

GNASEKARAN, P; MAHMOOD, M; SUBRAMANIAM, S. 2016. Ultrastructure study of *Vanda* Kasem's Delight orchid's protocorm-like body. *Horticultura Brasileira* 34: 333-339. DOI - <http://dx.doi.org/10.1590/S0102-05362016003005>

## Ultrastructure study of *Vanda* Kasem's Delight orchid's protocorm-like body

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### ABSTRACT

Growing orchids has been classified as an international business since it covers 8% of the world floriculture trade. Thus, large-scale micropropagation of orchid using tissue culture techniques and improvement of some essential traits, such as resistances to various diseases and pests, and tolerances to environmental stresses, such as low temperatures and low light intensities, via genetic engineering acknowledged the orchids as one of the top ten cut flowers. Protocorm-like bodies (PLBs) are excellent explants for clonal propagation, artificial seeds development and genetic engineering since they are organized and easily regenerable somatic embryos that propagate rapidly. *Vanda* Kasem's Delight hybrid orchid's PLBs derived from shoot tip culture were examined in order to understand the fundamental ultrastructure of PLB. Examination of the cross sections of PLB revealed that PLB is made of a discrete bipolar structure that contains anterior and posterior meristems. Actively dividing meristem cells and outer layer lined by several rows of small and isodiametric cells with a dense cytoplasm and a prominent nucleus were also observed via cytological studies. Scanning Electron Microscope investigations revealed that the surface of PLBs is occupied by vertically positioned branched trichome appendages and regularly spaced stomatal openings with two guard cells. Organelles such as mitochondria of various sizes, shapes and biconvex chloroplast in the cytoplasm were revealed by Transmission Electron Microscope analysis. Hence, inheriting actively dividing cells, and bestowed with elements related to transpiration, photosynthesis and energy synthesizing power house makes PLB a suitable explant for micropropagation and genetic engineering studies.

**Keywords:** *Vanda* Kasem's Delight, morphology, scanning electron microscope, transmission electron microscope.

### RESUMO

#### Estudo da ultra-estrutura do protocormio da orquídea *Vanda* Kasem's Delight

O cultivo de orquídeas é considerado um negócio internacional, abrangendo 8% do comércio mundial da floricultura. Assim, a micropropagação em larga escala da orquídea, usando técnicas de cultura de tecidos e melhoria de alguns tratamentos essenciais, tais como resistências a várias doenças e pragas, e tolerâncias a estresses ambientais (por exemplo baixas temperaturas ou intensidades de luz), através da engenharia genética, classifica as orquídeas entre as dez mais importantes flores de corte. Estruturas semelhantes a protocormios (PLBs) constituem explantes excelentes na propagação clonal, desenvolvimento artificial de sementes e engenharia genética, uma vez que são embriões somáticos organizados, facilmente regeneráveis e de rápida propagação. PLBs dessa orquídea híbrida em estudo, derivados da cultura do meristema, foram examinados visando compreender a sua ultra-estrutura fundamental. O estudo das seções transversais revelou que o PLB é feito de uma discreta estrutura bipolar que contém meristemas anterior e posterior. Através do estudo citológico foram observadas células meristemáticas ativamente em divisão além de uma camada exterior revestida por várias camadas de células pequenas e isodiamétricas com um citoplasma denso e um núcleo proeminente. No microscópio eletrônico de varredura observou-se que a superfície da PLB é ocupada por apêndices de tricomas ramificados, posicionados verticalmente, além de aberturas estomáticas, com duas células guarda, regularmente espaçadas. Organelas como mitocôndrios de vários tamanhos e formas além de cloroplastos biconvexos no citoplasma foram reveladas pelo microscópio eletrônico de transmissão. Assim, a observação de células em divisão ativa, e elementos relacionados com a transpiração e fotossíntese faz do PLB um explante adequado para estudos de micropropagação e engenharia genética.

**Palavras-chave:** *Vanda* Kasem's Delight, morfologia, microscópio eletrônico de varredura, microscópio eletrônico de transmissão.

(Recebido para publicação em 25 de abril de 2015; aceito em 2 de fevereiro de 2016)

(Received on April 25, 2015; accepted on February 2, 2016)

The development of the orchid industry is very rapid especially in Association of Southeast Asian Nations (ASEAN) such as Malaysia, Singapore, Indonesia and Thailand. Orchids are commercially important plants cultivated as cut flowers and

pot plants around the world (Chang & Chang, 2000; Kabir *et al.*, 2012) primarily due to their exotic values such as spectacular variation in colour, size, intriguing shapes, fragrance and long shelf life (Chugh *et al.*, 2009).

The genus *Vanda* comprises about

35 species that are monopodial, mostly epiphytic and distributed mainly in tropical Asian regions (Shrestha *et al.*, 2010). *Vanda* Kasem's Delight (VKD) orchid is nearly 9 cm in diameter which may produce 12 blooms per spike. VKD blooms are very vibrant with stunning

and intense purplish blue coloration that may appear pinkish under sunlight. VKD is quite a pricey orchid variety due to the aesthetic attributes such as huge flat blossoms with rich and dark coloration. The attraction of orchid lovers towards VKD calls for the improvement of this breed in terms of longer shelf life than usual.

Hence, there is a necessity to examine ultra structure of VKD protocorm-like bodies (PLB) to analyse the suitability of it as a starting material for the trait improvement studies via genetic engineering. Therefore, the objective of the following study is to describe the ultrastructure of VKD orchid's PLBs via histological, scanning electron microscope (SEM) and transmission electron microscope (TEM) studies. It's the very first time of conducting ultrastructure studies in VKD PLBs.

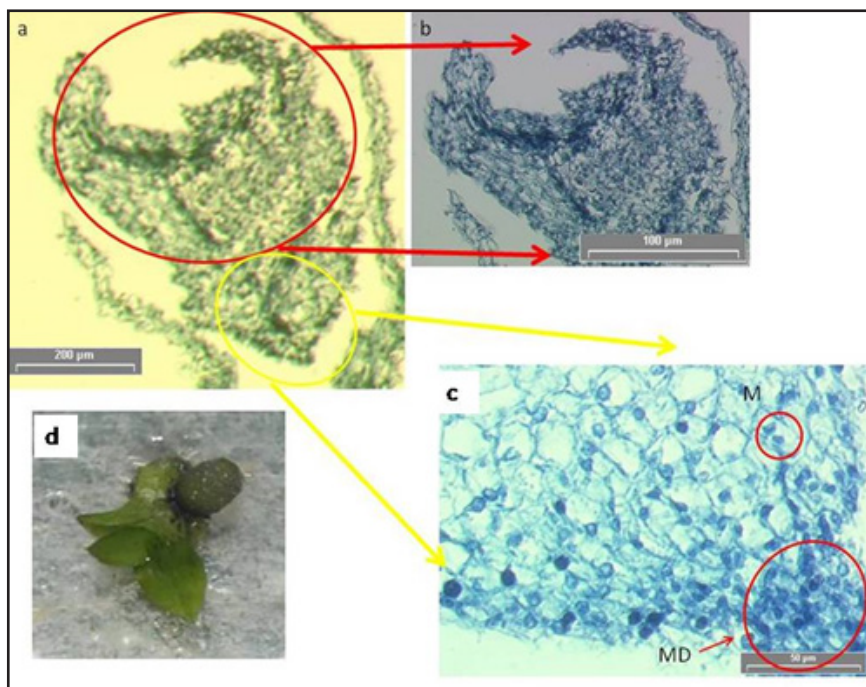
## MATERIAL AND METHODS

**Plant material** - Protocorm-like bodies (PLBs) of *Vanda Kasem's Delight* (VKD) initiated via shoot tip culture were subcultured from a long standing tissue culture by maintaining on Vacin & Went medium (1949) supplemented with 15% coconut water and 30% tomato extract. The pH (CyberScan PC 510 pH/mV/Conductivity/TDS/°C/°F Bench Meter, Eutech 73 Instruments, Singapore) of Vacin & Went medium in this study was adjusted to 4.8-5.0 prior to autoclaving (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan). The culture was incubated at 25°C under 16 h photoperiod with cool white fluorescent light (supplied by Philips TLD fluorescent light tubes of 36 W, 150 µmol/m<sup>2</sup>/s). The PLBs were then subcultured every 4 weeks on modified Vacin & Went medium as mentioned previously to produce large quantities of explants for transformation. Healthy, greenish and rapidly growing PLBs were used for ultrastructure studies.

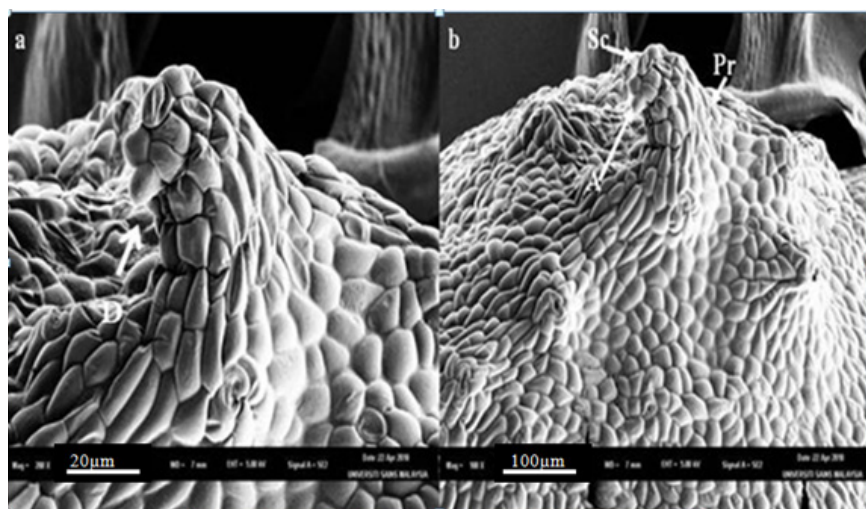
**Histology procedure** - Three 12-week old PLBs were fixed in formalin-acetic acid-ethanol (FAA) fixative solution for 24 hours. Fixed samples were washed under running tap water for

another 24 hours. The PLBs were then dehydrated in a graded series of ethanol. On day 1, samples were dehydrated

in 50% *tert*-butyl alcohol (TBA). On day 2, samples were first dehydrated with 70% ethanol for 7 hours followed



**Figure 1.** Micrographs of the cross sections analysis of the *Vanda Kasem's Delight* (VKD) Protocorm-like bodies (PLBs) {fotografia microscópica de seção transversal de estruturas semelhantes a protocormios (PLBs) da orquídea *Vanda Kasem's Delight* (VKD)}. (a) Apical meristems of a bipolar PLB (meristema apical de PLB bipolar); (b) Enlarged view of apical meristems of a PLB section at higher magnification (meristema apical em seção ampliada de PLB); (c) Cell division and development of meristematic dome (divisão celular e desenvolvimento do domo meristemático); (d) Plantlet regenerated from PLB (plântula regenerada de PLB). Cell with two nuclei (M) {célula com 2 núcleos (M)}, Meristematic dome (MD) {domo meristemático (MD)}. Penang, Universiti Sains Malaysia, 2010.



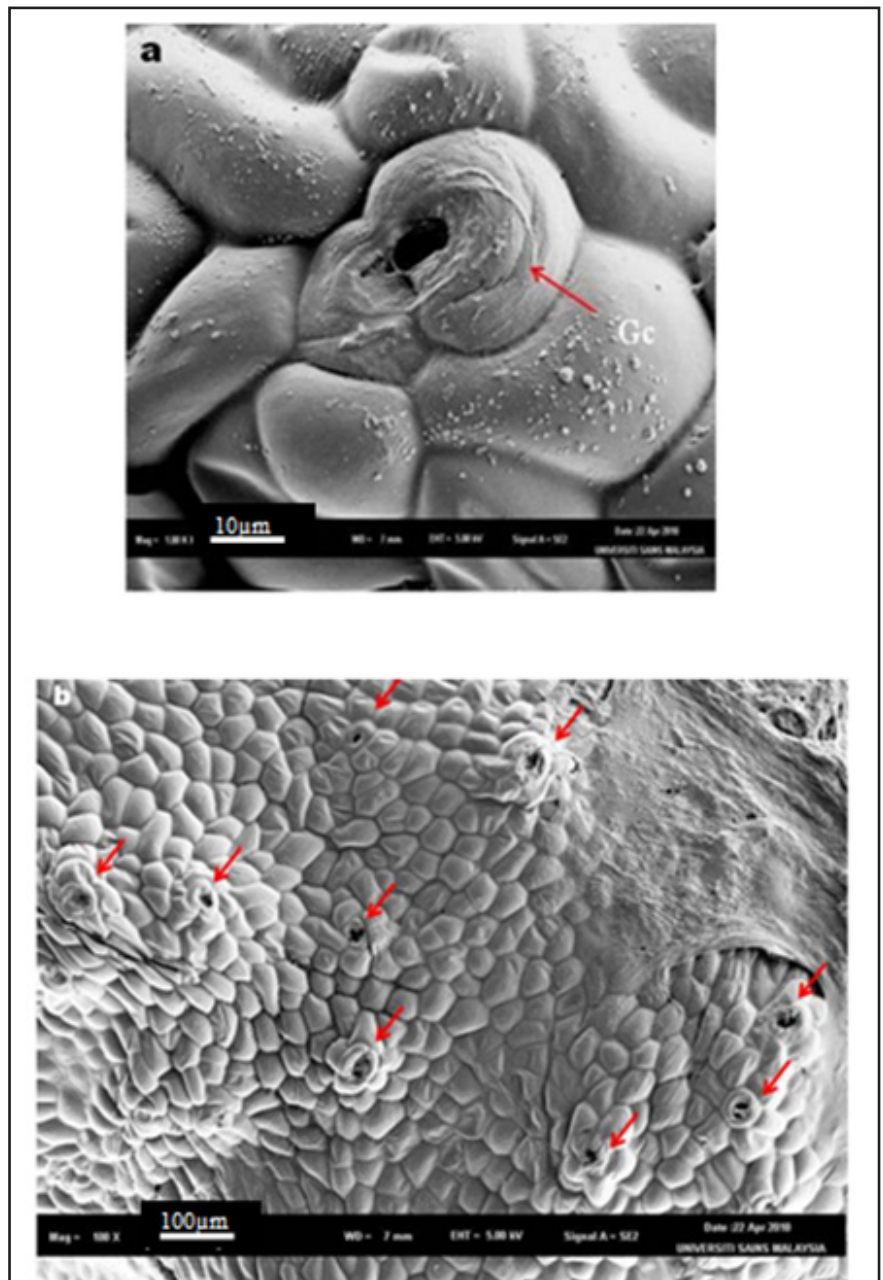
**Figure 2.** Scanning electron microscope (SEM) photographs of PLB {fotografias de PLB em microscópio eletrônico de varredura (SEM)}. (a,b) Apical part of protocorms showing the development of apex and scale; D= depression (depressão), A= apex (ápice), Sc= scale, Pr= meristematic dome (promeristem) {domo meristemático (promeristema)}. Penang, Universiti Sains Malaysia, 2010.

by 85% ethanol TBA. On day 3, 4, 5, and 6, samples were dehydrated for 24 hours in ethanol TBA 98%, ethanol TBA 100%, TBA I, and TBA II respectively. Dehydration was followed by clearance step (day 7) whereby the samples were immersed in xylene-substitute for 2 hours. During the wax absorption step samples were first immersed in a mixture of xylene-substitute and wax for 6 hours and then transferred to pure wax solution and left overnight. The following day, samples were treated twice with new wax solution and left overnight. Wax absorption was carried out in incubator at 60°C. On day 9, melted wax was poured into mould to form wax blocks and the samples were immediately positioned inside the wax block. These blocks were then cut into thin slices under microtome cutter (Leica RM 2135) and the thin slices were allowed to rest on the clean glass slides. Glass slides containing sample slices were left in the incubator (Memmert, Germany) at 60°C overnight. This was followed by staining procedure which was carried down in a transparent vertical glass jar with lining to hold the slides. Overnight dried slides were first dewaxed by rinsing in Histo-Clear solvent (National Diagnostics, USA) for 10 minutes. This was followed by rehydration of the slides in a series of reducing ethanol concentrations (100%, 90%, 70%, and 50%) for 2 min each. The slides were then stained with safranin for 20 min and rinsed in 70% and 80% ethanol for a minute respectively. This was followed by fast green staining for 2 mins. Care need to be taken as fast green tend to counter stain safranin as it's a strong stain. The slides were then dehydrated twice in 95% ethanol for a total of 2 minutes and finally rinsed in 100% ethanol for 2 min. The slides were then rinsed twice with Histo-Clear for a total of 6 min to wash the slides. The slides were then allowed to dry before mounting with cover slip using Shandon Histomount xylene substitute Mountant (Thermo Scientific). Stained slides were then viewed under light microscope (Olympus BX50, Olympus Optical Co. Ltd., Japan) fitted to a JVC K-F55B colour video camera (JVC Victor Company of Japan, Limited, Japan)

and analysed with the Docu Version 3.1 image analysis system (Soft Imaging System, GmbH, Münster, Germany).

**SEM tissue preparation - freeze drying technique** - Three PLBs were placed on a planchette on a damp filter paper lined Petri dish. The Petri dish was brought into fume hood and a few drops

of 2% osmium tetroxide ( $\text{OsO}_4$ ) were added to the filter paper. The Petri dish was closed immediately and kept in the fume hood for 2 hours. This is known as vapour fixation. Once the samples had been vapour fixed, they were plunged into liquid nitrogen and transferred on to the peltier-cooled stage of the freeze dryer for about 10 hours at varying



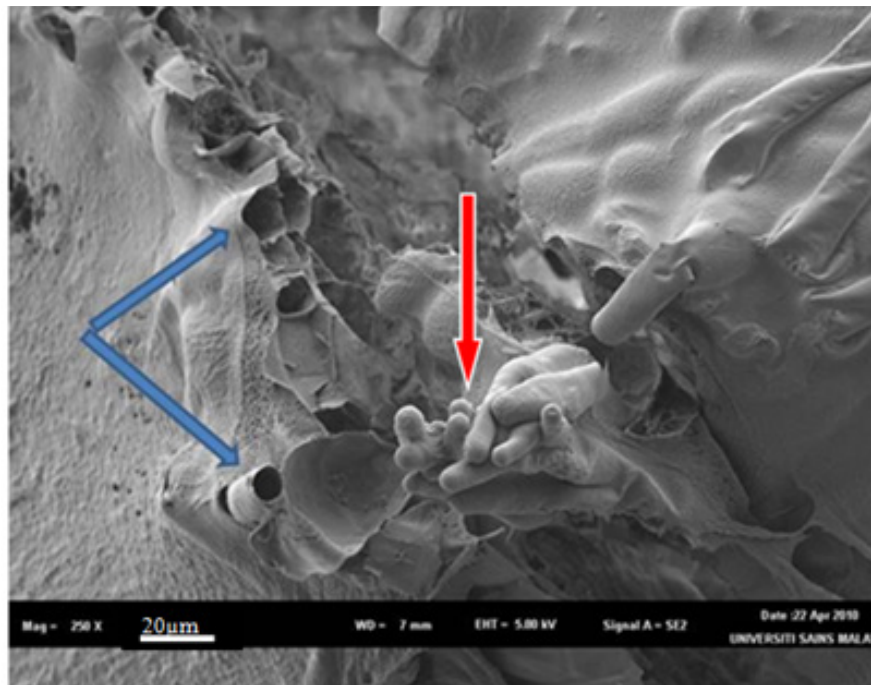
**Figure 3.** SEM photographs of stomata on surface of PLB (fotografias em SEM de estômatos na superfície de PLB). (a) Epidermal layer of PLB showing the presence of stomata which are surrounded by guard cells (camada epidermal de PLB apresentando a presença de estômatos circundados por células guarda); Gc= guard cells (células guarda). (b) Arrows indicate a greater density of stomata which are randomly distributed on the PLB surface (flechas indicam maior densidade de estômatos distribuídos aleatoriamente na superfície de PLB). Penang, Universiti Sains Malaysia, 2010.

temperature. The prepared samples were stored in a desiccator if not viewed immediately. The specimens were then sputter coated with 5-10 nm gold particles before viewing it using a Leo Supra 50VP Field Emission scanning electron microscope (Carl Zeiss SMT, Germany).

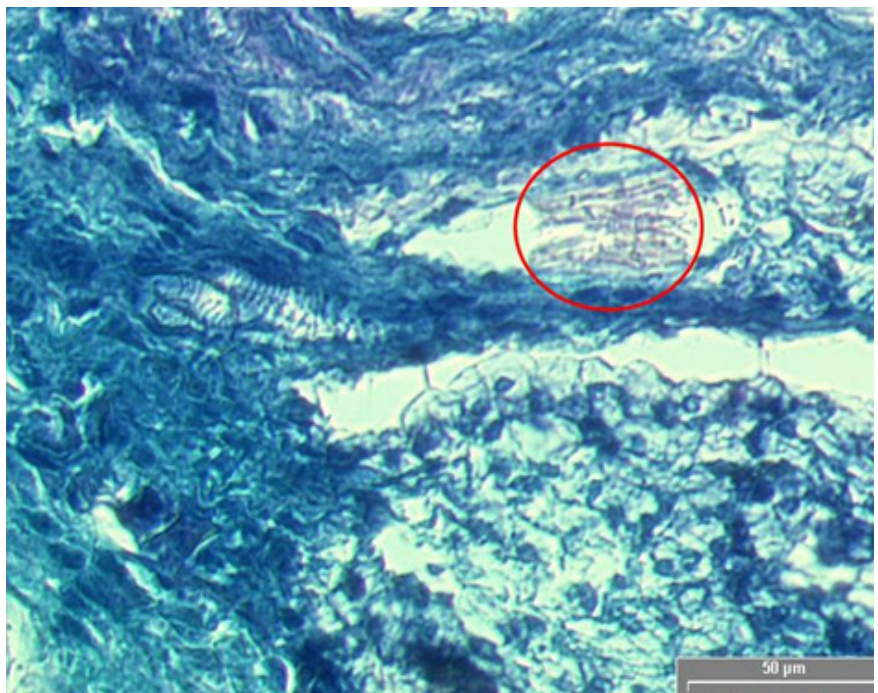
**TEM tissue preparation** -Three PLBs were fixed in McDowell-Trump fixative at 4°C for 24 hours. This fixative contains 4% formaldehyde and 1% glutaraldehyde in a 0.1 M phosphate buffer, and a pH of 7.2-7.4. Mix 86 mL distilled water, 10 mL Fisher F-79 37-40% formaldehyde, 4 mL 25% biological grade glutaraldehyde, 1.16 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  and 0.27 g NaOH in the stated order to prepare the McDowell-Trump fixative. The following day, the PLBs were washed in 0.1 M phosphate buffer three times at 10 minutes interval and post fixed in 1% osmium tetroxide prepared in the same buffer as above at room temperature for 2 hours. Then the PLBs were washed in distilled water twice at 10 minutes interval. The PLBs were dehydrated in a graded ethanol series [50% ethanol (15 minutes), 75% ethanol (15 minutes), 95% ethanol (15 minutes, twice), 100% ethanol (30 minutes, twice), and 100% acetone (10 minutes, twice)]. Resin containing a mixture of acetone and Spurr's resin was prepared at a ratio of 1:1. The resin-acetone mix was infiltrated into the samples in a rotator for 15-30 minutes followed by infiltration in Spurr's mix overnight in the rotator. Samples were infiltrated in a new change of Spurr's mix for another 5 hours in the rotator. Samples were embedded in polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Embedding was done by placing samples in a mould containing melted resin. Samples were cured at 60°C for 12-48 h. Samples were then sliced using ultramicrotome and gathered on carbon planchette. Sliced ultrathin samples were stained with lead citrate and uranyl acetate and viewed under TEM.

## RESULTS AND DISCUSSION

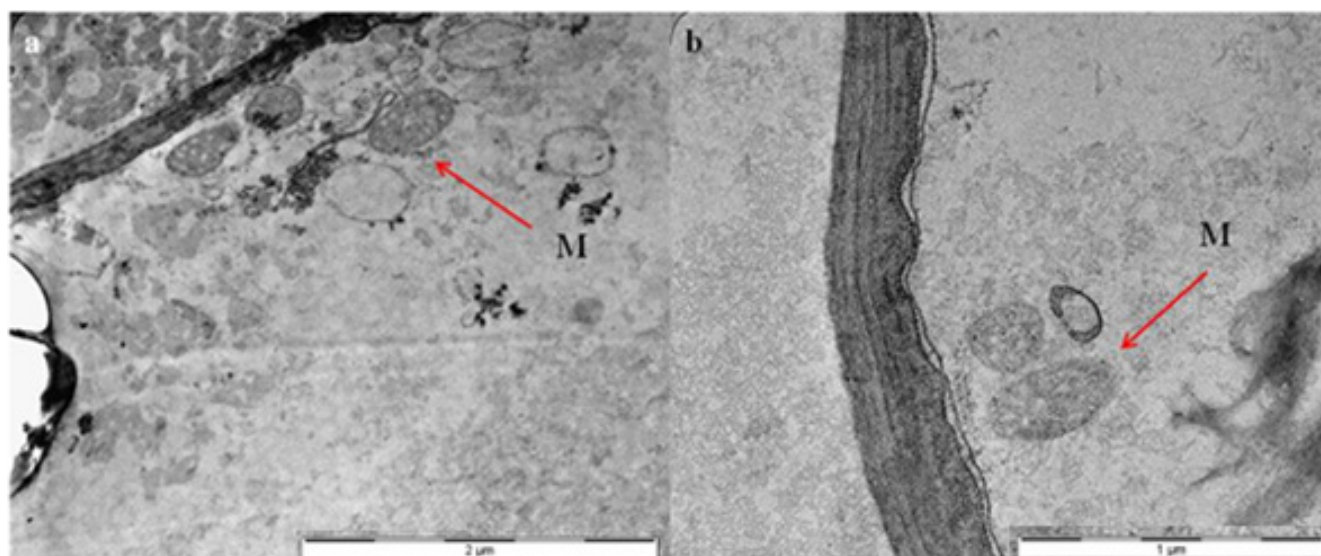
PLBs are composed of mass of



**Figure 4.** SEM photograph of elongated projections of a cluster of branched trichomes from the surface of the PLB (fotografia SEM de projeções alongadas de um grupo de tricomas ramificados na superfície de PLB). Certain trichomes collapsed because could not stand the tension force during SEM sample preparation (alguns tricomas não resistiram à força de tensão submetidos durante o preparo das amostras para SEM). Red arrow indicates intact trichome while blue arrows indicate broken trichomes (flechas vermelhas indicam tricomas intactos; flechas azuis indicam tricomas danificados). Damaged trichomes indicate that they are hollow in cavity and suggest that they could be fluid filled (tricomas danificados indicam que eles são ocos, sugerindo possivelmente conter líquido). Penang, Universiti Sains Malaysia, 2010.



**Figure 5.** Longitudinal section of a PLB revealed the presence of non-cytoplasmic inclusion, known as raphids (seção longitudinal de PLB revelou a presença de inclusão não-citoplasmica, conhecida como raphids). Penang, Universiti Sains Malaysia, 2010.



**Figure 6.** Transmission electron microscope (TEM) photographs of various shapes of mitochondria lining the cell wall {fotografia por microscópio de transmissão electrónica (TEM) de vários formatos de mitocondrios próximos à parede celular}; M= mitochondria (mitocondrios). Penang, Universiti Sains Malaysia, 2010.

differentiated cells that are regarded as orchid embryos (Ng & Saleh, 2011) which develop with two discrete bipolar structures, where the upper region becomes the shoot while the bottom region becomes the root meristem (Jones, 2009). A notable anterior pole and posterior pole was observed on the PLB (Figure 1a). The bipolar nature of the 12-week old VKD PLB indicated the presence of apical meristems in VKD PLB (Figure 1a). Meristematic zone occupies 1/3 of the length of the PLB (Figure 1b). The bipolar PLB eventually differentiated anterior and posterior regions into shoot and root system respectively (Figure 1d). Thus, meristematic PLB can be induced to regenerate into whole plantlets on plant growth regulator (PGR)-free medium (Ng & Saleh, 2011). Furthermore, the shoots develop directly from the primary and secondary meristems of PLB without an intervening explant-organogenesis phase (Ng & Saleh, 2011). Thus, absence of phytohormones and explant-organogenesis phase reduce the opportunity for somaclonal variation.

VKD PLB is made of cells that are actively involved in cell division. Cells with two nuclei beneath the epidermis layer were observed in PLB proving active mitosis (Figure 1c). In addition, Figure 1c shows the histochemical

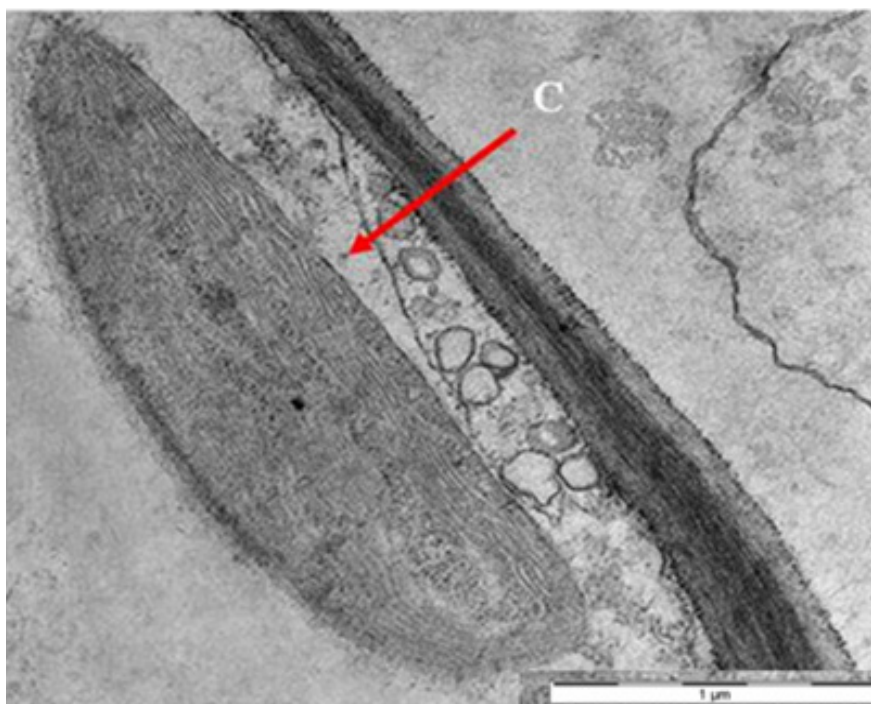
gradient established between the two ends of PLB. Histological observation on PLB revealed two types of cells. Based on Figure 1c, there was difference in size of cells within PLB. The outer layer consists of several rows of small and isodiametric cells with a dense cytoplasm and a prominent nucleus (Figure 1c). Such conditions were also observed and reported by Vega *et al.* (2009) in somatic embryo of *Oryza sativa* cv. 5272. Meristematic cells are also characterized by the tendency of their large nuclei to retain stain as observed in the meristematic dome (Figure 1c). Contrarily, the inner cell layers are more voluminous than those of apical end. Inner layers are made of large cells with small nucleus and large vacuole (Figure 1c).

Examination of upper portion of PLB at higher magnification revealed a cap-like structure which covers the small anterior apical meristem (Figure 1b). The cap-like structure is known as leaf primordium which eventually developed into first leaves (Figure 1d). Interestingly, SEM analysis of anterior meristematic pole of PLB showed elongated cone-shaped meristematic dome (Figure 2a and b). A more intense meristematic activity at one side of the dome displaced the apical end forming a hook-like structure depicting

depression. Leroux *et al.* (1997) observed a similar depression on the protocorm of *Cypripedium acaule* which was caused by the displacement of the apical end. The depression determined the length of the apex and the scale. It is obvious in Figure 2a and b that the elongated scale folded over the apex located at the bottom of the depression on the adaxial side. The first true leaves on Figure 1d appeared after the apical dome subsided into the body of PLB.

SEM observation of the external surface of PLB revealed the regularly spaced stomatal openings (Figure 3a). They were anomocytic and possess two guard cells with no subsidiary cells (Figure 3b). Generally, care should be taken since cultured plants with non-functional stomata, weak root systems and poorly developed cuticles may cause mortality upon the transfer to *ex vitro* conditions (Mathur *et al.*, 2008). Furthermore, high stomata density on VKD PLBs provides increased rates of stomatal conductance with improved transpiration efficiency.

Randomised projections of branched trichome on the surface of PLB were observed under SEM observation (Figure 4). Trichomes are bush-like appendages on the surface of plant tissues, which range in size from a few microns to several centimetres (Tissier,



**Figure 7.** TEM photograph of chloroplast along the length of cell wall (fotografia TEM de cloroplasto ao longo da parede celular); C= chloroplast (cloroplasto). Penang, Universiti Sains Malaysia, 2010.

2012). They can be single-celled or multicellular, curved or straight, non-secretory or glandular, to name a few (Werker, 2000). Trichomes develop as projections from protodermal cells (Wagner, 1991). Trichomes are the very first structure to appear during differentiation of cultured cells (Wagner, 1991). Trichomes may serve as absorption or conduction tube since they are known to accommodate fluids inside (Gorb *et al.*, 2007). Broken trichomes in Figure 4 show that trichomes are hollow in cavity which could be fluid filled.

A longitudinal sectioning of a PLB revealed the presence of non cytoplasmic inclusion, known as raphids (Figure 5). Raphids are bundles of long needle-like (prismatic) calcium oxalate monocrystals and may occur in ordinary parenchymatous cells or special cells called idioblasts (Gomes & Lombardi, 2010). Raphids are mostly observed in young and actively growing tissues (Prychid & Rudall, 1999). They act as depositories of calcium ions and metabolic waste product (Sugimura *et al.*, 1999) and play extracellular structural roles like fortifying the cell wall.

Transmission electron micrographs of the PLB revealed the presence of clusters of round shaped mitochondria with prominent cristae (highly folded inner membrane) and intercrystal spaces (Figure 6a and b). TEM details of the PLBs cross section show chloroplast with the broad side of it parallel to the cell wall. Furthermore, plastoglobuli appearing as black spot was detected in the elongated chloroplast of PLBs (Figure 7). Observations indicate that VKD PLBs possess biconvex chloroplast and the thylakoid are almost entirely filling the stroma of the plastid (Figure 7). Presence of mitochondria and chloroplast indicates that PLB is a differentiated organ that will function as an intact plant.

It could be concretely concluded that, bipolar VKD PLB is a transitory orchid embryo which proved to contain mass of differentiated cells that regenerated into an intact plantlet. VKD PLB is characterized by small isodiametric outer cells and voluminous inner cells. The presence of stomata, trichome, raphids, mitochondria and chloroplast indicates that physiological process of an intact plant is supported in the system

of VKD PLB.

## ACKNOWLEDGEMENTS

This work was supported by FRGS Grant 2014 (203/PBIOLOGI/6711456) and USM Post-graduate Research Scheme (PGRS) grant.

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