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Short Note

# Histological analyses of PLBs of *Dendrobium* sonia-28 in the recognition of cell competence for regeneration and *Agrobacterium* infection

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**Abstract**

Protocorm-like bodies (PLBs), resembling the orchid embryo, are excellent target explants for many orchid experiments. Histology and scanning electron microscopy (SEM) were applied on PLBs of *Dendrobium* sonia-28. Histological observations revealed that globular masses arose from the epidermal cell layers of the PLBs, giving rise to new PLBs. The primordial and young leaves differentiated from the shoot apical meristem, transforming PLBs into plantlets. The SEM analysis revealed an abundance of stomata and a scarcity of trichomes on the PLB surface, favourable for *Agrobacterium* infection.

**Keywords**: Freeze Drying; HMDS; Scanning electron microscope; Toluene; Xylene.
**Abbreviations:** BAP-6-benzylaminopurine; HMDS- hexamethyldisilazane; PLBs- protocorm-like bodies; SEM-scanning electron microscopy; TBA- *tert*-butyl-alcohol.

## Introduction

Any plant tissue targeted for *Agrobacterium*-mediated transformation must be competent for bacterial attachment and infection. Hence, it is necessary to investigate the competence of a target tissue before attempting transformation. Protocorm-like bodies (PLBs) are undifferentiated tissue masses showing resemblance to an early stage of orchid embryo development (Tisserat and Jones, 1999). The PLBs are now considered as excellent target explants for many orchid biotechnology experiments including clonal propagation (Tisserat and Jones, 1999), breeding, genetic transformation (Chai et al., 2002) and cryopreservation (Ranjetta et al., 2009). Plantlets can be easily regenerated and produced from PLBs, with reduced intermediate culture periods (Mishiba et al., 2005). Many transformation protocols had successfully developed for genetic engineering of orchids targeting PLBs of *Cymbidium* (Chin et al., 2007), *Vanda* (Shrestha, 2007), *Cattleya* (Zhang et al., 2010), and *Dendrobium* (Quan et al., 2011) as initial explants. Histology is an integral step in the study of plant morphogenesis. A good histological examination reveals information on the changes in the plant body's organisation and cellular morphogenesis (Yeung, 1999). Histological observations can also assist in the selection and establishment of an optimised culture system that produces plant cells competent for genetic transformation (Creemers-Molenaar et al., 1994). The exact point of origin of the competent cells may be further subjected to various mutagenesis techniques to enhance the bacterial transformation efficiency (Mendoza et al., 1993). The treatment with clearing agents such xylene and toluene is an essential step to determine the preservation of plant tissue. Meanwhile, the SEM is a reliable technique used in the analyses of tissue surfaces. The SEM is a well-established technique in fields such as plant identification and characterisation, the study of plant tissue function and mechanism, and the investigation of plant interactions with stress and pathogenic factors (Elad et al., 1982; Cochard et al., 2000; Sandalio et al., 2001). The hexamethyldisilazane (HMDS) and drying technique has been found to be suitable in treating delicate tissue (Jung et al., 2010) whereas the freeze drying method offers lesser chemical changes and shorter time was also found to be great alternative of drying biological sample (Lee and Chow, 2011). This study aimed to produce both histological and SEM procedures for PLBs of *Dendrobium* sonia-28 that could provide information on the compatibility of the PLB cells for regeneration and *Agrobacterium* infection.

## Results

### *The histological observation of PLBs of Dendrobium Sonia-28*

After the dehydration process, PLBs which are treated with xylene for clearing prior paraffin wax embedding, displayed severe cellular breakage and shrinkage (Fig. 1A). Toluene-treated PLBs maintained their original structure and only displayed slight structural changes (Fig. 1B, C). Histological observations of the PLBs revealed that the external layer of the tissues was composed of densely-arranged meristematic cells. This thin epidermal meristematic cell layer consisted of between one to three rows of smaller cell layers (Fig. 1B, C). The inner cell layer was composed of large vacuolated parenchymal cells possessing nuclei. After one week of cultivation, the epidermal layer was observed to be actively dividing, displaying dense cellular arrangement. Small masses of tissues protruded from the epidermal meristematic cell layer (Fig. 2A). Actively dividing meristematic cells were observed to possess large-sized nuclei and cytoplasm,

which were darkly-stained compared to the rest of the cells. The tissues swelled into globular-shaped structures in the second week of cultivation as the embryonic cells underwent continuous cellular division (Fig. 2B). The embryonic cells expanded and enlarged into new PLBs in the third week of cultivation (Fig. 2C). After four weeks of culture, the cells located within the PLBs stopped dividing with the progression of the maturation and expansion stages (Fig. 2D). Some of the PLBs were able to detach themselves from the primary tissue at this stage. The densely-arranged shoot apical meristem region also displayed active cellular division (Fig. 2E). Some PLBs displayed fully-developed primordial and young leaves at the fourth week of culture (Fig. 2E).

***Surface morphological observations of PLBs***

In the SEM analyses, the HMDS technique was revealed to be unsuitable for the delicate PLB tissues. Most parts of the PLBs were fractured, torn and distorted due to the intense dehydration involved in the procedure (Fig. 3A, B). The freeze drying method preserved the original PLB structure, with minor changes observed in the samples (Fig. 3C). The stomata were present in abundance on the PLB surface (Fig. 3D, E). Trichomes were observed to be protruding from the PLB surface forming thick hair-like bodies (Fig. 3F). The number of trichomes distributed on the PLB surface were very little ranging from three to five clusters with each cluster consist of one to three trichomes. *Agrobacterium tumefaciens* were observed to be attached on PLB surfaces uncovered by barriers such as trichomes (Fig. 4).

**Discussion**

The PLBs of *Dendrobium* sonia-28 are delicate tissue. Even though xylene is commonly used as a clearing agent for plant tissue permeability (Schmidt et al., 1996; Moller et al., 2009) however, it appeared to be harsh to the PLBs of *Dendrobium* sonia-28, which are composed of soft tissue and high water contents, as it caused rapid shrinkage of the samples. On the other hand, toluene preserved the cell structure of the PLBs. Toluene is therefore favourable as a clearing agent in the histological observations of PLBs of *Dendrobium* sonia-28.
The division and arrangement of cells in PLBs of *Dendrobium* sonia-28 resemble that of somatic embryos in rice (Vega et al., 2009) and the nodular meristemoid in *Sclerocarya birrea* (Moyo et al., 2009). The compactly-arranged outer cell layers of the PLBs, consisting of between 1-3 rows of cells, are small in size compared to the large-sized inner cells, with the cells possessing nuclei that were easily observed. Protocorm-like bodies are delicate when compared to the embryonic calli of rice, which possess at least 9-12 rows of the outer cellular layer (Vega et al., 2009). The PLBs used as the starting material is henceforth termed as 1° PLBs, and subsequent PLB proliferations from the 1° PLBs are termed as 2° PLBs as termed by Texeira de Silva and Tanaka (2010). Various authors have concurred that PLB formation occurred on the epidermal layer of orchid tissues such as the callus, protocorm, shoot tip or leaf (Tee et al., 2010). The ease in the direct induction of 2° PLBs from the epidermal layer of 1° PLBs is advantageous for mass production of the plants and in the development of transgenic cell lines. Pena et al. (2004) demonstrated that the *Agrobacterium* transformation of citrus occurred mainly on the callus formation sites of the explants. Even though Jung et al. (2010) demonstrated that HMDS drying technique caused lesser damage with lesser shrinkage in dinoflagellates delicate tissue compared to critical point drying (CPD)

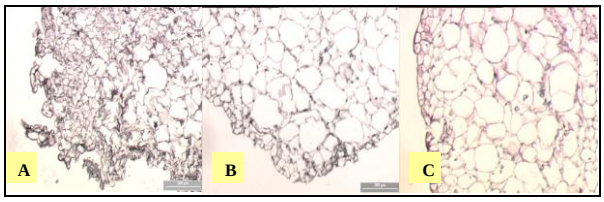


**Fig 1.**The effect of different clearing agents on the cellular structures of root apical meristem of PLBs of *Dendrobium* sonia-28. (A) Effect of xylene causing shrinkage to PLB cells; and (B and C) Effect of toluene which preserved the cell structure of the PLBs. Bar = 200µm.

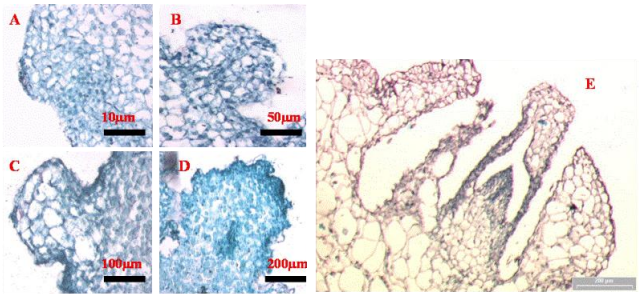


**Fig 2.** Histology of PLB development in *Dendrobium* sonia-28. (A) Small protruding epidermal layer in the 1st week; (B) Dense propagation of embryonic cells in the 2nd week.; (C) Enlargement and expansion of embryonic cells in the 3rd week.; (D) 2° PLBs in the 4th week; and (E) Shoot apical meristem emerged at the 4th week.

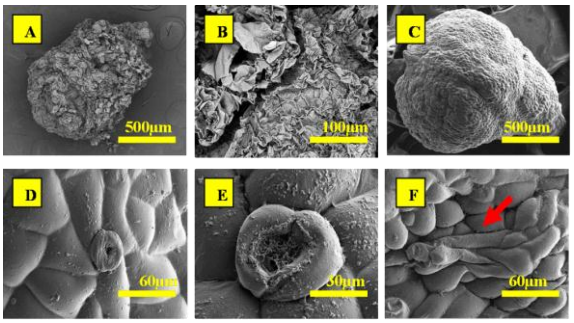


**Fig 3.** Scanning electron microscopy of PLBs of *Dendrobium* sonia-28 using HMDS and freeze drying dehydration techniques: (A and B) A PLB processed in HMDS. The HMDS treatment caused severe damage to the PLB; (C and D) A PLB processed using the freeze drying method. PLB treated using freeze drying remain preserved; (E) Stomatal opening on PLB increase the chance of infestation of *Agrobacterium tumefaciens*; and (F) Collapsed trichomes [red arrow] on a PLB treated using freeze drying. Scarcity of trichomes reduces PLBs defense against *Agrobacterium* attachment.

technique, the HMDS dehydration procedure was damaging to the PLBs of *Dendrobium* sonia-28.. Bray et al. (1993) demonstrated that HMDS was less efficient in the dehydration of biological specimens when compared to the CPD and Peldri II methods. The freeze drying method, involving rapid cooling at ultra low temperatures, causes the rapid exit of water out of the PLBs. Saravacos (1967) reported that efficient and rapid drying is achieved in the freeze drying method when compared to air drying as the

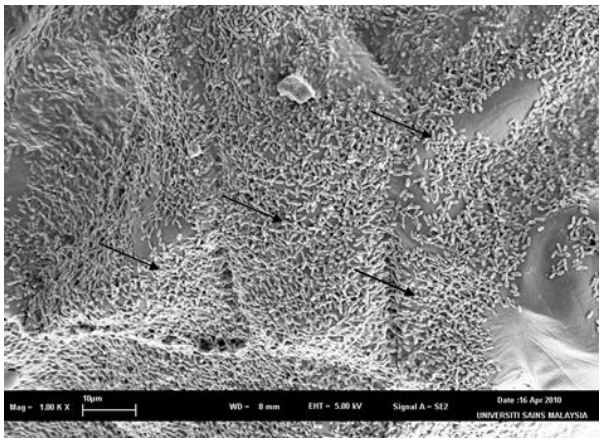


**Fig 4.** *Agrobacterium* attachment on the surface of a PLB of *Dendrobium* sonia-28. Arrows= *Agrobacterium tumefaciens* which remain attached on the PLBs surface after rigorous washing.

diffusivity of water vapours is higher under ultra low temperatures. Based on the SEM observations, PLBs are leafy in nature and possess trichomes. The number of trichomes observed was ranging from three to five clusters, which could be advantageous for *Agrobacterium* infection during transformation studies (Kumar et al., 2004). A large presence of trichomes could impede *Agrobacterium* attachment to the plant surface (Kumar et al., 2004). Stomatal openings were also observed to be distributed throughout the tissue. The high number of stomata could increase transpiration efficiency, essential in the growth of the PLBs. The stomata could increase the chances of *Agrobacterium* infestation during transformation studies when cultured *in vitro* in an opened state (Melotto, 2006). In conclusion, this study sheds light on the morphology and development of PLBs of *Dendrobium* sonia-28 produced through *in vitro* culture. Based on the histological and SEM observations, 2° PLBs are easily induced from the epidermal layer of the 1° PLBs, hence being suitable as target explants for rapid propagation and regeneration purposes.

## Materials and methods

### *Plant material and culture condition*

Protocorm-like bodies of *Dendrobium* sonia-28 were cultured on half-strength Murashige and Skoog (1962) semi-solid medium containing 2% (w/v) sucrose and 1mg/L BAP. The PLBs were incubated at 25±2ºC under a 16-hour photoperiod.

### *Histological observations of PLBs*

The PLBs samples were collected after four weeks of subculture. The PLBs were separated without wounding them and cultivated as single PLBs in half-strength MS semi-solid medium. After cultivation, single PLBs were collected every week for four weeks and were directly subjected to histology. The PLBs were fixed in FAA (40% formaldehyde: glacial acetic acid: 70% ethanol at 5:5:90). The tissues were then dehydrated in a *tert*-butyl-alcohol (TBA) series (50%, 75%, 85%, 95%, 100%, and absolute TBA). Xylene and toluene were evaluated as clearing agents to replace ethanol, which is miscible with paraffin wax. Subsequently, the tissues were embedded in paraffin wax and sectioned at 10-11µm thickness. Finally, the tissues were stained with 50% safranin-O and fast green prior to observation. The macroscopic quality of the PLBs was observed.

### *Morphological observations of PLBs through scanning electron microscopy (SEM)*

Protocorm-like bodies were subjected to two dehydration methods: hexamethyldisilazane (HMDS) and freeze drying. In the HMDS technique, the PLBs were fixed overnight in McDowell-Trump fixative and post-fixed with 1% osmium tetroxide for an hour. The PLBs were then dehydrated in an alcohol series and immersed in the HMDS solution. The freeze drying method involved vapour fixing of the samples with 1% osmium tetroxide for an hour, followed by freeze drying of the samples in liquid nitrogen slush. Attachment of *Agrobacterium tumefaciens* strain LBA 4404 on PLB surface was performed following Perez-Hernandes (2000) prior observed under SEM.

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