Journal of Sustainability Science and Management
Volume 16 Number 5, July 2021: 22-34
eISSN: 2672-7226

TAXONOMIC AND PHYSIOECOLOGICAL SIGNIFICANCE OF THE FLORAL-SURFACE MICROMORPHOLOGY OF *VANDA HELVOLA* AND *VANDA DEAREI* (ORCHIDACEAE)

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Submitted final draft: 14 August 2021 *Accepted: 23 September 2020* http://doi.org/10.46754/jssm.2021.07.002

Abstract: The *Vanda* orchid is appreciated for its large, beautiful, scented flowers. Anatomical enumeration of the most compatible *Vanda* species holds interesting and under-investigated aspects. We conducted an in-depth investigation into the floral-surface micromorphology of *V. helvola* and *V. dearei*, and assessed the significance in taxonomic delimitation using scanning electron microscopy (SEM) to feasibly answer questions on the reported dubious origins of certain *Vanda* species and hybrids. Floral trichomes, including papillae structures, stomata, epicuticular ornamentation and waxes, were enumerated and compared between the selected species. Findings based on the photomicrographs revealed that *V. helvola* and *V. dearei* were comparable based on the diversity and dissemination of trichomes, stomata and epicuticular ornamentation. Noteworthy was the heterogeneous occurrence of distinguishing barrel-shaped glandular trichomes with aperture, which might also function as nectaries, and branched glandular and biseriate trichomes on the labellum of *V. helvola*, but was absent in *V. dearei*. In contrast, an aggregation of conical papillae occurred on the spur of *V. dearei*, but lacking in *V. helvola*. We also employed SEM observations to understand the role of surface microstructures in physio-ecological functions. The glandular trichomes and stomata were presumed to be secretory structures for the unique scents of *V. helvola* and *V. dearei*.

Keywords: Ecological demands, epicuticular, floral anatomy, Malaysia, species delimitation, vandaceaous orchids.

Introduction

The genus *Vanda* R.Br. of the Orchidaceae family comprises 73 species that may be found from the Himalayas to South China, across Sri Lanka to Southeast Asia, and Papua New Guinea to Northern Australia (Gardiner & Cribb, 2013; Govaerts *et al*., 2020). Many species are considered endemic in southeast Asian archipelagos (Gardiner *et al*., 2013). The generic name "Vanda" originates from Sanskrit, and was proposed by Williams Jones in 1975, which was then used to describe the type species *Vanda tessellata* (Roxb.) Hook. *ex* G.Don (Hodgson *et al*., 1991). Vandas are previously divided into two groups appertaining to their vegetative structures — the strap-leaved *V. tricolor* Lindl. and the terete-leaved *V. teres* (Roxb.) Lindl. [= *Papilionanthe teres* (Roxb.) Schltr.] and *V. hookeriana* Rchb.f. [= *Papilionanthe hookeriana* (Rchb.f.) Schltr.] (Lim *et al*., 1999).

Morphologically, the *V. tricolor* group has strap-like leaves, besides being distichous, stem-clasping, keeled, green above and light beneath with the apex erose. Meanwhile, the *V. teres* group differs by having terete leaves as thick as a standard pencil, with the basal parts surrounding the stem (Hodgson *et al*., 1991). The former group has been declassified from *Vanda* and placed into a separate genus, the Papilionanthe Schltr. The inflorescence of *Vanda* orchids is characterised as erect and unbranched, culminating in few to many colourful large flowers, but mostly yellow-brown with markings to attract carpenter bees (Teoh, 1980). All species have a stout column, saccate spur and three-lobed labellum (Pridgeon, 1999). Sepals and petals are usually equal in size and round.

However, classification of *Vanda* has been described as a "taxonomic black hole" requiring "a complete revision" (Christenson, 1987; Gardiner, 2012). *Vanda* species exhibit

remarkably low genetic divergence, in spite of the vivid morphological variations seen across the genus and its broad geographical range. For instance, *V. helvola* occurs in Borneo and Peninsular Malaysia, whereas *V. dearei* from the same section, sect. Deltaglossa, occurs exclusively in Borneo (Gardiner *et al*., 2013). In Malaysia, *V. helvola* and *V. dearei* are the most utilised species for hybridisation and closely related with almost similar vegetative structures. *V. helvola* is the most widespread species in the genus, which is commonly found in the montane and riverine forests of Malaysia. A special feature of this species is its large flat, triangular deltoid and yellow labellum (Seidenfaden & Wood, 1992). *V. dearei* is a strap-leaved orchid which can be found at lowlands, hills and riverine forests. It is special for its large, strongly fragrant and pure yellow flowers devoid of spots that bloom frequently (Chan *et al*., 1994). These morphological characters make ranks it among the most preferred and ecstatic orchid species, and extensively used as a progenitor of many merited hybrids (Loh *et al*., 1978). However, the species itself is rare and threatened in the wild due to over collection.

Profiling work to detail the plant's morphology, phylogeny, floral microstructure and cytology in distribution population size, and micropropagation of the *Vanda* species are now deemed useful. With a genus as horticulturally important as *Vanda*, the potential for natural hybridisation and introgression between cultivated specimens is high, especially if the morphological differences between species are not well understood (Gardiner *et al*., 2013). The floral-surface microstructure of the flower part can provide valuable taxonomic and systematic evidence to discriminate between orchids with dubious genetic origins. This study represents the first comprehensive survey of floral-surface micromorphological characteristics of *V. helvola* and *V. dearei* via scanning electron microscopy (SEM) observations to assist in an identification of the mother parents of their hybrids, and to understand its physio-ecological significance in relation to scent production and pollination mechanism.

Materials and Methods

Samples of florets for each species were collected from living plants during a convenience sampling and assigned a voucher number as stated in Table 1. Collected specimens were processed according to the technique outlined in Bridson & Forman (2000) and deposited in the Herbarium of Universiti Putra Malaysia (UPM) in Serdang, Selangor. The flowers (Figure 1) were dissected and identified based on the illustrations and descriptions of Seidenfaden & Wood (1992) and Chan *et al*. (1994). Accepted names and current distribution were validated through the KEW World Checklist of Selected Plant Families (WCSP) (Govaerts *et al*., 2020).

Floral-surface micromorphology examination was conducted using SEM in the Anatomy Lab and Scanning Electroscope Room in the Faculty of Agriculture, UPM. The Copenhagen-preserved flower parts were cut into a number of 1 cm slices, put into separate vials and fixed in formalin acetic acid. Excised segments were then post-fixed in 1 % osmium tetroxide overnight. Afterwards, the experiment was continued with the dehydration process. Samples were passed through ethanol in a series of concentrations; 50 %, 75 %, 90 % 95 %, and 100 %, each for 30 minutes, and the final step was repeated in 100 % ethanol for another 30 minutes. The samples were then transferred into specimen baskets and subjected to critical point drying using a liquid CO_2 critical dryer for about 70 minutes.

Table 1: *Vanda* species examined including their locality, habitat and voucher

Species	Type Locality	Habitat	Voucher Deposited	Collector's name
V. helvola	Pahang	Hill forest	SC001	Rusea Go
V. dearei	Sabah	Hill forest	SC002	Rusea Go

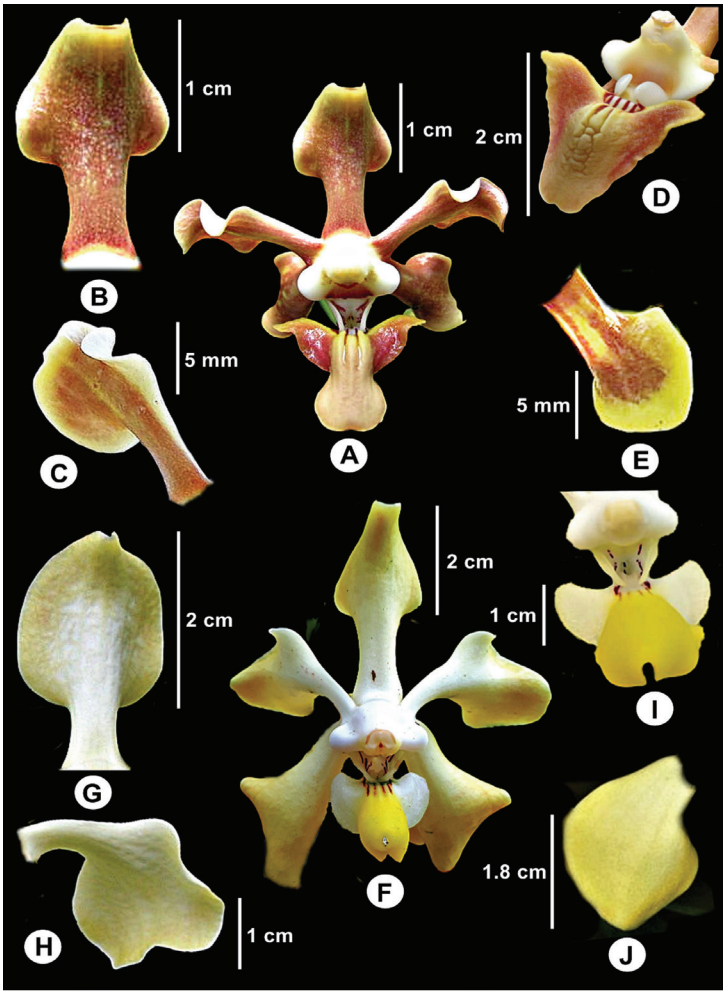

Figure 1: *Vanda helvola* (A-E); A: Flower, B: Dorsal sepal, C: Petal, D: Labellum and column, E: Lateral sepal. *Vanda dearei* (F-J); F: Flower, G: Dorsal sepal, H: Petal, I: Labellum, J: Lateral sepal

Dried samples were mounted onto the stub by means of double-sided carbon adhesive tabs and were gold-coated in sputter coater. Samples viewing were done under a JEOL-JSM 5610 LV scanning electron microscope (Jeol Ltd, Tokyo, Japan). The floral-surface microstructures of the anther, column, dorsal sepal, lateral sepals, petals, labellum, pollinium and spur were observed at various magnifications. The microstructures observed were trichomes, including papillary structures, stomata, epicuticular ornamentation and waxes. Stomatal architecture was described by referring to an illustration and terminologies of basal angiosperms stomata in Carpenter (2005) based on the number, form and arrangement of specialised epidermal cells associated with stomatal cells. Comprehensive terminologies of trichome morphology were described according to Adedeji *et al*. (2007) and Angulo & Dematteis (2014). Enumeration of trichomes required four steps: (1) examination of the overall surface appearance (indumentum); (2) investigation of the morphology of individual trichomes; (3) study of the trichome complement; and, (4) histological description of trichomes (glandular or non-glandular, unicellular or multicellular, uniseriate or biseriate) based on the clear partitioning of cells on the well-developed

trichomes.

Parameter measurements were done using a ruler under a clear magnification, and the values obtained were multiplied with the magnification scales. For the individual stomatal parameters, measurement methods in Savvides *et al*. (2011) and Shope & Mott (2006) were employed in the current study. Epicuticular ornamentation was described following Piwowarczyk (2015), Ghimire *et al*. (2018) and Kong & Hong (2018), and description of epicuticular waxes was based on Wilkinson (1979). The micromorphological features were comparatively described following outline in Ghazalli *et al*. (2019).

Results

Floral parts could be homogenous that consisted of the same type of microstructures. or heterogeneous with different microstructures, and some parts might possess unique type(s) of epicuticular sculpture. Each species was enumerated based on epicuticular ornamentation, stomata type, stomata formation, stomata frequency, stomata ornamentation, stomata size, trichome distribution and trichome type. The micromorphological descriptions of trichomes, and epicuticular ornamentation were described in Tables 2 and 3.

Table 2: Type and morphology of simple, branched, uniseriate and multiseriate trichomes on the floral parts of *V. helvola* and *V. dearei*

Type	Morphology description
I	glandular, multicellular stalk, unicellular head, short (< 30 µm), capitate
II	glandular, short (< 300 µm), barrel-shaped glandular trichomes with aperture
III	glandular, biseriate, multicellular head, long (300-1000 µm), pedunculate
IV	glandular, biseriate, multicellular head, short (< 300 µm), club-shaped
V	glandular, biseriate, branched, Y-shaped, long (300-1000 µm)
VI	glandular trichomes with single secretory cell at the tip, short (> 300 µm), multicellular stalk, unicellular head
VII	non-glandular, unicellular, short (< 300 µm), flattened, strap-shaped
Papillae	globular or tall, striated

Table 3: Type and morphology of epicuticular ornamentation on the floral parts of *V. helvola* and *V. dearei*

Type	Morphology Description
I	laevigate and polyhedral-striated outer periclinal wall; furrowed, straight and rounded anticlinal wall
II	foveate outer periclinal wall; fibrillary, undulate and sinuate anticlinal wall
III	flat outer periclinal wall; furrowed, straight and rounded anticlinal wall
IV	laevigate outer and polygonal periclinal wall; furrowed, straight and rounded anticlinal wall
V	laevigate, elongated and rectangular outer periclinal wall; furrowed, straight and rounded anticlinal wall
VI	microfossulate outer periclinal wall; fibrillary and undulate anticlinal wall
VII	laevigate and irregular shape with undulate striae outer periclinal wall; furrowed, straight and rounded anticlinal wall
VIII	laevigate and elongated rectangular with undulate striae outer periclinal wall; furrowed, straight and rounded anticlinal wall
IX	laevigate and elongated rectangular outer periclinal wall; furrowed, straight and rounded anticlinal wall

Species Enumeration and Comparative Study under SEM Examination

a. *V. helvola* Blume (Figure 2) — *Waxes:* scattered, warty-granulated, flake-like and cylindric. *Epicuticular ornamentation:* Type I, II, III, IV, V, VI and IX. *Stomata type:* Actinocytic and tetracytic. *Stomata formation:* paraficial, in parallel or random formation, sunken in the epidermal wall. *Stomata frequency and distribution:* sparsely occurred on anther and column. *Stomata ornamentation:* tetracytic — guard cells surrounded by four subsidiary cells with two laterals and two polars, each being present on the four sides, two laterals cells lie parallel to guard cells; actinocytic — raised above epidermal layer with ledge cells surrounded by a circle of at least five radially elongated subsidiary cells. Guard cells and ledges distinguishable from the neighbouring stomatal apparatus. *Stomatal cuticular striation:* striated. *Stomatal size:* tetracytic — L (38.46-125 *μm*) × W (35.39-83.33 *μm*); actinocytic — L (40.91-86.36 μm) × W (56.82-90.91 μm). *Trichome distribution:* present on column, labellum and spur. Labellum possessed barrel-shaped papillae or nectaries with aperture on its mid-lobe. Side lobes bear heterogeneous hairs with unbranched and branched glandular trichomes. On the middle abaxial surface, the spur bears homogenous conical papillae. *Trichome type:* glandular — Type I, II, III, V and VI. *Pustular glands:* present. *Papillae:* present.

b. *V. dearei* Rchb.f. (Figure 3) — *Waxes:* scattered, warty-granulated, flake-like and cylindric. *Epicuticular ornamentation:* Type I, II, IV, VIII and IX. *Stomata type:* Stephanocytic. *Stomata formation:* paraficial, in parallel or random formation, semi-raised on the epidermal surfaces or sunken in. *Stomata frequency and distribution:* sparsely occurred on anther and column. *Stomata ornamentation:* comprise a more or less well-defined rosette of four or more weakly specialised subsidiary cells. Guard cells and ledges distinguishable from the neighbouring stomatal apparatus. *Stomatal cuticular striation:* striated. *Stomatal size:* L (54.55-116.67 *μm*) × W (50-138.89 *μm*). *Trichome distribution:* present on column, labellum and spur. Spur had dense aggregation of conical papillae as nectaries at interior side wall and homogenous trichome type at the centre. *Trichome type:* non-glandular — Type VII; glandular — Type I, III and IV. *Pustular glands:* present. *Papillae:* present.

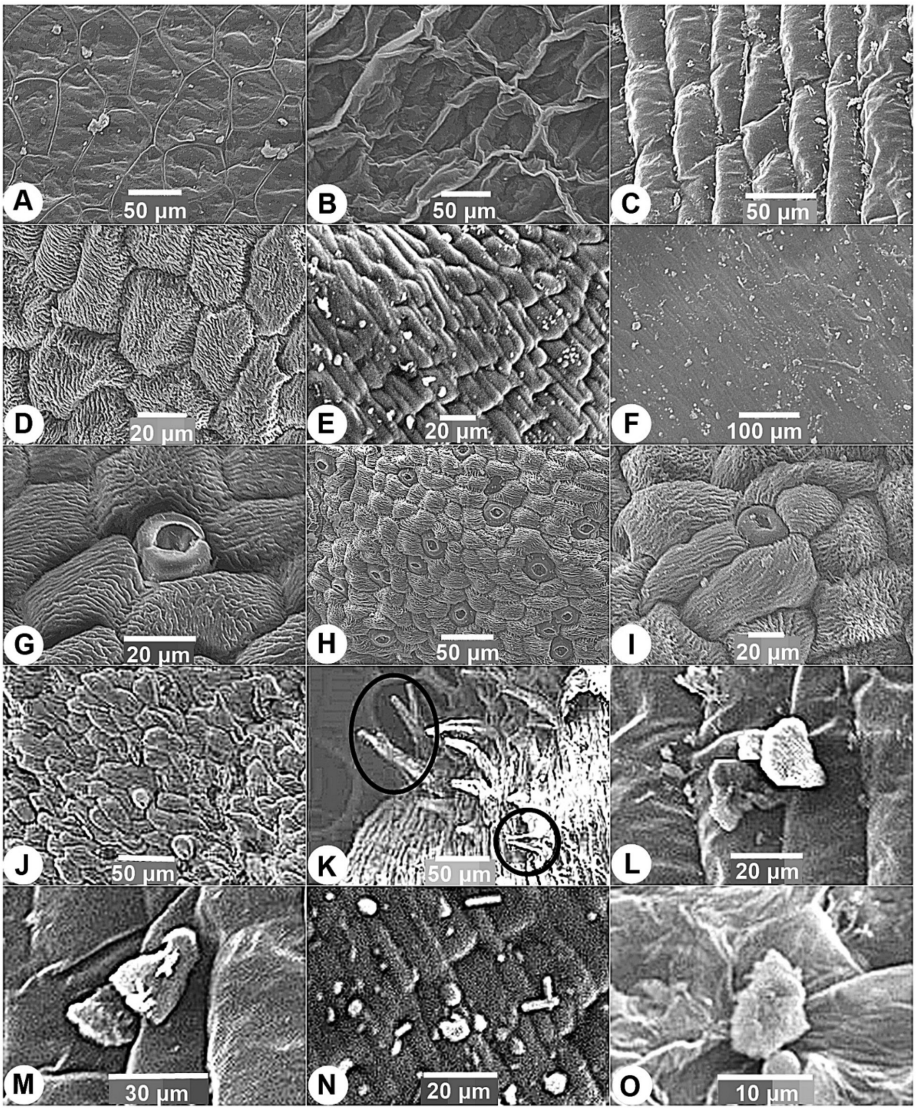

Figure 2: SEM observations of epicuticular ornamentation (A-F), stomata (G-I), trichomes (J&K) and epicuticular waxes (L-O) on floral parts of *V. helvola*; A: Type III epicuticular ornamentation with flat anticlinal wall; B: Type II foveate epicuticular ornamentation; C: Type V laevigate epicuticular ornamentation; D: Type I epicuticular ornamentation with laevigate and polygehdral-striated anticlinal wall; E: Type IX epicuticular ornamentation with laevigate and elongated rectangular outer periclinal wall; F: Type IX epicuticular ornamentation with unclear periclinal and anticlinal walls; G: Tetracyctic stoma; H: Actinocytic stomata; I: Actinocytic stoma; J: Type II barrel-shaped glandular trichomes with aperture; K: Type V branched and Type VII glandular trichomes (circled); L: Warty-granulated waxes; M: Warty-granulated waxes; N: prismatic crystals intersperse on the surface; and, O: Warty-granulated waxes.

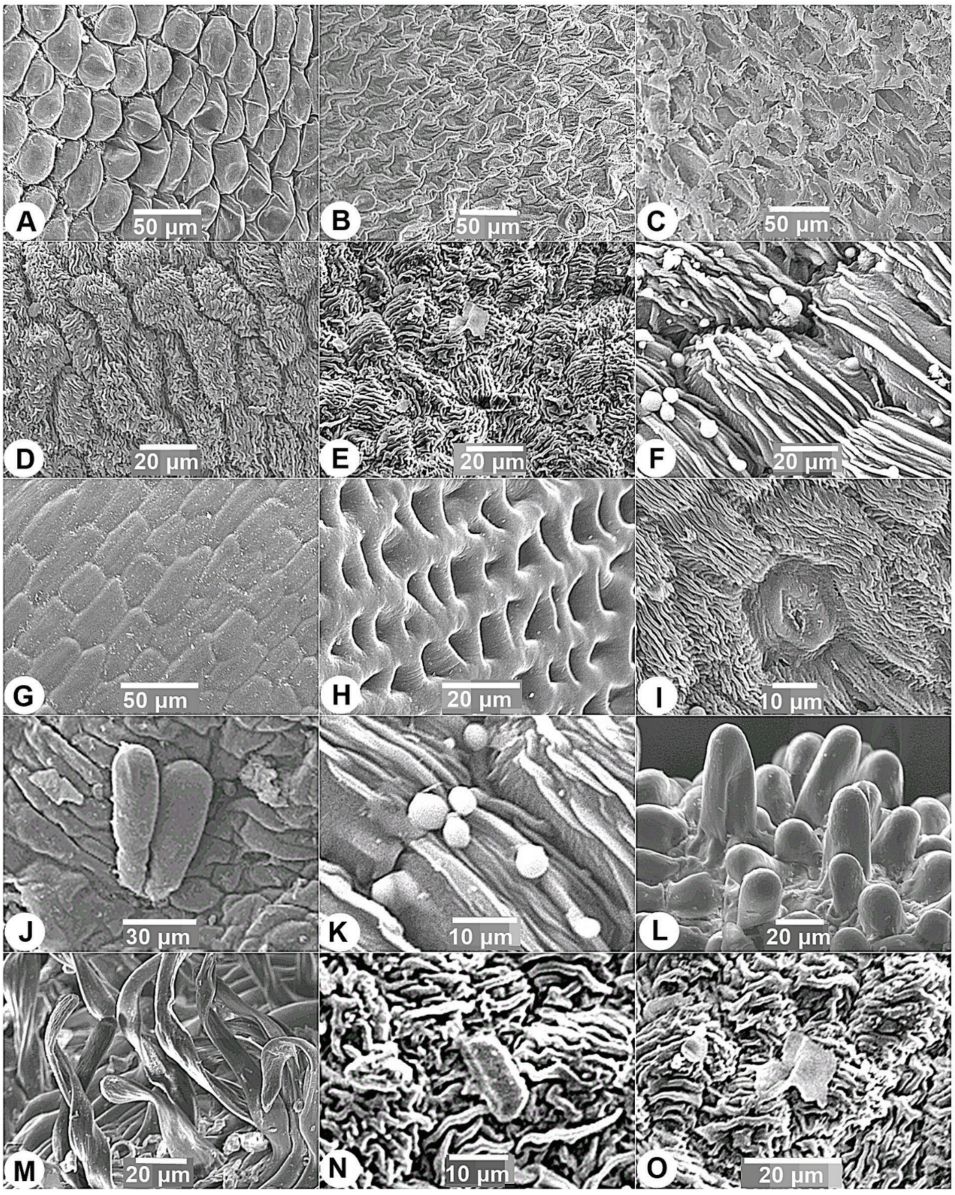

Figure 3: SEM observations of epicuticular ornamentation (A-H), stomata (I), trichomes including papillae (J-M) and epicuticular waxes (N&O) on floral parts of *V. dearei*; A: Type IV epicuticular ornamentation with laevigate outer and polygonal periclinal wall; B: Type II epicuticular ornamentation with foveate outer periclinal wall; C: Type II epicuticular ornamentation with foveate outer periclinal wall; D: Type I epicuticular ornamentation with laevigate and polyhedral-striated outer periclinal wall; E: Type VII epicuticular ornamentation with laevigate and irregular shape with undulate striae outer periclinal wall; F: Type I epicuticular ornamentation with laevigate and polyhedral-striated outer periclinal wall; G: Type IX epicuticular ornamentation with laevigate and elongated rectangular outer periclinal wall; H: Type VI epicuticular ornamentation with microfossulate outer periclinal wall; I: Stephanocytic stoma; J: Type IV club-shaped glandular trichomes; K: Barrel-shaped papillae; L: Papillae on spur; M: Type VII non-glandular trichomes on spur; N: Warty-granulated wax; and, O: Flake-like wax.

Comparative study on the floral-surface micromorphology of V. helvola and V. dearei

Non-glandular trichomes were observed in both studied species, which varied in structure and distribution on their floral parts, although, glandular trichomes were absent in both species. Stomata were present in floral parts of *V. helvola* and *V. dearei* at a very low frequency and sporadic distribution. Epicuticular ornamentation was prominent and distinctive on the epidermal surface of the selected species, differing in anticlinal and periclinal wall characteristics. The features and occurrence of each micromorphology characteristics are described in Tables 4 and 5.

Table 4: Features and distribution of floral-surface micromorphology characteristics of *V. helvola* and *V. dearei* (trichome)

Species	Floral Parts	Trichome type	Occurrence of non-glandular trichome	Occurrence of glandular trichome	Occurrence of papillae
V. helvola	Anther	Absent	Absent	Absent	Absent
	Column	I	Absent	Low	Low
	Dorsal sepal	Absent	Absent	Absent	Absent
	Lateral sepals	Absent	Absent	Absent	Absent
	Petals	Absent	Absent	Absent	Absent
	Labellum	II, V, VI	Absent	High	Absent
	Pollinium	Absent	Absent	Low	Absent
	Spur	III, VI	Absent	Very high	Very high
V. dearei	Anther	Absent	Absent	Absent	Absent
	Column	I, IV	Absent	High	Low
	Dorsal sepal	Absent	Absent	Absent	Absent
	Lateral sepals	Absent	Absent	Absent	Absent
	Petals	Absent	Absent	Absent	Absent
	Labellum	VII	High	Absent	Absent
	Pollinium	Absent	Absent	High	Absent
	Spur	III, VII	High	Absent	High

Table 5: Features and distribution of floral-surface micromorphology characteristics of *V. helvola* and *V. dearei* (epidermal, stomata).

Species	Floral Parts	Epicuticular ornamentation	Anticlinal wall features	Epicuticular striation	Stomata type	Stomata subsidiary and epidermal cells ornamentation	Peristomatal rim ornamentation	Pattern of stomatal distribution
V. helvola	Anther	I	Furrowed	Striated	Tetracytic	Clear	Clear	Parallel
	Column	I, II	Furrowed, fibrillary	Striated, rugulate	Actinocytic	Clear	Clear	Random
	Dorsal sepal	II, III	Furrowed, fibrillary	Smooth, rugulate	Absent	Absent	Absent	Absent
	Lateral sepals	II, IV, V	Furrowed, fibrillary	Smooth	Absent	Absent	Absent	Absent
	Petals	II	Fibrillary	Smooth, rugulate	Absent	Absent	Absent	Absent
	Labellum	I	Furrowed	Striated	Absent	Absent	Absent	Absent
	Pollinium	VI, IX	Furrowed, fibrillary	Smooth	Absent	Absent	Absent	Absent
	Spur	I	Furrowed	Striated	Absent	Absent	Absent	Absent
V. dearei	Anther	I, II, VIII	Furrowed, fibrillary	Striated, rugulate	Stephanocytic	Clear	Clear	Random
	Column	I, II, IV	Furrowed, fibrillary	Striated	Stephanocytic	Clear	Clear	Parallel
	Dorsal sepal	IV	Furrowed	Smooth	Absent	Absent	Absent	Absent
	Lateral sepals	II	Fibrillary	Smooth	Absent	Absent	Absent	Absent
	Petals	IV	Furrowed	Smooth	Absent	Absent	Absent	Absent
	Labellum	I	Furrowed	Striated	Absent	Absent	Absent	Absent
	Pollinium	VI, IX	Furrowed, fibrillary	Smooth	Absent	Absent	Absent	Absent
	Spur	I, II, IX	Furrowed, fibrillary	Striated, rugulate	Absent	Absent	Absent	Absent

Discussion

V. helvola and *V. dearei* were comparable based on the type and distribution of the epicuticular ornamentation, stomata and trichomes (Tables 4 and 5). Trichomes occurred in the column, labellum and spur of *V. helvola* and *V. dearei*. Glandular trichomes function as the emission layer prescribed by the accumulation of lipid-rich substances, probably precursors or the scent itself (Choi & Kim, 2013), which explained the scented flowers of both species with glandular trichomes occurring on the labellum and spur. Nectar production also commonly occurred within the spur orchid species, as in *Platanthera chlorantha* (Custer) Rchb. and *Gymnadenia conopsea* (L.) R.Br. Nonetheless, other flower parts, such as petals and bracts, could also function as nectaries, as in *Platanthera stricta* Lindl. and *Disa elegans* Sond. ex Rchb.f. (Patt *et al*., 1989; Hobbhahn *et al*., 2013). Toh *et al*. (2017) further suggested that both the epidermal layers of the petals and sepals of *Vanda* Mimi Palmer were preferential sites of scent production and emission.

Presumably, the presence of glandular trichomes and stomata may also offer a food reward to ensure pollinators revisited the plant (Stpiczyñska *et al*., 2018). Micromorphological studies justified that successful flower-pollinator interactions occurred through the floral micromorphological features, such as nectar secreting trichomes, odour-producing osmophores, or secretory epidermal cells under the cuticle in the labellum and spur (Stpiczyńska, 1993, 2003; Anton *et al*., 2012). Common types of epidermal conical papillae characterised by its wide base, concave, lateral walls and rounded tip (Davies & Turner, 2004) found on the column and spur of both studied species might also play a significant role as a secretory structure (*Şeker et al*., 2016).

Branched glandular trichomes with single secretory cell at the tip occurring on the labellum of *V. helvola* provided morphological significance to differentiate the studied species. Also, stomata diversity offered points on taxonomic delimitation of *V. helvola* and *V. dearei*. *V. helvola* had tetracytic stomata on the anther and actinocytic stomata on the column, whereas *V. dearei* had stephanocytic stomata on the anther and column. Random dispersal of warty-granulated, cylindric and flake-like epicuticular waxes without any unique types on each floral part provided no significance taxonomic value for the studied species as in Corybas (Besi *et al*., 2019) and Paphiopedilum (unpublished account) species.

Though it might offer protection from desiccation and herbivorous insects (Davies & Turner, 2004), or reward pollinators with lipids and protein (van der Pijl & Dodson, 1966; Dressler, 1993; Davies *et al*., 2003). Contrariwise, the multi-pattern epicuticular ornamentation on the floral parts offered a diagnostic value to discriminate the studied species at species level. Some morphological features shared by both species might indicate relatedness, such as lack of non-glandular trichomes and glabrous epidermal cell of sepals and petals in both species.

Conclusion

Profiling on floral microstructure and selecting the most suitable media formulation for micropropagation would ensure sustainable use of these two favourite horticulture species. Given the great similarity and hybridisation potential between *Vanda* species, the comparative investigations of the structure of the trichomes, stomata and epicuticular ornamentation in *V. helvola* and *V. dearei* could provide better understanding of the phylogenetic and taxonomy of the genus. Labellum and spur with glandular trichomes and stomata functioned as the emission sites for either nectar and scent production in *V. helvola* and *V.dearei*. Further understanding on the usefulness the waxy compound produced by *V. helvola* and *V. dearei* flowers in relation to pollination mechanism would require an investigation into the chemical compounds.

Acknowledgements

We like to thank the Forestry Department for granting the permit to access the studied areas. This work was sponsored by the UPM-KRIBB (Korea Research Institute of Bioscience and Biotechnology) grant (Vot. 6384300), UPM-Putra Grant (No. 9413603), Young Academic Scheme issued by Universiti Putra Malaysia and IPTA Academic Training Scheme under the Ministry of Higher Education. We appreciate all the people who contributed to the accomplishment of this study, particularly Lam Shun Jia, Cheong Fui Ying, Lai Yian Ee, Tan Yan Bei, Ting Siew Yang, Ng Yong Jin, Tan Mui Ching, Dr. Kenny Khor, Dr. Farah Alia Nordin (plant Taxonomist, Universiti Sains Malaysia), Madam Nur Qamarina Salman and Mr Arif.

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