion that accumulated beneath the cuticle stained intensely for proteins with both Ponceau 2R and CBB (Fig. 2h). Treatment with IKI (Fig. 2e) and with PAS revealed the presence of small quantities of starch grains, mainly in subepidermal parenchyma, whereas ruthenium red revealed that mucilage was mainly associated with the cell walls. The cuticle of the palisade-like epidermal cells showed great affinity for Sudan III, and in relatively thick, hand-cut sections, entire palisade-like cells stained intensely following treatment with this reagent (Fig. 2f). The cuticle also stained with SBB, but the secretion present beneath the blistered and detached cuticle remained unstained.

Scanning electron microscopy observations confirmed that the secretion accumulated mainly at the proximal end of the labellum, where the groove was widest (Fig. 4a–b). Epidermal cells lining the groove had a thick and striate cuticle (mean thickness = 1 μm), the striae arranged parallel to the long axis of the labellum (Fig. 4d). Cuticular blisters and small, abundant cuticular pores were observed proximally on the labellum (Fig. 4a–d), but they were absent centrally and distally. Globules of secretion were present proximally in the groove, close to the cuticular blisters, as well as distally (Fig. 4e–f).

The cytoplasm of palisade-like epidermal cells was electron-dense and highly granular, but lacked oil droplets (Fig. 7a–f). Numerous long and short profiles of rough endoplasmic reticulum (RER)

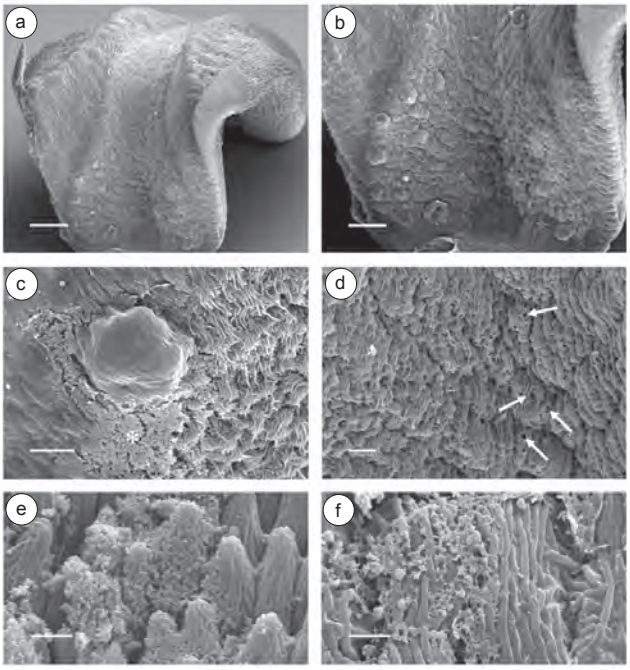

Fig. 4. *Bulbophyllum dissitiflorum*, SEM. (**a**) Adaxial proximal part of labellum with longitudinal median groove. (**b**) Detail of proximal part of labellum with cuticular blisters. (**c**) Cuticular blister surrounded by granular secretion (asterisk). (**d**) Palisade-like cells with pores (arrows) and striate cuticle in proximal part of labellum. (**e–f**) Epidermal cells in median part of labellum coated with granular secretion. Scale bars = 300 μm, 200 μm, 30 μm, 30 μm, 10 μm, 3 μm, respectively.

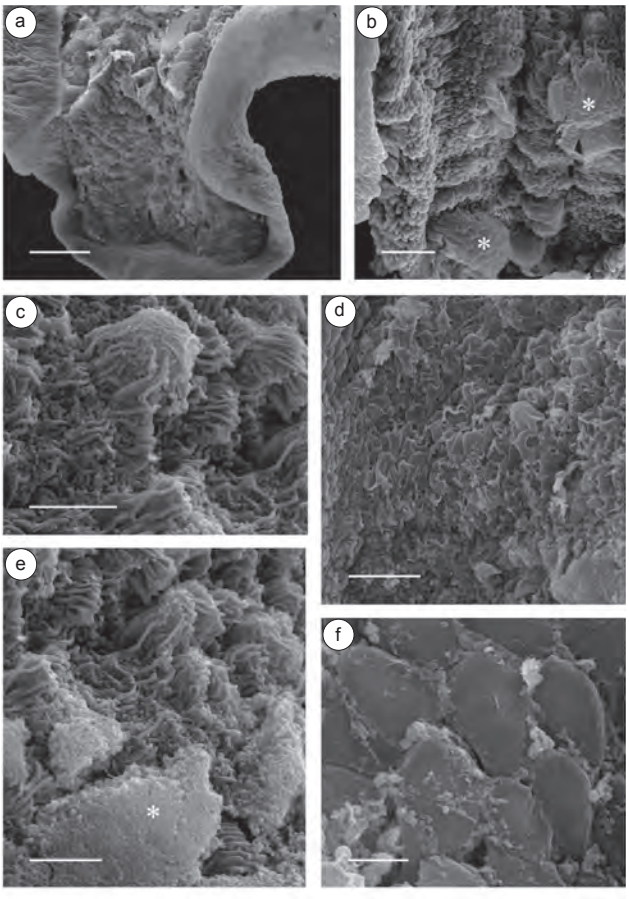

Fig. 5. *Bulbophyllum lilacinum*, SEM. (**a**) Proximal part of labellum showing median longitudinal groove with copious, amorphous secretion and, centrally, at the lower edge of the image, the point of articulation with the column-foot. (**b**) Palisade-like epidermal cells with cuticular blisters (asterisks). (**c**) Palisade-like cells and cuticular blister with striate cuticle. Note the presence of granular secretion between the cuticular striae. (**d**) Epidermal cells coated with granular material. (**e**) Confluent masses of secretory granules (asterisk) coating palisade-like cells. (**f**) Secretory granules at junctions of epidermal cells lacking cuticle. Scale bars = 200 μm, 100 μm, 20 μm, 50 μm, 20 μm, 10 μm, respectively.

and smooth endoplasmic reticulum (SER) were present, but RER predominated and dictyosomes and protein bodies (0.5 μm diameter) were scattered throughout the cytoplasm. Small vesicles gathered in the parietal cytoplasm, frequently within invaginations of the plasmalemma (Fig. 7a, c). They also occurred in the periplasmic space, and on the external surface of cell walls, as a component of the food-reward material (Fig. 7c). This material was visible as large, heterogeneous deposits on the surface of the cells (Fig. 7b). Small, elongate leucoplasts in both palisade-like cells and subepidermal parenchyma contained an electron-dense stroma and few tubular lamellae, and either lacked starch or contained minute starch grains (Fig. 7a, b, d). Small plastoglobuli were only rarely observed. Variously sized vacuoles contained electron-dense, diffuse or globular material. Numerous plasmodesmata were present in the primary pit-fields of cell walls of contiguous, palisade-like epidermal cells and those of the subepidermal parenchyma (Fig. 7f). The cuticle lacked micro-channels.

BULBOPHYLLUM LILACINUM

The racemose inflorescence is pendulous and compact (Fig. 1b). The flower sepals are mainly colored pale buff-pink, but heavily marked with purple, often as interrupted, longitudinal stripes. The petals are white and the labellum is similar in color to the sepals, except for its median adaxial region, which is bright yellow. The column is paler yellow, but again marked with purple (Fig. 1b, d). The stelidia are white and forwardly pointing, and have pointed tips. The flower produces a distinctive fragrance reminiscent of over-ripe fruit. The mobile, linguiform labellum has a wide, median longitudi-

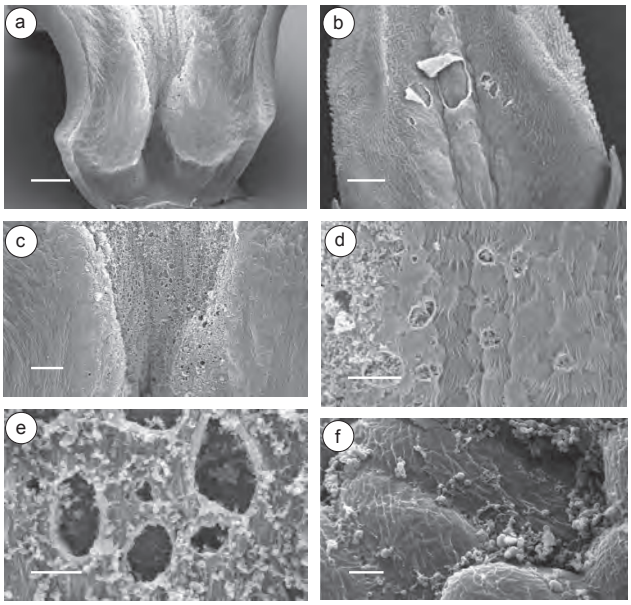

Fig. 6. *Bulbophyllum tricorne*, SEM. (**a**) Adaxial view of proximal part of labellum. (**b**) Median part of labellum showing longitudinal groove or sulcus, the cuticle partly absent. (**c**) Proximal part of labellum showing numerous fine pores in cuticle. (**d**) Pores in cuticle overlying epidermal cells, and subcuticular accumulation of granular secretion. (**e**) Detail of cuticular pores showing secretion upon and beneath cuticle. (**f**) Granular secretion on cell walls of epidermal cells devoid of cuticle. Scale bars = 300 μm, 300 μm, 100 μm, 30 μm, 10 μm, 3 μm, respectively.

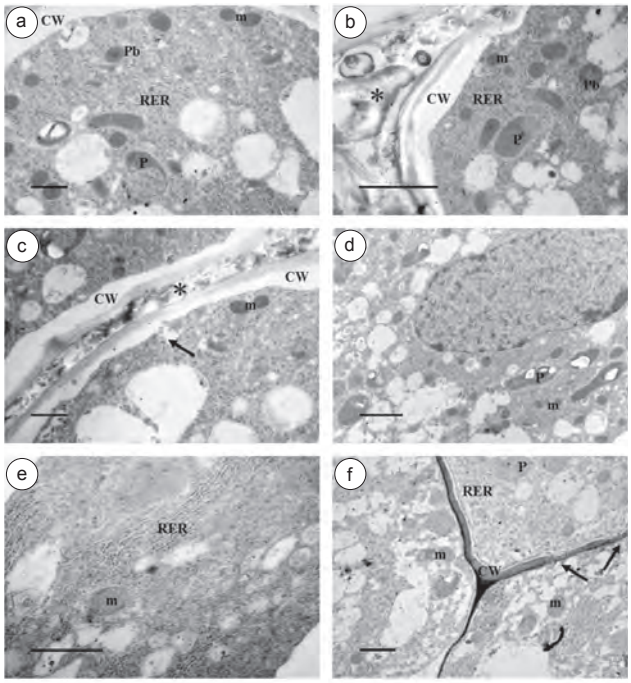

Fig. 7. *Bulbophyllum dissitiflorum*, TEM. (**a**) Section through palisade-like epidermal cell showing parietal, granular cytoplasm with irregularly shaped plastids, mitochondria, protein body and RER profiles. (**b**) Secreted, heterogeneous material (asterisk) deposited between palisade-like cells with parietal cytoplasm containing plastids, RER, protein body and mitochondria. (**c**) Secreted material (asterisk) accumulated between palisade-like cells. Note secretory vesicles in periplasmic space and also the larger cytoplasmic vesicle (arrow) associated with the plasmalemma. (**d**) Large, central nucleus surrounded by plastids, mitochondria and small vacuoles. Small starch grains occur in the plastids. (**e**) Detail of cytoplasm with numerous RER profiles and small vacuoles containing globular or fibrous material. (**f**) Part of palisade-like cell and two adjoining subepidermal cells. Note long RER profiles, and primary pit-fields with plasmodesmata in cell wall (arrows). Scale bars = 2 μm, 2 μm, 2 μm, 2 μm, 1 μm, 2 μm, respectively. CW = cell wall; m = mitochondrion; P = plastid; Pb = protein body; RER = rough endoplasmic reticulum.

nal groove or sulcus flanked by two pronounced, raised ridges (Figs. 1d, f; 2b). Secreted material was located in the proximal part of the median groove (Fig. 2b).

The labellar groove is lined with palisade-like epidermal cells of mean dimensions 61.0 x 17.8 μm. Their thin, outer cellulosic cell walls have a relatively thick cuticle (Figs. 2d; 3b, e–f). These cells contain dense cytoplasm and a prominent nucleus (Fig. 3b). The cytoplasm of the isodiametric, subepidermal parenchyma cells (mean diameter = 32.0 μm) is also dense, with a centrally located nucleus and numerous perinuclear plastids. Three collateral vascular bundles occur in the ground parenchyma, together with scattered idioblasts containing raphides. The latter also occur in the subepidermal parenchyma (Fig. 3b, e, f).

Both the entire labellar groove and its secretion, both upon and beneath the cuticle, stained for proteins with CBB and with Ponceau 2R (Fig. 2b, g). However, the surface secretion neither stained selectively for mucilage with ruthenium red nor for lipids with Sudan III, whereas treatment both with this latter reagent and with SBB revealed the presence of numerous, small, oil droplets in the palisade-like epidermal cells and the subepidermal parenchyma. Larger oil droplets were present in raphide-containing idioblasts and, on occasion, fusion of oil droplets formed a peripheral layer within these cells (Figs. 2d, 3e). Treatment with IKI and PAS showed that starch was present mainly in subepidermal parenchyma, and only small, individual grains were observed in epidermal cells (Fig. 3f). Mucilage was mainly associated with cell walls.

Scanning electron microscopy revealed that the secretion was particularly abundant towards the wide proximal end of the labellar groove, and cuticular blisters resulting from subcuticular accumulation of secretion were also often found here (Fig. 5a–c). The cuticle (mean thickness = 1 μm) was striate, but generally, the striae were neither as evenly, nor as equally distributed as in *B. dissitiflorum*, and were less obvious on the surface of blisters. At the proximal end of the groove (towards the base of the labellum), pores were visible in the cuticle, and the cuticle had partly peeled away exposing the underlying cell walls (Fig. 5d). Secreted material was deposited on the surface of epidermal cells either as individual or as large, confluent masses of granules (Fig. 5c-e). Likewise, examination of palisade-like epidermal cells lacking an intact cuticle revealed that secretion was also present on the smooth outer surface of cell walls, especially at cell wall junctions (Fig. 5f).

Transmission electron microscopy observations showed that the granular cytoplasm of the palisade-like epidermal cells contained abundant mitochondria, together with RER and SER profiles (Fig. 8a–c), and these were particularly numerous in the parietal, apical part of the cells. The parietal cytoplasm also contained dictyosomes and numerous, variously sized vesicles, the latter sometimes occurring in invaginations of the plasmalemma. Of course, these 'invaginations', as elsewhere, may not be invaginations of the plasmalemma at all, but rather, vesicles fusing with the latter and discharging their contents into the periplasmic space. Electron-dense protein bodies (0.5 μm mean diameter) which may contain globoids (Fig. 8b–c) and much smaller (0.05–0.14 μm diameter), individ-

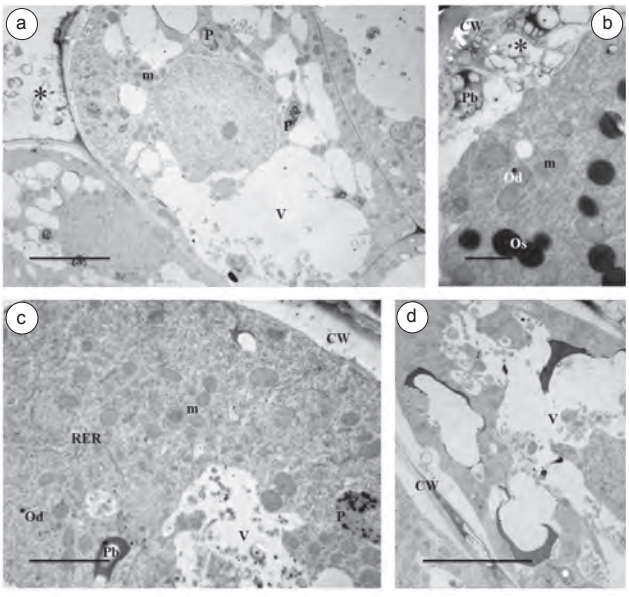

Fig. 8. *Bulbophyllum lilacinum*, TEM. (**a**) Palisade-like cell with centrally located nucleus surrounded by vacuoles of various sizes, mitochondria, and plastids with numerous plastoglobuli. Note granular secreted material on surface of cell wall (asterisk). (**b**) Heterogeneous, granular material and protein body in periplasmic space (asterisk). Mitochondria, spherical osmiophilic inclusions, short profiles of endoplasmic reticulum and vesicles are present in the parietal cytoplasm. (**c**) Short RER profiles, protein body and mitochondria in parietal cytoplasm, the vacuole containing amorphous material. (**d**) Vacuolar profiles of palisade-like cell emphasized by deposition of osmiophilic material on tonoplasts. Note intravacuolar globular material. Scale bars = 5 μm, 2 μm, 1 μm, 5 μm, respectively. CW = cell wall; m = mitochondrion; Od = oil droplet; Os = osmiophilic inclusion; P = plastid; Pb = protein body; RER = rough endoplasmic reticulum; V = vacuole.

ual, osmiophilic, spherical, homogeneous bodies were scattered throughout the cytoplasm, the latter probably representing oil droplets that may coalesce. Other spherical, indeterminate osmiophilic inclusions (as much as 0.85–3.3 μm diameter) were also present (Fig. 8b). Vacuolar contents comprised diffuse, amorphous material and small vesicles (Fig. 8c–d). Intravacuolar osmiophilic material (lipid – see Fig. 3e) was also often associated with, and followed the contours of the tonoplast (Fig. 8d). Palisade-like cells contained plastids (probably chromoplasts based on morphology and the yellow coloration of the labellum) with numerous plastoglobuli (Fig. 8a, c), but starch was only occasionally present in palisade-like cells. Globular, secreted material was visible within the periplasmic space and upon the surface of the cell wall (Fig. 8a–b). Plasmodesmata were present in radial cell walls of palisade-like cells and in tangential walls abutting those of subepidermal parenchyma cells (Fig. 8a). Cuticular micro-channels were seemingly absent.

BULBOPHYLLUM TRICORNE

In this species, the racemose inflorescence is pendulous and lax, with spirally arranged, well-spaced flowers that have a very mildly unpleasant, but non-descript scent. The sepals are golden-brown, striped red, and the petals bright yellow (Fig. 1c). The labellum is very mobile (Fig. 1g), golden-brown centrally along the longitudinal axis, with dark-red vertical walls and incurved margins, and two pronounced red-black keels alongside the median longitudinal groove (Figs. 1c; 2c). The incurved part of the abaxial surface of the labellum is mainly dark-red. The abaxial and adaxial sepal surfaces appeared to glisten, as did the labellar surface, including keels, the labellar groove and the ovary. This was presumably due to the relatively thick floral cuticle (1.5 μm). The stelidia are white, prominent and downwardly curved, and the column is mainly yellowish-white. A prominent dark-red tooth projects from beneath the stigmatic surface. It was not possible to test for lipids, proteins and mucilage by immersing entire fresh flowers in appropriate reagents owing to the dark pigmentation of the flower and thus, the tests were performed on fixed material.

The epidermis lining the adaxial, secretion-filled, median longitudinal groove of the labellum is composed of palisade-like cells of mean dimensions 49.9 x 15.1 μm (Fig. 3c). These contain dense cytoplasm with a centrally located nucleus and several small vacuoles. The subepidermal layer or hypodermis comprises small isodiametric parenchyma cells (mean dimensions = 21.0 μm) and larger idioblasts (mean dimensions = 38.0 x 44.5 μm), the latter containing raphides. The vacuolar contents of idioblasts stained strongly with MB/AII (Fig. 3c) and with the PAS reaction. The ground parenchyma also contained idioblasts with raphides, together with collateral vascular bundles.

Histochemical tests revealed that the secretion present in the median-longitudinal groove of the labellum stained strongly for protein with CBB (Fig. 2c) and Ponceau 2R, but only slightly for mucilage with ruthenium red. The labellar groove and its associated secretion did not, however, stain selectively for lipids with Sudan III. In hand-sectioned material, both the cytoplasm of secretory epidermal cells and the secreted globular material stained for proteins. Sudan stains did not reveal the presence of lipids in the palisade-like epidermal cells, nor in the hypodermal cells. Again, mucilage was associated with cell walls, and starch occurred mainly in subepidermal cells.

Scanning electron microscopy showed the surface of the labellar groove to be relatively glabrous (Fig. 6a–b). Cuticular distension was present only at the central region of the groove, and here, the cuticle had peeled away to reveal the outer walls of the epidermis (Fig. 6b). Most of the centrally located epidermal cells in the groove had a finely striate cuticle, whereas the cuticle of the outermost cells had more strongly defined striae (Fig. 6d). The cuticle of the palisade-like cells at the central part of the groove contained numerous pores of variable size (5–30 μm), and these were coated with globules of secreted material (Fig. 6c–e). Secretion was also visible on the exposed surface of parts of the outer cell walls of palisade-like cells that lacked cuticle (Fig. 6f).

Transmission electron microscopy observations revealed the presence of cuticular micro-channels. Numerous RER and SER profiles, together with mitochondria and dictyosomes were observed in the cytoplasm (Fig. 9a–f). Multivesicular bodies were present parietally (Fig. 9b), as well as ovoid plastids (chromoplasts) containing numerous plastoglobuli and/or starch grains. Both relatively large (0.5–2 μm) and small (0.28–0.40 μm), osmiophilic bodies (Fig. 9a, c, d) also occurred in the cytoplasm, often in close proximity to plastids and connected to ER sacs (Fig. 9c). Whereas the larger bodies were ovoid, and often heterogeneous, the smaller bodies were spherical and homogeneous, and since some of the former appeared to contain globoids (Fig. 9d), they were interpreted as protein bodies, the smaller bodies probably representing oil droplets. These oil droplets were on occasion observed to coalesce. Indeterminate, osmiophilic inclusions (as much as 1.8 μm diameter) were also present (Fig. 9c). Again, intravacuolar vesicles and diffuse material were present (Fig. 9b). The secreted material present upon the surface of palisade-like cells was heterogeneous.

To summarize, the fragrance-secreting tissues of some *Bulbophyllum* spp. (including those of sect. *Racemosae*) cannot easily be distinguished based on micromorphology alone. Furthermore, identification of such tissues by histochemistry is frustrated in that both fragrance terpenoids and lipid-rich food-rewards (both secreted by epidermal cells) have an affinity for lipophilic stains, and interpretation of TEM images can prove difficult since both lipid-rich food-rewards and fragrances are likely to be synthesized by a similar complement of organelles. Nevertheless, the food-reward of all three species investigated here, unlike that of most *Bulbophyllum* spp. studied to date, was proteinaceous and seemingly lacked lipid. This enabled the use of these taxa as suitable models for investigating fragrance-secreting tissues. In each case, the palisade-like epidermal cells contained plastids with plastoglobuli and starch grains, as well as protein bodies (large, heterogeneous, often with globoids). The plastids of non-fragrant *B. dissitiflorum*, however, were small, scant, elongate or irregular in shape and contained few plastoglobuli and starch grains, whereas those of fragrant *B. lilacinum* and *B. tricorne* were larger, more abundant, ovoid and contained numerous plastoglobuli and generally larger, more abundant starch grains. Similar plastids were present also in sub-epidermal cells. Only the palisade-like cells of fragrant spe-

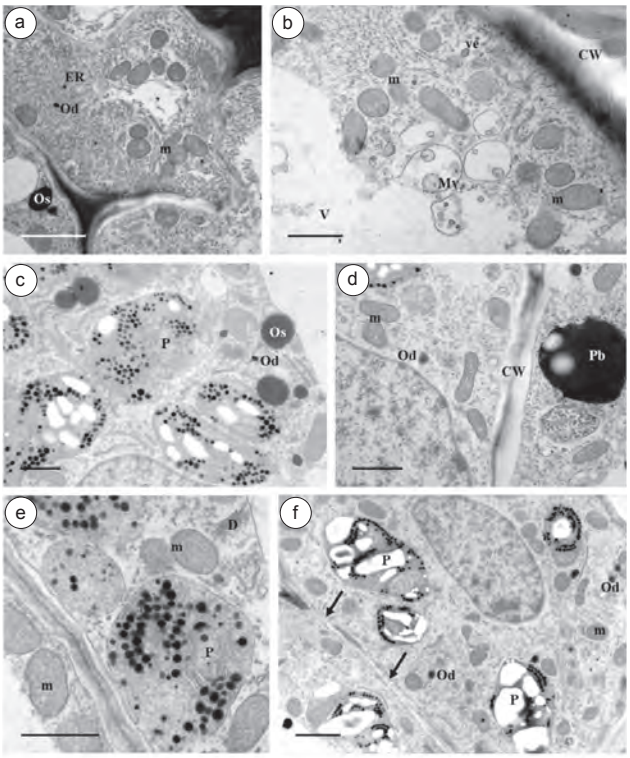

Fig. 9. *Bulbophyllum tricorne*, TEM. (**a**) Electron-dense cytoplasm of palisade-like cell with numerous profiles of endoplasmic reticulum and mitochondria. (**b**) Vesicles, multivesicular bodies, numerous mitochondria and ER profiles in parietal cytoplasm. (**c**) Plastids containing numerous plastoglobuli and starch grains. Osmiophilic inclusions and oil droplets present in cytoplasm. (**d**) Electron-dense protein body containing globoids, also granular cytoplasm with peripherally located mitochondria and oil droplets. (**e**) Plastids with dense stroma and numerous plastoglobuli. Cytoplasm with numerous vesicles, mitochondria and dictyosomes. (**f**) Cytoplasm of palisade-like cell showing nucleus, mitochondria, profiles of ER, vesicles, oil droplets, and plastids containing starch and plastoglobuli. Note plasmodesmata (arrows) connecting contiguous cells. Scale bars = 2 μm, 1 μm, 2 μm, 1 μm, 1 μm, 2 μm, respectively. CW = cell wall; D = dictyosome; ER = endoplasmic reticulum; m = mitochondrion; Mv = multivesicular body; Od = oil droplet; Os = osmiophilic inclusion; P = plastid; Pb = protein body; V = vacuole; ve = vesicle.

cies contained oil droplets (individual oil droplets are small and homogeneous) and indeterminate osmiophilic inclusions. Cuticular pores and, on occasion, cuticular micro-channels were present. On this basis, the palisade-like cells of the two last species, possibly in conjunction with sub-epidermal cells, are considered to function in the production of fragrance.

DISCUSSION

LABELLUM ANATOMY

To date, very few anatomical studies of the labellum have been conducted on *Bulbophyllum* (de Pádua Teixeira et al., 2004; Nunes et al., 2014, 2015), and there have been even fewer investigations of its ultrastructure and secretions (Davies and Stpiczyńska, 2014; Kowalkowska et al., 2015; Stpiczyńska et al., 2015). The labellar morphology of the representative members of sect. *Racemosae* that form the subject of the present study (*Bulbophyllum dissitiflorum*, *B. lilacinum* and *B. tricorne*) and that of previously investigated members of the section (*Bulbophyllum careyanum*, *B. morphologorum*, *B. orientale* and *B. wangkaense* – Davies and Stpiczyńska, 2014) is very similar, the labellum being linguiform in each case, with an adaxial median longitudinal sulcus containing verrucae, and the internal anatomy being more or less identical. Similar labellar morphology also occurs in Neotropical and certain African members of the genus (de Pádua Teixeira et al., 2004; Nunes et al., 2014, 2015; Stpiczyńska et al., 2015). Based on the little information we currently have for *Bulbophyllum*, the labellum, as viewed in transverse section, generally comprises an epidermis, a subepidermis (or hypodermis) of small, isodiametric, starch-laden parenchymatous cells, and central ground parenchyma (referred to as mesophyll by Nunes et al., 2014, 2015) containing collateral vascular bundles and idioblasts with bundles of raphides. The median, longitudinal groove is lined with palisade-like epidermal secretory cells. Accumulation of secreted food-rewards on the outer tangential wall of the epidermal cells results in distention and blistering of the often highly striate cuticle and their eventual discharge, forming masses or confluent sheets of granules on the surface of the labellum (Davies and Stpiczyńska, 2014). In some species there is evidence of cuticular pores and cracks, and occasionally, microchannels. Thus, in many respects, the labellar morphology and anatomy of species of Asian *Bulbophyllum* sect. *Racemosae* studied to date (Davies and Stpiczyńska, 2014) resemble those of certain Neotropical and African members of the genus (de Pádua Teixeira et al., 2004; Nunes et al., 2014, 2015; Stpiczyńska et al., 2015). Indeed, de Pádua Teixeira et al. (2004) have commented on the highly conservative character of the labellum in *Bulbophyllum*.

FOOD-REWARDS AND THEIR SECRETION

Contrary to expectation, the food-rewards of representatives of sect. *Racemosae*, unlike those of many other *Bulbophyllum* species examined to date, including Neotropical and African species (e.g., de Pádua Teixeira et al., 2004; Stpiczyńska et al., 2015), consisted mainly of protein and tested negatively for lipids (Davies and Stpiczyńska, 2014 and the present paper). Indeed, Davies and Stpiczyńska (2014) state that generally, "hardly any intracellular lipid droplets were observed" in the secretory epidermal cells of *B. careyanum*, *B. morphologorum*, *B. orientale* and *B. wangkaense* (sect. *Racemosae*). By contrast, Nunes et al. (2015) also recorded the presence of protein in the secretory epidermal cells of the Neotropical sect. *Napelli*. Rchb.f., but unlike sect. *Racemosae*, small droplets of lipid were present here in the ground cytoplasm (mesophyll). In their earlier work on the Neotropical sect. *Didactyle* (Lindl.) Cogn. (Nunes et al., 2014), these same authors tabulated that proteins were present in the secretory epidermis and that lipids occurred as small droplets in the mesophyll (ground parenchyma). They also stated that epidermal cells of the callus of *B. popayanense* Kraenzl. contain lipid and later recorded in the same report that the epidermis and three to five subepidermal secretory layers of *B. popayanense*, *B. weddellii* (Lindl.) Rchb.f., *B. involutum* Borba, Semir & F. Barros, *B. exaltatum* Lindl., *B. meridense* Rchb.f., *B. tripetalum* Lindl. and *B. perii* Schltr. (names all inferred from their Fig. 4) contain elevated levels of cytoplasmic protein. De Pádua Teixeira et al. (2004), however, reported that "in *B. epiphytum* Barb. Rodr., *B. glutinosum* (Barb. Rodr.) Cogn. and *B. regnellii* Rchb.f., the papillose epidermal cells had … many lipid droplets" and that the cavity (groove or sulcus) cells of *B. involutum*, *B. ipanemense* Hoehne and *B. weddellii* stained with osmium tetroxide and Sudan Black B, indicating the presence of lipid material. The last authors, however, did not test for protein. Therefore, although the food-rewards of all *Bulbophyllum* spp. recorded to date contain elevated levels of protein, it would appear that sect. *Racemosae* is atypical in that its food-rewards seemingly lack lipid.

Contrary to previously investigated species of sect. *Racemosae*, where mucilage provided a vehicle for the secretion of protein onto the surface of the labellum, this material was not detected (except

for minute quantities in *B. tricorne*) on the labellar surface of the species studied here, nor was it reported for those Neotropical and African species of *Bulbophyllum* examined to date (Nunes et al., 2014, 2015; Stpiczyńska et al., 2015). In the present study, however, despite its general absence from the surface secretion, mucilage was detected in walls of the secretory cells of all species. We speculate that mucilage, when present in the surface secretion, is produced by dictyosomes and, once secreted onto the labellar surface, although not nutritious in itself, may absorb atmospheric moisture, swell and glisten, causing the floral reward to appear more obvious and more attractive to pollinators, or that it might cause the secretion to adhere more tightly to the labellar surface or delay evaporation, thus increasing the time that the food-reward is available to pollinators.

PROTEIN BODIES VERSUS OIL DROPLETS

It was sometimes difficult to distinguish between protein bodies and oil droplets in these species under TEM, and when it was not possible to do so with any degree of certainty, they were referred to as 'osmiophilic inclusions', owing to their great affinity for osmium tetroxide. These measured as much as 0.85–3.30 μm in diameter.

Protein bodies are usually formed inside vacuoles (e.g., Herman and Larkins, 1999; Bassham, 2002; Jiang and Rogers, 2001) and vary greatly in size and morphology depending on a range of variables, including the species in question, the organ investigated and the degree of maturation. Consequently, they cannot easily be described, as a quick look through the literature will clearly demonstrate. In the species investigated here, they were often ovoid, heterogeneous, occasionally having an electron-translucent centre and a more electron-dense periphery, or *vice versa*. Electron-transparent globoids were sometimes present and, rarely, it was possible to see that protein bodies were bound by an electron-dense membrane, as observed for the endosperm of *Zea mays* L. (Reyes et al., 2011) and for *Chenopodium quinoa* Willd. (Burrieza et al., 2014). In our studies, protein bodies ranged from 0.5–2.0 μm in diameter. Conversely, individual oil droplets were usually much smaller (0.05–0.4 μm diameter), spherical, entirely homogeneous and lacked a bounding membrane, but were frequently seen to coalesce.

FRAGRANCE-SECRETING CELLS

There is no clear distinction on morphological grounds between typical palisade-like epidermal cells and labellar fragrance-secreting cells in representatives of sect. *Racemosae*. Gross testing and histochemical investigation of tissue sections for lipid mainly proved negative or weak. This perhaps can be attributed to a lack of sufficient quantities of these compounds in the secretion to give a positive reaction, or alternatively, that oil droplets visible using TEM represent volatile constituents of fragrances that evaporate readily from the labellum surface or are leached from sections by organic solvents during tissue processing for light microscopy. Our current knowledge of the chemical composition of these substances is summarized in Pridgeon et al. (2014).

The occurrence of oil droplets and numerous large, ovoid plastids containing many plastoglobuli and several starch grains in the cytoplasm of *B. lilacinum* and *B. tricorne* (fragrant flowers), and the absence of oil droplets and the presence of relatively few, smaller, elongate or irregular plastids containing few plastoglobuli and occasional starch grains in *B. dissitiflorum* (non-fragrant), would indicate the involvement of oil droplets and plastids in scent production. This hypothesis is further supported by our previous studies on sect. *Racemosae*, where all four species investigated produced fragrances (sometimes malodorous), and in each case, the epidermal cells contained well developed plastids (Davies and Stpiczyńska, 2014). Similar plastids also occur in the osmophores of *Stanhopea* J. Frost ex Hook. (Curry et al., 1991; Antoń et al., 2012), *Cycnoches chlorochilon* Klotzsch (Antoń et al., 2012) and various pleurothallid orchids (Pridgeon and Stern, 1983, 1985), including *Acianthera prolifera* (Herb. ex Lindl.) Pridgeon & M.W. Chase, a myophilous species that produces nitrogenated volatile compounds (Melo et al., 2010).

Small oil droplets that stained with SBB, as described for *B. lilacinum*, also occurred both in osmophore cells of the petal appendages and the secretory cells lining the labellar groove of *B. wendlandianum* Kraenzl. Dammer (Kowalkowska et al., 2015), currently assigned to sect. *Cirrhopetaloides* Garay, Hamer & Siegerist. Other features indicating the possible role of these epidermal cells in fragrance production in sect. *Racemosae* were cuticular pores similar to those described for the osmophores of *Restrepia* Kunth (Pleurothallidinae; Pridgeon and Stern, 1983), and micro-channels as found in the osmophores of *Stanhopea* (Curry et al., 1991) and *Chloraea membranacea* Lindl. (Chloraeinae; Sanguinetti et al., 2012). In *B. lilacinum*, a layer of osmiophilic material lined the tonoplast, as occurs both in the osmophore vacuoles of *Restrepia* and the vacuolate plastids of *Scaphosepalum* Pfitzer (Pridgeon and Stern, 1983, 1985). This material consists primarily of lipid as, in *B. lilacinum*, it stained strongly with SBB.

CONCLUSION

In all species of sect. *Racemosae* investigated to date, proteinaceous food-rewards that seemingly lack lipid are produced and, as would be expected, abundant arrays of RER predominate and protein bodies are present, the secretory epidermal cells stain strongly for protein. Since there is no clear morphological distinction between these cells and fragrance-producing cells, in the absence of lipid-rich food-rewards, lipophilic staining are useful in recognizing cells involved in scent production in these species.

Differences in plastid structure may reflect differences in metabolic processes. For example, the plastids of non-fragrant *B. dissitiflorum* were relatively scant, elongate or irregular in shape and contained few plastoglobuli, the starch grains being infrequent and small. By contrast, the secretory cells of fragrant *B. lilacinum* and *B. tricorne* contained larger, ovoid plastids with numerous plastoglobuli and larger starch grains, and SER was relatively abundant possibly indicating a greater capacity for the production of volatile oils (terpenoids). Plastids similar to those recorded here, as well as being present in the osmophores of fragrant orchids (e.g., Pridgeon and Stern, 1983, 1985; Curry et al., 1991), are also known to occur in certain members of sub-tribes Maxillariinae (Davies et al., 2003; Davies and Stpiczyńska, 2009) and Oncidiinae (Davies et al., 2014), where they are considered to be involved in the production of lipid-rich food-rewards; this material, once secreted onto the labellum, is gathered by insect pollinators (Braga, 1977; Flach et al., 2004) and possibly fed to their larvae. However, since elevated concentrations of lipid were not detected in the food-rewards of any of the subjects of the present paper, we propose that here, oil droplets probably exclusively represent droplets of fragrance. Starch was reported for the subepidermal parenchyma of all investigated species, and hydrolysis of subepidermal starch reserves in conjunction with mitochondrial activity is thought to provide the metabolic energy required for both food-reward and fragrance production, and their respective secretory processes (Vogel, 1990; Nepi, 2007). Moreover, the abundance of small vesicles alongside the plasmalemma of secretory cells would indicate subsequent vesicle-mediated transport of secretory products to the point of secretion.

Thus, it would appear that the palisade-like labellar epidermal cells of members of sect. *Racemosae* play a dual role in the attraction of potential pollinators, both that of fragrance secretion (as evidenced by an affinity for Sudan stains, large plastogobuli-laden plastids, oil droplets and abundant SER) and the secretion of proteinaceous food-rewards (as evidenced by histochemistry, protein bodies and numerous RER profiles). Since, however, the majority of *Bulbophyllum* spp. studied to date produce lipid-rich food-rewards, it is possible that unlike sect. *Racemosae*, their plastids and other organelles involved in lipid metabolism are implicated both in the production of food-rewards and that of fragrances. This indicates that the secretory epidermal cells of *Bulbophyllum* retain a degree of plasticity at maturity and may have a dual role (possibly multiple roles) in secretion, as was proposed by Davies and Turner (2004) for *Maxillaria* Ruiz & Pav.

AUTHORS' CONTRIBUTIONS

Both authors contributed to the conception and design of this investigation, the acquisition, analysis and interpretation of data. The authors confirm that they have no conflict of interest.

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