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Original Article

Development of *in vitro* culture system for proliferation of protocorm-like bodies of *Oncidium* Golden Anniversary orchid

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Abstract

Oncidium orchid has high demand and economic value especially in floriculture industry. This study aimed to optimize a suitable medium for rapid proliferation of protocorm-like bodies (PLBs) of *Oncidium* Golden Anniversary orchid. Results from this study confirmed that ½ MS semi-solid medium displayed the highest growth index and total numbers of PLBs. Inclusion of 20g/L sucrose and 1mg/L BAP produced the highest PLB proliferation rate, while adding charcoal gave no significant effect. Half-moon PLB size contributes to the highest growth rate on using a thin cell layer (TCL) system. Hence, the best medium for *Oncidium* Golden Anniversary PLB proliferation was ½ MS semi-solid media supplemented with 20 g/L sucrose and 1 mg/L BAP.

Keywords: *Oncidium* Golden Anniversary, protocorm-like bodies, growth index, thin cell layer

1. Introduction

The Orchidaceae is one of the largest families of flowering plants and has more than 800 genera and 25,000 to 35,000 species (Khoddamzadeh *et al.*, 2011). *Oncidium* is one of the four best-known orchids in the world aside from *Dendrobium*, *Cattleya* and *Phalaenopsis* because of the highly valued horticultural flowers (Wang *et al.*, 2007). Orchids have become an economically important horticultural commodity in countries such as Malaysia, Thailand, Singapore, Australia, and several others (Chugh *et al.*, 2009). Shen and Chen (2012) reported that Malaysia and Taiwan are the major *Oncidium* producers in the world. This orchid has become an economically important crop and is cultivated for cut flowers and potted plants (Mengxi *et al.*, 2011). A common name for

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Oncidium is "Dancing Ladies" for the distinctive floral design that looks like ladies dancing in dresses. It has bright yellow flowers with red-brown markings on the petals borne in the shower of a hundred on each spray (Mahmood *et al.*, 2011). *Oncidium* Golden Anniversary is one of the popular orchid hybrids available in Malaysia. This hybrid orchid has high potential to be propagated at a rapid rate by using tissue culture technique.

Micropropagation of orchid using PLBs is more efficient than using seeds and adventitious shoots. As reported by Luo, Zha and Jiang (2003), a large number of PLBs can be generated in a short period, either on a solid or in a liquid culture medium. Many studies have revealed that one of the methods to improve the micropropagation process is based on culturing PLBs using optimized medium conditions (Luo, Wawrosch & Kopp, 2009; Shimura & Koda, 2004). The plant tissue culture success is mostly determined by nutrition and hormones provided in the media. Generally, an orchid's tissue culture medium is high in minerals, salt, growth regulators, vitamins, and water (Murdad, Latip, Aziz & Ripin, 2010). Carbon supplies energy and helps the plants photosynthesize their own food (Ferreira *et al.*, 2011).

Several studies have reported on the influence of plant growth regulators on tissue culture of orchids such as in *Oncidium* (Chen & Chang, 2000), *Phalaenopsis* (Khoddamzadeh *et al.*, 2011) and *Cymbidium* (Silva, 2014). Wu, Chen and Chang (2004) found that the growth rate and development of *Oncidium* 'Gower Ramsey' PLBs and plantlets were accelerated by using 2 mg/l kinetin and 0.5 mg/l zeatin. The ratio of auxin and cytokinin plays a role in PLB formation, which is genotype-specific (Arditti & Ernst, 1993). Thin cell layer (TCL) system is a simple and effective technique to easily perform mass clonal multiplication of an explant reproducibly (Silva, 2013). It offers an advantage over conventional *in vitro* culture methods in producing high-frequency plant regeneration (Pandey, Tripathi, Rai & Pandey-Rai, 2019; Zhao *et al.* 2007).

This is the first study on the establishment of an efficient proliferation system for *Oncidium* Golden Anniversary's PLBs and the optimization of thin cell layer technique for the promotion of rapid proliferation of PLBs.

2. Materials and Methods

2.1 Plant materials and culture conditions

Oncidium Golden Anniversary is a hybrid between *Oncidium sphacelatum* and *Oncidium Sarcatum* species, and was used as the starting material in this study. The Protocorm-like bodies (PLBs) of *Oncidium* Golden Anniversary orchid (Figure 1) were obtained from an orchid nursery in Serdang, Selangor, Malaysia. The PLBs were subcultured on half-strength MS medium (Murashige & Skoog, 1962) with 20 g/L sucrose and 2.75 g/L Gelrite at 25± 2ºC with 16-h photoperiod for 4 weeks.

2.2 PLB proliferation

Ten (10) newly formed PLBs were selected and cultured on MS media of different strengths namely ¼ MS, ½ MS, ¾ MS, and full MS, which were either semi-solid or liquid (80 rpm agitation on orbital shaker). The medium

Figure 1. *Oncidium* Golden Anniversary orchid. Scale bar = 1cm

giving optimum proliferation was then used in subsequent experiments. The half MS medium was supplemented with 15, 20 and 30 g/L of sucrose or alternatively glucose. Then, the media were tested for added Benzylaminopurine (BAP; 0, 0.5, 1, 1.5, 2 mg/L) in combinations with 1-Naphthaleneacetic acid (NAA; 0, 0.5, 1, 1.5, 2 mg/L). Inclusion of 1 or 2 g/L charcoal together with combination of 1 g/L charcoal and 1mg/L BAP were tested. The cultures were kept in culture room for 4 weeks at 25± 2ºC under 16-h photoperiod.

2.3 Thin Cell Layer (TCL)

The three (3) types of TCL technique used in this study were the whole PLBs (3-4 mm), half-moon PLBs (cut symmetrically) and transverse (tTCL) PLBs that were sectioned to 1-2 mm thickness (Figure 2). Explants were cultured on half-strength semi-solid MS medium added with 20 g/L sucrose, 1 mg/L BAP and 2.75 g/L (0.275%) Gelrite. The cultures were kept for 4 weeks at 25± 2ºC under 16-h photoperiod.

2.4 Statistical analysis

Data on PLB proliferation were recorded after four weeks based on growth index and number of newly formed PLBs per replicate. For growth index data, a 1g initial weight was measured and kept for four weeks to collect data used in the following formula:

$$\text{Growth index} = \frac{(\text{Final weight} - \text{Initial weight})}{(\text{Initial weight})}$$

Experiments were performed with ten (10) PLBs in each replicate, and with a total of three replicates. Results are reported for growth index and newly formed PLBs as mean ± SE. Data were subjected to analysis of variance (ANOVA) using SPSS software (version 21). Significant differences of means were determined by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

3. Results

3.1 Effects of physical state of medium and MS strength

Protocorm-like bodies (PLBs) proliferated in both semi-solid and in liquid media after 4 weeks. The growth index is based on fresh mass in Figure 3, and the number of

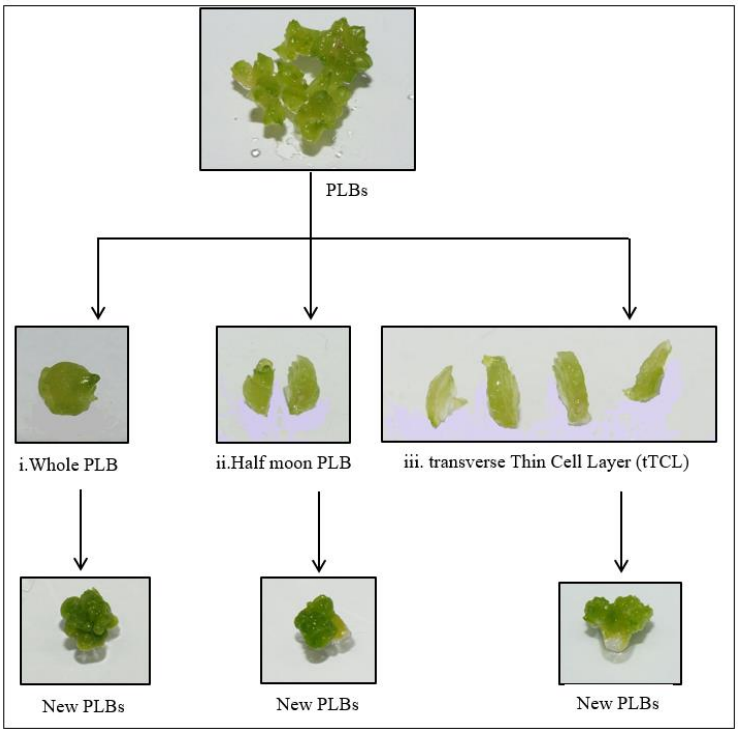

Figure 2. Schematic diagram of PLB proliferation from thin cell layer protocol using (i) whole PLB, ii) half-moon PLB, and iii) transverse thin cell layer (tTCL) of PLB

PLBs (Figure 4) on semi-solid media increased two- to three-fold compared to the liquid media. The PLBs cultured on semi-solid media remained green while PLBs in the liquid media slowly faded and turned brown. PLBs cultured in semi-solid media responded better than to liquid media. No significant difference was detected in the number of PLBs and their mass by strength of medium.

3.2 Effects of carbon source and its concentration

Oncidium Golden Anniversary orchid PLBs were cultured on half-strength MS medium added with different concentrations of glucose or sucrose (0, 15, 20 and 30 g/L). The maximum growth index (Figure 5) and maximum number of PLBs (Figure 6) were observed for 15 and 20 g/L of sucrose, as well at for 15 g/L of glucose (Figure 5). The absence of carbon and increasing concentration of glucose up to 30 g/L also decreased the growth index and the number of PLBs produced.

3.3 Effects of plant growth regulators (PGRs)

The effects of two (2) types of plant growth regulator (PGR) on the PLB proliferation of *Oncidium* Golden Anniversary are shown in Table 1. PLBs cultured on half-strength MS medium devoid of any plant growth regulators produced a relatively high growth index at 3.55 ± 0.29.

The number of PLBs was reduced when NAA was used in the culture medium without BAP. When 0.5 mg/L BAP was used together with or without NAA, the PLB growth index increased to be significantly higher than that of the control. The highest growth index was over 4.4 for 0.5 mg/L

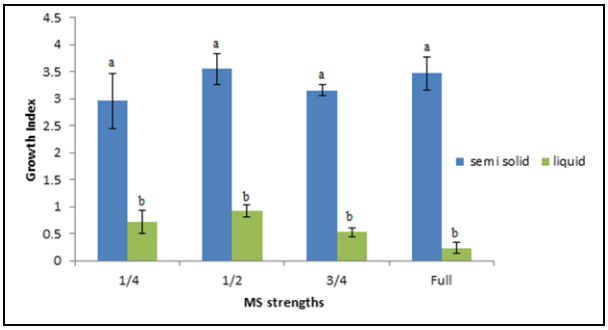

Figure 3. Effect of culture conditions and MS strength on growth index of PLBs after 4 weeks. Means with the same letters are not significantly different based on Duncan Multiple Range test at P≤0.05.

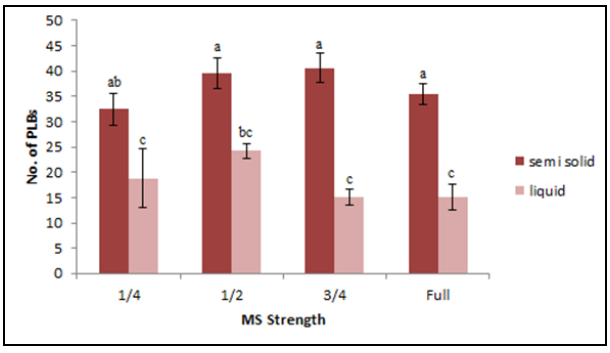

Figure 4. Effects of culture conditions and MS strength on number of PLBs after 4 weeks. Means with the same letters are not significantly different based on Duncan Multiple Range test at P≤0.05.

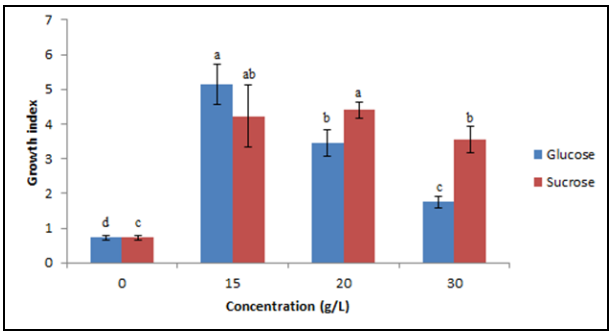

Figure 5. Effects of carbon source on growth index after 4 weeks. Means with the same letter are not significantly different based on Duncan Multiple Range Test at P≤0.05.

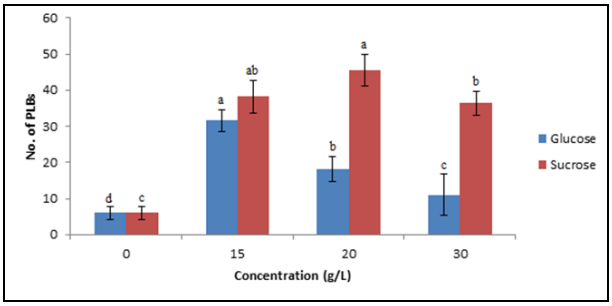

Figure 6. Effects of carbon source on number of PLBs after 4 weeks. Means with the same letter are not significantly different based on Duncan Multiple Range Test at P≤0.05.

Table 1. Effects of various plant growth regulators (PGRs) on growth index and number of PLBs after 4 weeks in culture

½ MS + PGRs (mg/L)		Growth Index	No. of PLBs per replicate	½ MS + PGRs (mg/L)
BAP	NAA			
0	0		3.55 ± 0.29 bcd	32.80 ± 1.16 abcd
	0.5		1.30 ± 0.02 f	26.00 ± 0.58 defgh
	1		1.89 ± 0.24 ef	21.67 ± 2.33 gh
	1.5		1.63 ± 0.22 f	20.67 ± 2.73 h
	2		1.60 ± 0.53 f	24.67 ± 1.76 efgh
0.5	0		3.97 ± 0.55 ab	37.00 ± 3.65 a
	0.5		4.79 ± 0.18 a	27.20 ± 1.07 defgh
	1		4.77 ± 0.33 a	29.80 ± 2.44 abcdef
	1.5		4.41 ± 0.18 ab	37.25 ± 3.09 a
	2		3.63 ± 0.18 bcd	29.80 ± 1.46 abcdef
1.0	0		4.80 ± 0.11 a	37.25 ± 2.73 a
	0.5		4.00 ± 0.28 ab	29.40 ± 1.33 bcdefg
	1		4.14 ± 0.33 ab	25.40 ± 1.96 defgh
	1.5		4.72 ± 0.50 a	21.80 ± 1.71 gh
	2		4.75 ± 0.14 a	27.40 ± 2.01 cdefgh
1.5	0		2.83 ± 0.59 cd	35.00 ± 5.18 ab
	0.5		3.57 ± 0.14 bcd	25.20 ± 1.62 defgh
	1		3.83 ± 0.09 abc	22.60 ± 1.08 fgh
	1.5		3.89 ± 0.13 ab	24.40 ± 2.40 efgh
	2		4.02 ± 0.27 ab	24.80 ± 0.66 efgh
2.0	0		2.77 ± 0.23 de	30.60 ± 1.63 abcde
	0.5		3.38 ± 0.43 bcd	26.60 ± 1.29 defgh
	1		3.80 ± 0.31 abcd	26.00 ± 1.92 defgh
	1.5		3.93 ± 0.31 ab	24.00 ± 2.12 efgh
	2		3.61 ± 0.18 bcd	25.80 ± 1.74 defgh

Values are given as mean±SE of 3 replicates. Means with the same letter are not significantly different based on Duncan Multiple Range Test at P≤0.05.

BAP with either 0.5, 1.0 or 1.5 mg/L NAA. The number of PLBs at these combinations was high from 27.20 ± 1.07 to 37.25 ± 3.09. With the supplement of 1.0 mg/L BAP regardless of NAA concentration, PLB growth index exceeded 4.0 and gave the highest number of PLBs. Inclusion of NAA with 1.0 mg/L BAP decreased the number of PLBs. A high growth index with a low number of PLBs implied that NAA increased the size of PLBs. Increasing the concentration of BAP to 2 mg/L affected PLB proliferation.

3.4 Effect of activated charcoal

Figure 7 shows the highest growth index with the inclusion of 2 g/L of activated charcoal in the MS medium. Treatment without activated charcoal displayed similar growth index as the addition of 1 g/L activated charcoal + 1 mg/L BAP. On other hand, PLB production was highest in the absence of activated charcoal (Figure 8). Addition of activated charcoal either alone or together with BAP showed no significant difference in the number of PLBs produced.

3.5 Effect of thin cell layer (TCL)

Aside from only using whole PLBs, also half-moon PLBs and transverse TCL (tTCL) PLBs were tested to compare the proliferation rates. Figure 10 shows that the half-moon PLBs produced a higher growth index (10.28) than the whole PLBs with no significant difference to tTCL. The PLB explants in three (3) types of TCL technique did not show any significant difference in number of PLBs produced, ranging from 33 to 41 PLBs per replicate (Figure 11). The PLB proliferation increased with culture time. The initial culture produced new PLBs after 4 weeks of culture (Figure 9 b and e). Subsequently, PLBs proliferated about four- to fiv-fold higher after 8 weeks (Figure 9 c, d, and f).

4. Discussion

Generally, a liquid medium offers optimum conditions and better aeration for nutrient uptake compared to solid medium, especially under bioreactor aeration or shaker agitation. However, the preferred conditions depend on many factors, including genus or species of plant. Puchooa (2004) studied the plantlet regeneration from *in vitro* grown leaf of *Dendrobium* orchid by optimizing different media, supplements, and conditions of the culture, and found that MS liquid medium added with 0.1 mg/l BA and 1.0 mg/l NAA produced 54 PLBs while solidified MS medium only produced 37 PLBs. Conversely, Park, Murthy and Paek (2002) reported that solid medium with 7% phyto agar in Hyponex media had improved PLB proliferation of *Phalaenopsis* by producing 18 PLBs compared to liquid cultures that only produced 5 PLBs. Chen and Chang (2001) studied *Oncidium* 'Gower Ramsey' to induce direct somatic embryogenesis on the leaf of the orchid and found the highest average number of 10.7 PLBs per explant on half-strength MS medium containing 20g/L sucrose, 2.8 g/L Gelrite and 1 mg/L TDZ. The current study also showed that semi-solid medium containing 2.75 g/L Gelrite could produce a higher PLB proliferation of up to 40 PLBs compared to liquid medium that only produced 25 PLBs. This semi-solid medium can support the PLBs so that they do not sink in, and provides

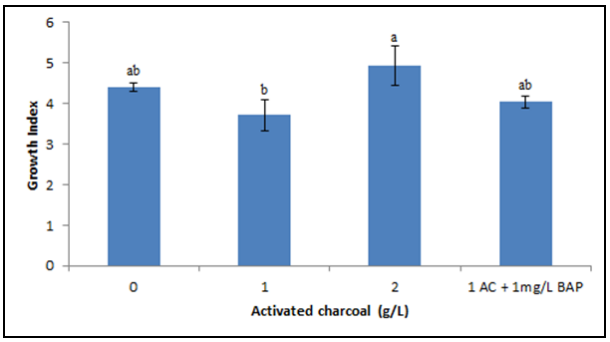

Figure 7. Effects of activated charcoal on growth index of PLBs after 4 weeks. Means with the same letter are not significantly different based on Duncan Multiple Range Test at P≤0.05.

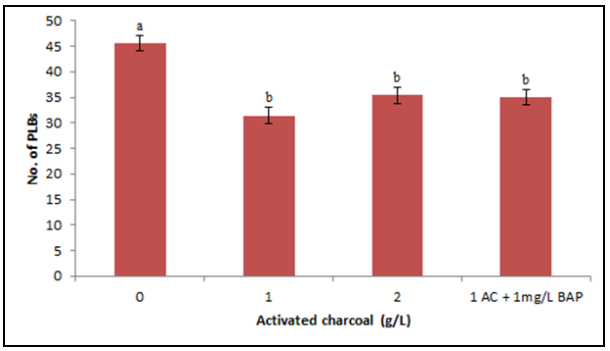

Figure 8. Effects of activated charcoal on number of PLBs after 4 weeks. Means with the same letter are not significantly different based on Duncan Multiple Range Test at P≤0.05.

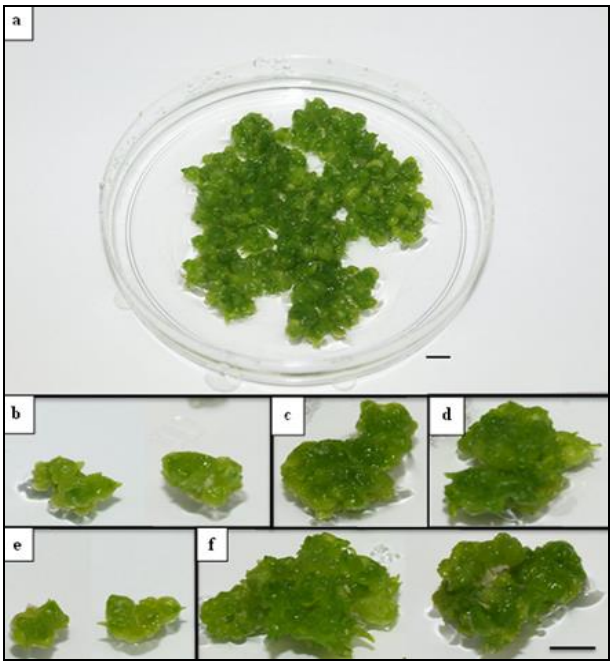

Figure 9. The production of *Oncidium* Golden Anniversary PLBs. a PLB proliferation on ½ MS medium supplemented with 1 mg/L BAP after 4 weeks, b PLBs from half-moon PLB after 5 weeks, c-d PLBs from half-moon PLB after 8 weeks, e PLBs from tTCL after 5 weeks, and f PLBs from tTCL after 8 weeks (*bar = 5mm*).

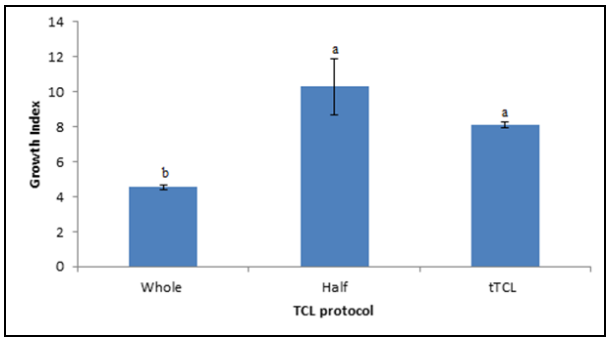

Figure 10. Effects of thin cell layer (TCL) protocol on growth index of PLBs after 4 weeks. Means with the same letter are not significantly different based on Duncan Multiple Range test at P≤0.05.

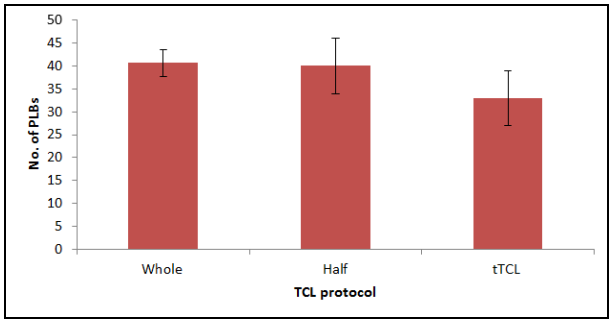

Figure 11. Effects of thin cell layer (TCL) protocol on number of PLBs after 4 weeks. Values are given as mean±SE of 3 replicates. Means with the same letter are not significantly different based on Duncan Multiple Range test at P≤0.05.

suitable conditions for PLB proliferation, contrary to the norm that liquid medium would give a better proliferation. Exposure of *Oncidium* PLBs to excess liquid led to hyperhydricity even though the liquid cultures were agitated on a shaker.

The different culture media with organic additives showed significant effects on the growth and development of PLBs of *Oncidium* Golden Anniversary orchid. The full strength MS medium has a high ionic concentration from nutrient salts. Therefore, ½ MS could sufficiently support rapid production of protocorms in orchids (Samalaa, Te-Chatob, Yenchonb & Thammasiric, 2014). Cardoso and Ono (2011) reported that shoot number of *Brassocattleya* orchid was high at 5 shoots per explant in reduced strength MS medium at lower concentrations of potassium and nitrogen. Maximum capacity of cell growth could be inhibited if the accumulation of potassium and nitrogen were high in the medium. Thus the ½ MS medium was suitable for PLB proliferation in this study, producing 40 PLBs while the ¼ MS medium produced 32 PLBs. Similar results were obtained in previous studies using different orchids such as in *Oncidium* which gave 10.7 PLBs (Chen & Chang, 2000), *Phalaenopsis bellina* (9 PLBs) (Khoddamzadeh *et al.*, 2011), *Grammatophyllum speciosum* (6.87 PLBs) (Samalaa *et al.*, 2014) and *Dendrobium* Broga Giant (12.3% proliferation rate) (Uddain, Gnasekaran, Zakaria, Lynn & Subramaniam, 2015).

Sugar has always been considered as a critical component of plant tissue culture medium. Sugars play a role as precursors needed for fatty acid and amino acid biosynthesis, besides being used as an intermediary in

respiratory metabolism and as a substrate for synthesis of complex carbohydrates (Bonga & Aderkas, 1992). A report by Borisjuk, Rolletschek, Wobug and Weber (2003) confirmed that both glucose and sucrose specifically improved cell development promoting the accumulation of reserves in plant embryos. Hong, Chen and Chang (2008) mentioned that the utmost amount of embryos in *Oncidium* was attained at 30 g/L of sucrose for cv. Gower Ramsey and at 20 g/L of glucose for cv. Sweet Sugar with 34 and 31 embryos, respectively. Furthermore, Nambiar, Tee and Maziah (2012) reported that sucrose enhances the proliferation of *Dendrobium* Alya Pink PLBs to 6.5 g PLBs compared to glucose which gave 0.9 g PLBs. Similar findings were obtained in this study with higher PLB proliferation recorded for addition of sucrose compared to glucose, with 45 and 20 PLBs, respectively. Sucrose is transported across the plasma membrane efficiently, making it broadly used in plant tissue cultures as the primary carbohydrate source to provide energy to the cells (Swamy, Sudipta, Balasubramanya & Anuradha, 2010).

Interaction between cytokinins and auxins plays an important role in control of many phases of differentiation, cell growth, and organogenesis in organ cultures. The requisite concentration of each plant growth regulator varies depending on the species cultured. For *Oncidium* Golden Anniversary, NAA increased PLB size when it was used with BAP at 1.0 mg/L, above which PLBs could reach growth index 3.8. BAP alone could improve PLB proliferation at a concentration between 0.5 and 1.0 mg/L up to 37 PLBs. BAP at 2.0 mg/L supported higher proliferation and growth rate of PLBs, except in combination with higher NAA concentrations. Hence, endogenous auxins are sufficient for the optimum development of PLBs. The effects of plant growth regulator(s) on the rate of PLB multiplication can vary by contents in the medium. Few studies have compared auxin and cytokinin effects in the tissue culture and have confirmed similar results as in this study. Chen *et al.* (2005) highlighted that exogenous auxins such as 2,4-D, IAA, IBA and NAA were found to inhibit direct somatic embryogenesis in *Oncidium* 'Gower Ramsey'. However, the inclusion of cytokinins (1 mg/l TDZ) supported the development of somatic embryogenesis in orchid to 10.7 embryos per explant (Chen & Chang, 2001). Saiprasad and Polisetty (2003) found that addition of 1.0 mg/L BAP in MS culture medium promoted a larger number of plantlets for the *Cattleya leopoldii* species besides *Oncidium* and *Dendrobium* orchid genera.

Activated charcoal is frequently used in orchid seeds' germination (Kauth *et al.*, 2008). The charcoal plays important roles in reducing browning and adsorbing complexes that are released from plant tissues together with vitamins, metal ions and PGRs. In addition, it can promote growth and minimize the deleterious effects of phenolics by binding complexes that are released from damaged explants, which alter and darken culture media (Thomas, 2008). In this study, the addition of activated charcoal increased mass growth of PLBs (growth index 5), while charcoal free medium promoted a higher number of PLBs (45) in MS medium in the absence of any plant growth regulators.

In this study, newly formed PLBs did not significantly differ by the type of PLBs used (whole PLB, half-moon PLB and tTCL PLB) that all produced 40 PLBs. Growth index from whole PLBs was lower (4.5) than from half-moon and tTCL PLB (10). The initial size of PLBs used is important and influences the plant growth. Silva (2013) reported that larger PLBs (more than 6 mm) might have already started to form adventitious roots and shoots. That the PLBs could be in the advanced developmental stage thus minimized the PLB-inducing possibility of new PLBs. Smaller PLBs have more severe tissue damage and produced less PLBs due to reduced surface area. Higher rate of PLB proliferation can be produced from this system and at the same time, it can reduce the consumption of sources of the PLBs since one whole PLB can be excised for a few explants. Dhir and Shekhawat (2014) used transverse thin cell layer (tTCL) explants of *C. bulbosa* to develop a rapid and a proficient protocol for direct adventitious shoot bud induction on medium containing 8.8 µM BA, producing 15.6 shoots per explant. This system also has been studied on other plants such as *Cymbidium* (Silva, 2013), *Dendrobium candidum* Wall ex Lindl. (Zhao *et al.*, 2007) and *Boerhaavia diffusa* (Pandey *et al.*, 2019). Usually tTCLs are always used as an explant because this includes a small number of cells from different tissue types such as cortical, epidermal, cambium, parenchyma cells, perivascular and medullar tissue, and therefore is an efficient propagation system (Silvia, 2003).

5. Conclusions

Effective medium for the proliferation of PLBs of *Oncidium* Golden Anniversary was obtained from half-strength Murashige and Skoog (MS) semi-solid medium by enriching it with 20 g/L sucrose and 1 mg/L BAP, for using either whole PLBs, semi-moon PLBs or transverse thin cell layers.

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