Emir. J. Food Agric. 2014. 26 (1): 73-81
doi: 10.9755/ejfa.v26i1.17447
http://www.ejfa.info/

PLANT SCIENCE

Microscopical analysis of *in vitro Mokara* Broga Giant orchid's PLBs

Jessica Jeyanthi James Antony[1], Jeevandran Sundarasekar[1,2], Xavier Rathinam[2], Kasi Marimuthu[2] and Sreeramanan Subramaniam[1*]

[1]School of Biological Sciences, Universiti Sains Malaysia (USM), Georgetown, 11800, Penang, Malaysia
[2]AIMST University, JalanBedong, Semeling 08100, Bedong, Kedah Darul Aman, Malaysia

Abstract

Mokara orchids comes with variety of hybrids that have unique and highly variable types of characteristics such as petal colour, shape, size and other floral characteristics that make it very important economically and aesthetically. This study was conducted to evaluate on its histological and scanning electron microscopy analyses that make its PLBs to be a desirable biotechnological explant in propagation of this orchid as well as its importance in many biotechnological researches. Histological observation in Mokara Broga giant indicated the presence of meristematic tissues that are vital for biotechnological research such as genetic transformation, cryopreservation, micropropagation and others. It also indicated the presence of shoot primordia. Scanning electron microscopy analysis also indicated the developmental characteristics of PLBs in detail. Both analyses showed that the maturation of PLBs and gradual formation of shoot leaf primordial occurs through somatic embryogenesis and PLBs are vital explant in many plant tissue culture researches such as genetic transformation, cryopreservation, micropropagation and others.

Key words: Orchids, PLBs, Histology, Scanning Electron Microscope

Introduction

Orchidaceae, a family with 20,000 to 30,000 species and is the biggest family in the plant kingdom (Godo et al., 2010). Orchids are one of the most attractive clusters of ornamental plants and numerous novel cultivars have been formed by interspecific and intergeneric hybridization in order to produce plants with exotic and elegant flowers. Due environmental disruption, succession of natural habitats and overexploitation of horticultural, many orchid species are threatened today (Godo et al., 2010).

The most widespread use of orchids is as ornamental cut flowers. However, only few orchid plants are attractive enough for direct use as ornamentals. Orchids hybrids are used as ornamental due to they have beautiful foliage, shiny, multicoloured and decorated with prominent veins in elaborate patters. Orchids from several genera such as *Mokara*, *Dendrobium, Anoectochilus*, *Goodyera*, *Ludisia* (Haemaria) and *Macodes* are used widely. Orchids used indoor and outdoor landscapes are often employed to generate a special effect such as exotic surroundings, feeling of luxury and unique environment (Arditti, 1992; Dalayap et al., 2011). Generally, the flowers were made into corsages. In the orchid industry, trade in plants has always been and remains a significant aspect. However, in the past, this trade was limited to species, seedlings and matured plants. The establishment of clonal propagation procedures and hybridization of orchid such as *Mokara* hybrid has improved this market (Arditti, 1992; Dalayap et al., 2011). In return, it had brought a large numbers of outstanding cultivars which are mass-propagated by growers. Orchids are also used as a form of national flower such as *Peristeriaelata* in Panama (Arditti, 1992).

Mokara (*Mokara spp.*), commonly identified as "Smile Orchid" and it is native to Asia, where it has been first discovered and cultivated. It is a *Vandaceous* orchid resulting from a trigeneric hybridization between the *Ascocentrum*, *Vanda* and *Arachnis* orchids. The first *Mokara* hybrid was produced in Singapore in 1969 and was called *Mokara* Wai Liang, named after C.Y. Mok. Since then, several varieties were created where many of the hybrids have exclusive and highly variable star-shaped flowers (Dalayap et al., 2011). This orchid

Received 28 July 2013; Revised 19 September 2013; Accepted 29 September 2013; Published Online 11 October 2013

*Corresponding Author

Sreeramanan Subramaniam
School of Biological Sciences, Universiti Sains Malaysia (USM), Georgetown, 11800, Penang, Malaysia

Email: sreeramanan@usm.my;sreeramanan@gmail.com

has flowers with the largest number of colors compared to other orchids like purple, pink, blue, red, orange, yellow and coral with each and every colour has its own variety. The flower comprises of three sepals, which are usually diverse from the petals in shape but not in colour (Dalayap et al., 2011). There are also three petals but one has been significantly altered into a lip or labellum, which is a very complex structure and quite different from the other two sepals (Soon, 1989; Dalayap et al., 2011). This orchid group is therefore vital economically due to these unique floral characteristic.

In vitro propagation is the utmost popular method used for the multiplication of orchids as this method is found to be more effective than sexual propagation and other vegetative procedures (Talukder et al., 2003). However, tissue culture in orchids via indirect method which produce callus is not favoured due to lower growth rate and regular occurrence of necrosis in culture (Zhao et al., 2008). According to Chugh et al. (2009), the future of orchid propagation is most expected through multiplication of true-to- type plants through *in vitro* techniques. Several methods of propagation have been developed for orchids through *in vitro* culture of various parts including shoot tips, flower stalk nodes, buds, root tips and rhizome segments (Chen et al., 2000; Park et al., 2002b) with varying responses.

The most commonly used micropropagation techniques include direct and indirect shoot organogenesis and somatic embryogenesis. Somatic embryogenesis is more necessary biotechnological technique for large-scale clonal propagation of elite genotypes, development of synthetic seed technology, plant transformation (Bomal and Tremblay, 2000; Bandyopadhyay and Hamill, 2000) and conservation of threatened plant species. The most ideal regeneration systems for genetic transformation are direct and repetitive production of somatic embryos or de novo shoot organogenesis originating from single cells in the epidermis (Rugkhla and Jones, 1998). Whereas, genetic clones created through shoot organogenesis require further manipulation for root initiation, in somatic embryogenesis complete plants with a bipolar axis, vascular system and functional meristems are created in a single step (Bassuner et al., 2007). In somatic embryogenesis, somatic cells develop into plants in similar developmental stages to zygotic embryogenesis (Gomes et al., 2006).

Recently, numerous reports have described shoot organogenesis through somatic embryo-like structures from different explants, mainly leaves and hypocotyls. Histological observations of the embryo-like structures have established the initial globular-shaped morphological features (Haensch, 2004; Tian et al., 2008) during the early developmental stages of their growth, in synchrony with conventional somatic embryogenesis. These embryo-like structures have been mistaken for true somatic embryos (Salaj et al., 2005). Our present study describes the histological approach to determine the anatomical characteristics of somatic cells within the PLBs. To the best of our knowledge this is the first report on the histological analysis in *Mokara* Broga Giant.

Therefore, this study aims to evaluate the importance of the structural conditions by histological analysis and scanning electron microscope analysis of this orchid PLBs that confirms its structure as somatic embryos that makes it vital in the orchid propagation industry.

Materials and Methods

Establishment of *in vitro* culture of PLBs

In vitro culture of protocorm-like bodies (PLBs) of *Mokara* Broga Giant were initiated by aseptically culturing shoot tips of the plant in half strength semi-solid Murashige and Skoog (1962) media supplemented with 1mg/L BAP (BAP; DUCHEFA, Netherlands), 2% (w/v) sucrose and 2.75g/L GelriteTM (DUCHEFA, Netherlands). The pH (CyberScan PC 510 pH/mV/Conductivity/ TDS/°C/°F Bench Meter, Eutech 73 Instruments, Singapore) of all the media in this study was adjusted to 5.8 prior to autoclaving (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan). The resulting PLBs were grown at 25°C under 16 hours photoperiod (Philips TLD, 36 W, 150µmol.m^{-2}.s^{-1}). The PLBs were subsequently subcultured for every 4 weeks in half strength liquid Murashige and Skoog (1962) media supplemented with 1mg/L BAP and 2% (w/v) sucrose. In histological and scanning electron microscope analysis, 4 weeks old *Mokara* Broga Giant PLBs cultures were utilised.

Histological Analysis

Fixation

Mokara Broga Giant PLBs were fixed in FAA (95% ethyl alcohol: glacial acetic acid: formaldehyde: water, 10:1:2:7) for 1 week. Fixation is a process whereby the biological tissues are preserved from decay, stops the biochemical reactions and also increase the mechanical strength or stability of the treated tissues.

Dehydration

The PLBs were rinsed with distilled water a period of 1 hour with every 15 minutes of rinsing. PLBs were then transferred into alcohol tertiary-

butyl alcohol, TBA which was done with varying concentration of (TBA) (50-100%); 50% TBA for 2 hours, 70% TBA for 2 hours, 85% TBA for 2 hours, 98% TBA for 2 hours and 100% TBA for 3 hours. Following that, the PLBs were treated with TBA I and TBA II for 3 hours respectively, and then treated PLBs were left overnight.

Clearing

PLBs were subsequently exposed to xylene for a period of 10 minutes followed by treatment xylene and wax for 30 minutes and subsequently samples were treated with wax I, II, and III (xylene and Shandon Histoplast Pelletised Paraffin Wax, Thermo Scientific) for 1 hour respectively at 60°C in an oven (Memmert, Germany).

Blocking, slicing and staining of samples

PLBs samples were then blocked and sliced using 6 Micron Microtome (Leica RM 2135). Then, the samples were then stained with safranin and fast green. Safranin, a basic dye which stains acidic features of tissue such as nuclei and lignified secondary cell walls that surround mature xylem cells in vascular bundles. Fast Green, an acidic dye that stains the basic components of tissue such as the cytoplasm and cellulosic primary cell walls. Slides were observed by light microscope (Olympus BX41).

Scanning Electron Microscope Analysis

PLBs samples were processed by freeze drying technique and the specimens were observed using Scanning Electron Microscope (Leo Supra 55VP-Ultra High Resolution analytical FESEM).

Results and Discussion

Propagation of orchids by the development of secondary PLBs from protocorms or PLBs has been defined for several orchids including *Phalaenopsis* (Chen and Chang, 2004; Murdad et al., 2006), *Dendrobium* (Saiprasad et al., 2004), *Aerides* (Sheelavanthmath et al., 2005) and *Cymbidium* (Teixeira da Silva and Tanaka, 2006). Numerous comparative analyses of plant responses to various types of plant growth regulators and the addition of organic additives to the culture medium to promote *in vitro* growth and proliferation of orchid is a common practice (Ichihashi and Islam, 1999; Chai et al., 2002; Islam et al., 2003; Rahman et al., 2004; Arditti, 2008, George et al., 2008).

The use of organic additives and PGRs may add toward the development of a simple and economical plant culture (Islam et al., 2003). Numerous comparative analyses of plant responses to diverse types of cytokinin (BA and kinetin) have revealed that the presence of BA in the culture medium produces higher frequencies of induction and proliferation of PLB in some orchids, such as *Dendrobium nobile*, *Dendrobium Densiflorum*, and *Cymbidium aloifolium* (Nayak et al., 2002; Sheelavanthmath et al., 2005; Luo et al., 2008). However, Teixeira da Silva and Tanaka (2006) specified that kinetin was more effective in promoting the formation of PLB in *Cymbidium* hybrids when supplemented together with NAA to the culture medium. In contrast, Chen et al. (2002) described that both BA and kinetin were equally effective for PLB proliferation in *Epidendrumradicans*.

Instead, Chai et al. (2002) described that the addition of a suitable amount of organic additives to the culture media significantly promoted the growth of PLBs in *Phalaenopsis*. This review displayed that varying conditions are desirable for diverse types of orchids to form their optimum PLBs growth. The presence of meristematic cells in PLBs can develop from PLBs wound surface, maturing into shoot primordial and can be converted into plantlets through somatic embryogenesis (Figure 1).

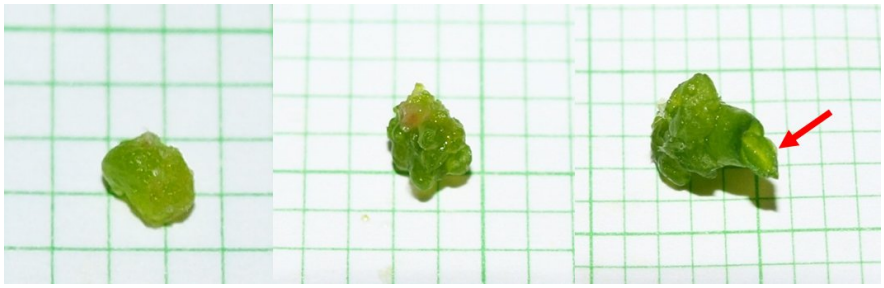

Figure 1. Direct induction and proliferation of protocorm-like bodies (PLBs) of *Mokara* Broga Giant in half strength MS medium (A), Multiplication of PLBs with secondary PLB formation (sPLB) forming cluster of PLBs (B), Proliferating PLBs forming shoots primordial (arrows) from the PLB base (C).

The histological observation showed that the protocorm-like body of *Mokara* Broga Giant orchid comprises of the shoot apical meristem (SAM) and leaf primodia of sheath leaves at the interior region and larger cells act as the storage area at the posterior region (Figure 2). Histological observation presented the presence of an area having dense cytoplasm located on the anterior side of the PLB, showing meristematic cells, embedded below it with parenchymatous tissue with thin-walled cells. This parenchymatic mass of cells protruded from the surface on the upper part of the protocorm and associated with vascular tissue (Figure 3).

Furthermore, dividing cells of the SAM were smaller in size and were more densely organized (Figures 2, 3). Further development of these cells gave SAM a shape of a dome. The shoot primordial (SP) was also noted and is different. They comprised of small cells, undergoing anticlinal and periclinal divisions, still surrounded by large, apparently isodiametric parenchyma cells (Figures 2, 3). SEM study observation using freeze drying method displayed the presence of enlarged globular PLBs shape which consists of epidermal cells with rough surface texture, which gradually tend to become rougher and wrinkled at the further developmental stages (4 weeks old) (Figure 3). The embryo axis also displayed the development of LP after formation of a constriction (C) at the top of the globular head (Figure 4). There are more than one constriction (C) can be detected on a single PLB head (Figure 4). The leaf primordial (LP) was also noted (Figures 4, 5). Even though wide research has been carried out on *in vitro* PLB in cultivated orchids, there are limited complete structural details known on the histological observation of PLBs (Park et al., 2002a).

In the histological analysis, we presented that the presence of parenchyma cells next to the meristematic cells. The parenchyma tissue close to the core played an evident storage function (Sgarbi et al., 2009). The histological and SEM studies carried out in the present work displays that the meristematic core was enveloped by a mass of parenchymatic cells. The existence of meristematic tissue at the leaf apex discloses a characteristic that is inherent to the Orchidaceae family (Churchill et al., 1973). This study offered the morphological development observed with naked eye is substantiated with the details based on histological as well as SEM observations.

Haensch (2004) described that *Pelargonium hortorum* observed that although globular and heart-shaped embryo-like regenerants were formed, histological analysis revealed that they lacked a defined root pole. In contrast to somatic embryogenesis where the globular-stage usually comprises of small cells with dense cytoplasm and large nuclei (Haensch, 2004), globular embryo-like structures had vacuolized parenchymatous cells (Salaj et al., 2005). Similar structures observed in other plant species have also been stated to as meristemoids, promeristems, meristemoid-like precursors (Hicks, 1994), protocorm-like bodies (Young et al., 2000; Tian et al., 2008), nodules (Batista et al., 2000; Xie and Hong, 2001; Ferreira et al., 2009) and nodular meristemoids (McCown et al., 1988). McCown et al. (1988) describe nodules as independent, spherical, dense cell clusters which are cohesively bound together and display consistent internal cell or tissue differentiation and loosely resemble protocorms. In contrast, a meristemoid can be described as a cluster of cells acting together as a meristematic centre (McCown et al., 1988). Hence, a histological approach is vital in distinguishing true somatic embryos and nodular meristemoids. Many published reports on somatic embryogenesis have drawn conclusions on the basis of morphological appearance only and should be reviewed critically (Bassuner et al., 2007). Histological approaches can offer critical information to allow the application of the most suitable *in vitro* plant regeneration methods (Woo and Wetzstein, 2008).

Histological and SEM observations through the presence of meristematic cells clearly presented that PLBs can develop from explants wound surface and can be converted into plantlets through sequential organogenesis or somatic embryogenesis. Since the PLBs comprised of multiple meristematic centres, it can be differentiated into shoot, leaf and then into plantlets gradually. Tian et al. (2008) reported that differentiation of globular cells led to formation of meristematic centers, which developed into PLBs. The SEM observations also definite that PLBs clearly exhibit shoot primordial and a meristem dome. The formation of PLBs is a unique characteristic of Orchidaceae and the term specifies a structure from induction of globular swellings until SP occur, without a root apparatus (Batygina et al., 2003).

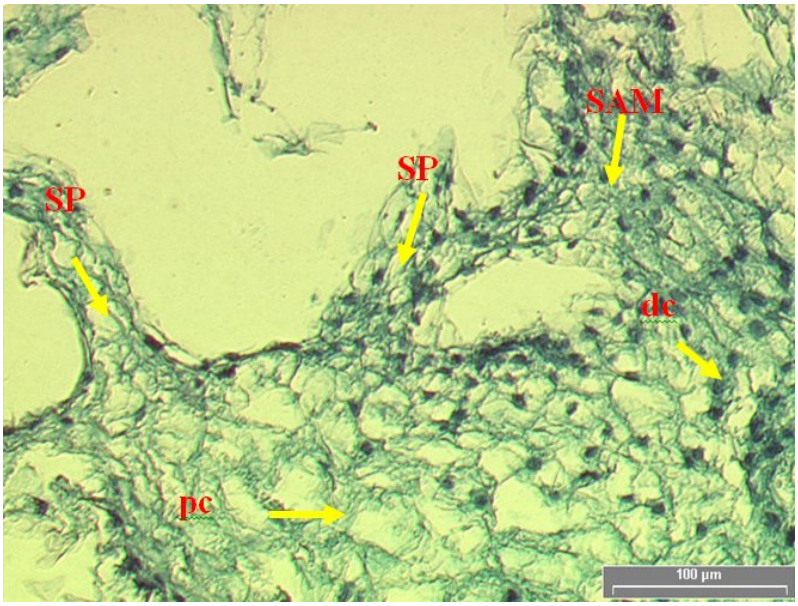

Figure 2. Histological section of *Mokara*Broga Giant's PLBs (100µm).
The cross section of PLBs, n; nucleus, dc; dense cytoplasm, SAM; shoot apical meristem, sp; shoot primordial, pc; parenchyma cell.

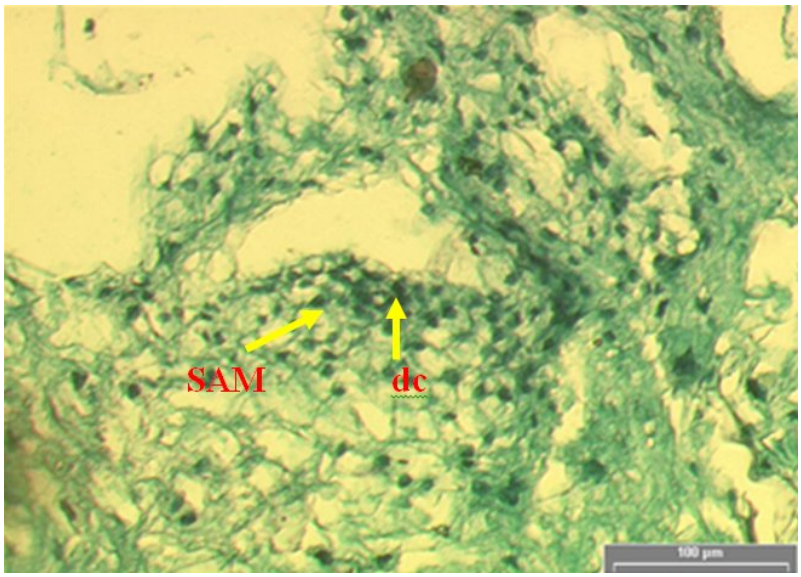

Figure 3. Histological section of *Mokara*Broga Giant's PLBs (100µm).
The cross section of PLBs showingSAM; shoot apical meristem, dc; dense cytoplasm.

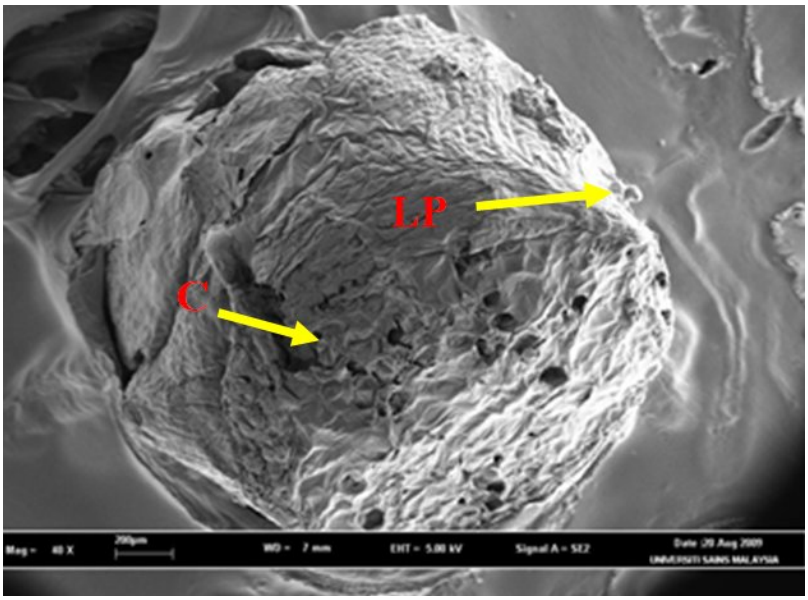

Figure 4. SEM of *Mokara*Broga Giant's PLBs (40X). The top view ofproliferating PLB at 4th week of culture showing constriction (C) and emergence of leaf primordia (LP).

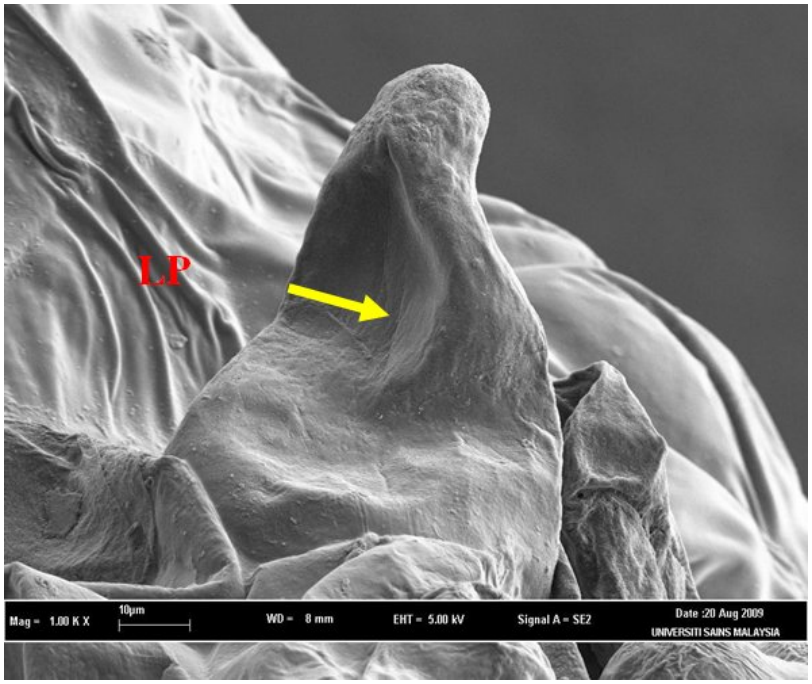

Figure 5. SEM of *Mokara*Broga Giant's PLBs (1.0 KX) showing the leaf primordial (LP).

Therefore, due to the existence of abundant meristematic tissues in PLBs, propagation of orchids using tissue culture techniques has been practiced for more than a century and has resulted in the production of uniform clones in many orchid genera. The formation of protocorms from germinated seed and the successive induction of protocorm-like bodies (PLBs) or callus from the protocorm, stem-node, shoot-tip, leaf, root-tip, or root-tuber explants has become a reliable technique for propagating orchids (Park et al., 2003; Anjum et al., 2006; Hong et al., 2008; Medina et al., 2009). Propagation through PLB formation is preferred by commercial growers of most orchid genera due to

the large number of PLBs that can be obtained within a moderately short period of time. The large-scale propagation of PLBs can also be accomplished using a bioreactor system (Park et al., 2000). PLBs are also the common target tissue for genetic transformation studies in orchids since they can proliferate rapidly and have high abilities to regenerate into complete plantlets (Liau et al., 2003; Sreeramanan et al., 2008). Furthermore, PLBs can also serve as an appropriate plant material for cryopreservation experiment (Yin and Hong, 2009). PLBs are well-differentiated tissues that are sometimes viewed as orchid embryos that develop with two discrete bipolar structures, namely, the shoot and root meristem. Consequently, these structures are capable to convert to plantlets certainly when grown on plant growth regulator (PGR)-free medium. Besides, the PLBs directly formed from meristem tissue will display an advance genetic stability than those produced by callus (Luo et al., 2008; Gantait and Uma Rani, 2012).

Conclusion

Therefore, the presence of abundant meristematic cells within the PLBs displayed importance in orchid propagation through orchid tissue culture which makes it to be a prospective explant for cryopreservation and genetic transformation studies.

Acknowledgements

This work was supported by Universiti Sains Malaysia Research Grant 2012 and Ph.D. Program.

References

Anjum, S., M. Ziaand and M. F. Chaudhary. 2006. Investigations of different strategies for high frequency regeneration of *Dendrobium malones* 'Victory'. Afr. J. Biotech. 5:1738-1743.

Arditti, J. 1992. Fundamental of Orchid Biology. John Wiley and Sons, Irvine, California. p.1-691.

Arditti, J. 2008. Micropropagation of orchids, 2nd edn. Blackwell Publishing Ltd., Maiden, MA, USA.

Bandyopadhyay, S. and D. Hamill. 2000. Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and comparisons with zygotic embryos found in mature seeds. Ann. Bot. 86:237–244.

Bassuner, B. M., R. Lam, W. Lukowitz and E. C. Yeung. 2007. Auxin and root initiation in somatic embryos of *Arabidopsis*. Plant. Cell. Rep. 26:1–11.

Batista, D., L. Ascensa˜o, M. J. Sousa and M. S. Pais. 2000. Adventitous shoot mass production of hop (*Humuluslupulus* L.) var. Eroica in liquid medium from organogenic nodule cultures. Plant. Sci. 151:47–57.

Batygina, T. B., E. A. Bragina and V. E. Vasilyeva. 2003. The reproductive system and germination in orchids. Acta. Biol. Cracov. Ser. Bot. 45:21–34.

Bomal, C. and F. M. Tremblay. 2000. Dried cryopreserved somatic embryos of two *Picea*species provide suitable material for direct plantlet regeneration and germplasm storage. Ann. Bot. 86:177–183.

Chai, M. L., C. J. Xua, K. K. Senthil, J. Y. Kim and D. H. Kim. 2002. Stable transformation of protocorm-like bodies in *Phalaenopsis*orchid mediated by *Agrobacterium tumefaciens*. Sci. Hortic. 96:213–224.

Chen, Y. C., C. Chang and W. C. Chang. 2000. A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. In Vitro Cell. Dev. Biol. Plant. 36:420–423.

Chen, L. R., J. T. Chen and W. C. Chang. 2002. Efficient production of protocorm-like bodies and plant regeneration from flower stalk explants of the sympodial orchid *Epidendrumradicans*. In Vitro Cell. Dev. Biol. Plant. 38:441–445.

Chen, J. T. and W. C. Chang. 2004. Induction of repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. formosashimadzu. In Vitro Cell. Dev. Biol. Plant. 40:290–293.

Chugh, S., S. Guha and R.U. Rao. 2009. Micropropagation of orchids: a review on the potential of different explants. Sci. Hortic. 122:507–520.

Churchill, M. E., E. A. Ball and J. Arditti. 1973. Tissue culture of orchids. I. Methods for leaf tips. New Phytol. 72:161–166.

Dalayap, R. M., M. A. J. Torres and C. G. Demayo. 2011. Landmark and outline methods in describing petal, sepal and labellum shapes of the flower of *Mokara* orchid varieties. Int. J. Agric. Biol.13:652–658.

Ferreira, S., D. Batista, S. Serrazina and M. S. Pais. 2009. Morphogenesis induction and organogenic nodule differentiation in *Populuseuphratica* Oliv. Leaf explants. Plant Cell. Tissue. Organ. Cult. 96:35–43.

George, E. F., M. A. Hall and G. Jan De Klerk. 2008. Plant propagation by tissue culture, 3rd edn. Springer SBS, Dordrecht.

Gantait, S. and S. Uma Rani. 2012. Rapid micropropagation of monopodial orchid hybrid (*Aranda* Wan CharkKuan 'Blue' x *Vanda*coeruleaGrifft. ex. Lindl.) through direct induction of protocorm-like bodies from leaf segments. Plant Growth Regul. 68:129–140.

Godo, T., M. Komori, E. Nakaoki, T. Yukawa and K. Miyoshi. 2010. Germination of mature seeds of *Calanthetricarinata* Lindl., an endangered terreserial orchid, by asymbiotic culture *in vitro*. In Vitro Cell. Dev. Biol. Plant. 46:323-328.

Gomes, F. L. A., F. F. Heredia, P. B. Silva, O. Faco and F. Campos. 2006. Somatic embryogenesis and plant regeneration in *Opuntiaficusindica* (L.) Mill. (Cactaceae). Sci. Hortic. 108:15–21.

Haensch, K. T. 2004. Morpho-histological study of somatic embryo like structures in hypocotyl cultures of *Pelargonium* x *Hortorum* Bailey. Plant Cell Rep. 22:376–381.

Hicks, G. S. 1994. Shoot induction and organogenesis *in vitro*: a developmental perspective. In Vitro Cell. Dev. Biol. Plant. 30:10–15.

Hong, P. I., J. T. Chen and W. C. Chang. 2008. Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of *Maudiae* type slipper orchid. Acta Physiol. Plant. 30:755–759.

Ichihashi, S. and M. O. Islam. 1999. Effects of complex organic additives on callus growth in three orchid genera, *Phalaenopsis, Doritaenopsis* and *Neofinetia*. J. Jpn. Soc. Hortic. Sci. 68:269–274.

Islam, M. O., A. R. M. M. Rahman, S. Matsui. and A. K. M. A. Prodhan. 2003. Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the *Doritaenopsis* orchid. Jpn. Agric. Res. Q. 37:229–235.

Liau, C. H., S. J. You, V. Prasad, H. H. Hsiao, N. S. Yang and M. T. Chan. 2003. *Agrobacterium tumefacien*-mediated transformation of an *Oncidium* orchid. Plant Cell Rep. 21:993–998.

Luo, J. P., Y. Wang, X. Q. Zha and L. Huang. 2008. Micropropagation of *Dendrobium densiflorum* Lindl. ex Wall through protocorm-like bodies: effects of plant growth regulators and lanthanoids. Plant Cell Tissue Org. Cult. 93:333–340.

Medina, R. D., E. A Flachsland, A. M. Gonzalez, G. Terada, M. M. Faloci and L. A. Mroginski. 2009. *In vitro* tuberization and plant regeneration from multimodal segment culture of *Habenaria bractescens* Lindl., an Argentinean wetland orchid. Plant Cell Tissue. Organ. Cult. 97:91–101.

McCown, B. H., E. L. Zeldin, H. A. Pinkalla and R. R. Dedolph. 1988. Nodule culture: a developmental pathway with high potential for regeneration, automated micropropagation and plant metabolite production from woody plants. In: J. W. Hanover and D. E. Keathley (Eds.), pp. 149–166. Genetic Manipulation of Woody Plants. Plenum, New York.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15:473–49.

Murdad, R., S. H. Kuik, K. S. Choo, A. L. Mariam, A. A. Zaleha and R. Rimi. 2006. High frequency multiplication of *Phalaenopsis gigantea* using trimmed bases protocorms technique. Sci. Hortic. 111:73–79.

Nayak, N. R., S. Sahoo, S. Patnaik and S. P. Rath. 2002. Establishment of thin cross section (TCS) culture method for rapid micropropagation of *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl. (Orchidaceae). Sci. Hortic. 94:107–116.

Park, S. Y., H. N. Murthy and K. Y. Paek. 2000. Mass propagation of protocorm-like bodies using bioreactor system and subsequence plant regeneration in *Phalaenopsis*. Plant Cell Tissue Organ Cult. 63:67–72.

Park, S. Y., E. C. Yeung, D. Chakrabarty and K. Y. Paek. 2002a. An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. Plant Cell Rep. 21:46–51.

Park, S. Y., H. N. Murthy and K. Y. Paek. 2002b. Rapid propagation of *Phalaenopsis*from floral stalk-derived leaves. In Vitro Cell Dev. Biol. Plant. 38:168–172.

Park, S. Y., H. N Murthy and K. Y. Paek. 2003. Protocorm-like body induction and subsequent plant regeneration from root tip cultures of *Doritaenopsis*. Plant. Sci. 164:919–923.

Rahman, A. R. M. M., M. O. Islam, A. K. M. A. Prodhan and S. Ichihas. 2004. Effects of complex organic extracts on plantlet regeneration from PLBs and plantlet growth in the *Doritaenopsis* orchid. Jpn. Agr. Res. Q. 38:55–59.

Rugkhla, A. and M. G. K. Jones. 1998. Somatic embryogenesis and plantlet formation in *Santalum album* and *S. spicatum*. J. Exp. Bot. 49:563–571.

Saiprasad, G. V. S., P. Raghuveer, S. Khetarpal and R. Chandra. 2004. Effect of various polyamines on production of protocorm-like bodies in orchid—*Dendrobium* 'Sonia'. Sci. Hortic. 100:161–168.

Salaj, J., B. Petrovska´, B. Obert and Pret'ova´. 2005. Histological study of embryo-like structures initiated from hypocotyl segments of flax (*Linumusitatissimum* L.). Plant Cell Rep. 24:590–595.

Sgarbi, E., M. Grimaudo and C. Del Prete. 2009. *In vitro* asymbiotic germination and seedling development of *Limodorum abortivum* (Orchidaceae). Plant. Biosyst. 143:114–11.

Sheelavanthmath, S. S., H. N. Murthy, B. P. Hema, E. J. Hahn and Y. Paek. 2005. High frequency of protocorm like bodies (PLBs) induction and plant regeneration from protocorm and leaf sections of *Aeridescrispum*. Sci. Hortic. 106:395–401.

Soon, T. E. 1989. Orchids of Asia. Times Book International, Times Center, Singapore.

Sreeramanan, S., B. Vinod, S. Sashi and R. Xavier. 2008. Optimization of the transient *Gus A* gene transfer of *Phalaenopsisviolacea*orchid via *Agrobacterium tumefaciens*: an assessment of factors influencing the efficiency of gene transfer Mechanisms. Adv. Nat. Appl. Sci. 2:77–88.

Talukder, S. K., K. M. Nasiruddin, S. Yesmin, L. Hassan and R. Begum. 2003. Shoot proliferation of *Dendrobium* orchid with BAP and NAA. J. Biol. Sci. 3(11):1058–1062.

Teixeira da Silva, J. A. and M. Tanaka. 2006. Multiple regeneration pathways via thin cell layers in hybrid *Cymbidium* (Orchidaceae). J. Plant. Growth Regul. 25:203–210.

Tian, C., Y. Chen, X. Zhao and L. Zhao. 2008. Plant regeneration through protocorm-like bodies induced from rhizoids using leaf explants of *Rosa* spp. Plant Cell Rep. 27:823–831.

Woo, S. M. and H. Y. Wetzstein. 2008. Morphological and histological evaluations of *in vitro* regeneration in *Elliottia racemosa* leaf explants induced on media with thidiazuron. J. Am. Soc. Hort. Sci. 133:167–172.

Xie, D. and Y. Hong. 2001. *In vitro* regeneration of *Acacia mangium* via organogenesis. Plant Cell Tissue Organ Cult. 66:167–171.

Yin, M. and S. Hong. 2009. Cryopreservation of *Dendrobiumcandidum* Wall. exLindl. protocorm-like bodies by encapsulation-vitrification. Plant Cell Tissue Organ Cult. 98:179–185.

Young, P. S., H. N. Murthy and P. K Yoeup. 2000. Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*. Plant Cell Tissue Organ Cult. 63:67–72.

Zhao, P., F. Wu, F. S. Feng and W. J. Wand. 2008. Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobiumcandidum* wall ex Lindl. In Vitro Cell Dev. Bio. Plant. 44:178–185.