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PLANT TISSUE CULTURE

Efficient micropropagation of *Dendrobium* sonia-28 for rapid PLBs proliferation

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Abstract

Effective propagation of protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 was achieved in this study. The highest PLBs proliferation within 21 days was observed in semi-solid media over liquid culture. Half strength of Murashige and Skoog (MS) media produced the highest PLBs growth rate percentage compared to full and double strength MS media in semi-solid culture. In sucrose treatment, the highest increased of PLBs growth rate percentage was in 10g.L^{-1}. PLBs growth rate percentage was the highest (14%) in media devoid of exogenous 6-benzylaminopurine (BAP) or naphthalene acetic acid (NAA). However, the addition of BAP and NAA in combinations of BAP (4.44 or 8.88 µM) and NAA (8.88 µM) resulted in increased PLBs growth rate to 14% compared to when added separately. The optimized micropropagation media and condition of PLBs proliferation are important for the production of large number of plant material required for the development of genetic modification and cryopreservation studies.

Key words: Tissue culture, Orchid, *Dendrobium* sonia-28, Protocorm-like bodiese

Introduction

Dendrobiums are highly valued in the flower industry as potted plant and cut flower (Khosravi et al., 2008). Micropropagation provided an important breakthrough for mass propagation of many orchid species which have highly heterozygous genotype and have extremely slow sexual reproduction capability (Kanjilal et al., 1999).

Over the years, MS medium (Murashige and Skoog, 1962) widely used in many orchid's micropropagation protocol including *Dendrobium* genus (Martin and Madassery, 2006; Fadel et al., 2010). Optimum media salt concentration is essential for *in vitro* orchid to provide sufficient nutrient require to promote metabolism and cell growth and to prevent toxic effect of media salt (Fadel et al., 2010). Sucrose has been accounted to be superior to other sugars for *in vitro* orchid growth (Jawan et al., 2010). Sucrose is easily available to cell and directly participate in glycolytic and penthose phosphate pathways for cell growth and was also found to act as osmotic role to the culture system (Zha et al., 2007).

Culture system has a significant impact on the proliferation of PLBs of *Phalaenopsis* and *Doritaenopsis* (Liu et al., 2006). Liquid system is continuously shaken on automated rotator or roller type bioreactors to supply plantlets with ample oxygen for optimum respiration and equal nutrient distribution for enhanced nutrient uptakes (Kanjilal et al., 1999; Mehrotra et al., 2007). In some plant species, gelling agents is essential to prevent hyperhydricity which is typically seen on plant cultured in excess of water (Ibrahim et al., 2005). The roles of benzylaminopurine (BAP) and naphthalene acetic acid (NAA) are often varies for PLBs proliferation. Efficient induction of PLBs of *Dendrobium* sonia was observed on MS media supplemented with 4.44 µM BAP alone (Martin and Madassery, 2006). NAA was found to enhance the size of existing PLBs of *Cymbidium* orchid (Fujii et al., 1999).

The main objective of this study is to micropropagate *in vitro* PLBs of *Dendrobium* sonia-28 through the modification of the strength of culture media, level of sucrose, liquid or semi-solid media, and plant growth regulators supplementation.

Materials and Methods

Plant materials

Dendrobium sonia-28 (*Dendrobium Caesar* × *Dendrobium Tomie Drake*) is a pink-coloured popular orchid hybrid in Malaysia as a cut flower.

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Protocorm-like bodies (PLBs) were initiated aseptically by culturing nodal segment of *Dendrobium* sonia-28 in a semi-solid media and incubated at 25±2°C under 16 hours photoperiod. The PLBs were subcultured every four weeks to avoid shooting and rooting. The PLBs that was subcultured twice were used in the following treatments.

Preparation of different media compositions

Semi-solid MS media containing different types of macro nutrient (NH_4NO_3, $CaCl_2 \cdot 2H_2O$, $MgSO_4$ $7H_2O$, KH_2PO_4, KNO_3), micronutrients, vitamins, $Na_2EDTA.2H_2O$, Benzylaminopurine (BAP) sucrose and 2.75 g L^{-1} Gelrite. To test the effect of MS medium (Murashige and Skoog, 1962) strength on PLBs growth, different strengths of MS media (half, full [control], and double strengths) were prepared in both liquid and semi-solid media. Half strength of MS semi-solid media contain NH_4NO_3 (5 ml.L^{-1}), $CaCl_2 \cdot 2H_2O$ (5 ml.L^{-1}), $MgSO_4 \cdot 7H_2O$ (5 ml. L^{-1}), KH_2PO_4 (5 ml. L^{-1}), KNO_3 (5 ml. L^{-1}), micronutrients (5 ml.L^{-1}), vitamins (5 ml.L^{-1}), $Na_2EDTA.2H_2O$ (5 ml.L^{-1}), BAP (10 ml.L^{-1}) and 2.75 g L^{-1} gelrite. Similarly, full strength and double strength semi-solid MS media prepared two and four times higher all components accordingly except Gelrite concentration. To determine the effect of sucrose concentration on PLBs growth, various concentrations of sucrose (0 [control], 10, 20, and 30g.L^{-1}) were supplemented in half-strength MS liquid or semi-solid media. To test the effect of plant growth regulators (PGRs) on PLBs, various concentrations (0 [control], 4.44, 8.88 and 13.38 µM) and combinations of BAP and NAA were supplemented in semi-solid half-strength MS media.

Determination of growth rate percentage of PLBs

One gram of PLBs, marked as the initial fresh weight (FW), was cultivated into each media preparation. Liquid cultures were placed on orbital shaker set at 120 rpm. The PLBs were incubated under plant tissue culture room condition at 25±2°C and 16 hours photoperiod. After 21 days of incubation, the PLBs were weighed to obtain the final weight. The relative growth rate of the PLBs was defined as percentage of growth in gram per day. Growth rate percentages were calculated using the following formula (Sopalun et al., 2010):

$$Growth\ rate\ percentages\ (\%) = \frac{(Final\ FW - initial\ FW)}{Initial\ FW * Days\ of\ incubation} \times 100\%$$

Statistical analysis

The experiment was designed in a completely Randomized Design (CRD). All data were analyzed by one-way ANOVA using Statistical Package for the Social Sciences (SPSS) Software version 17.0 (SPSS Inc., Chicago, IL, USA). Comparisons of the mean and standard deviations were determined by Duncan's multiple range tests at $p<0.05$ level of significance.

Results and Discussion

Effects of different MS strengths on PLBs growth rate

Growth rate percentage of more than 12% were recorded for protocorm-like bodies (PLBs) cultivated in half strength MS in semi-solid media (Figure 1a). Increasing the strength of MS media to full- and double strength significantly ($p<0.05$) decreases the growth rate percentages of PLBs to 6.2% and -1.4% (from 1 g of initial fresh weight to 0.93g final fresh weight), respectively (Figure 1a). Growth rate percentages of PLBs grown in half strength MS media in liquid culture system was 5.9% (Figure 1b). Growth rate percentage of PLBs was also decreased in liquid culture as the MS strengths were increased (Figure 1b).

Reducing MS media concentrations significantly increased PLBs formation of *Grammatophyllum* orchid (Sopalun et al., 2010) and *Brassocattleya* orchid (Cardoso and Ono, 2011). In contrast, some orchid such as *Vanilla planifolia* needed the rich salt content of full strength MS to enhance early stage of *in vitro* growth (Geetha and Shetty, 2000). Adjusting MS concentrations is essential for each plant species to obtain optimum *in vitro* growth and morphogenesis development (as reviewed by Cardoso and Ono, 2011). Most favourable MS salt provides sufficient nutrient required to promote metabolism and cell growth at the same time prevent toxic effect of media salt (Fadel et al., 2010).

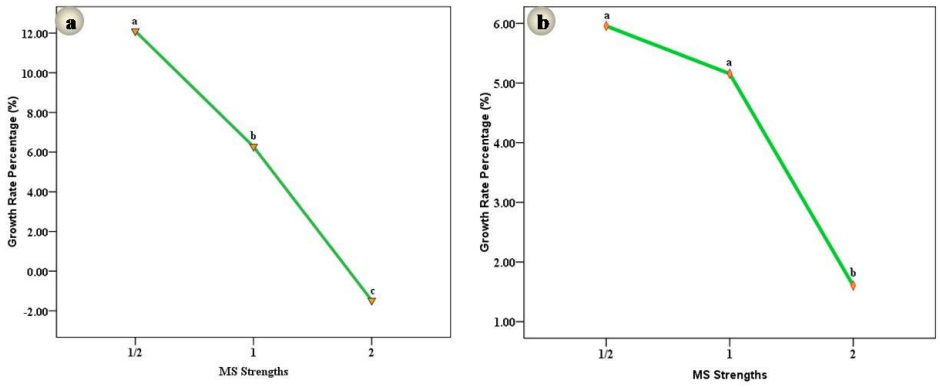

Figure 1. Growth rate percentage (Mean %±SD) of PLBs of *Dendrobium* sonia-28 in different MS strengths either in or liquid media. a=in semi-solid culture. b=in liquid culture. Means with different letter are significantly different (p<0.05).

Effects of different sucrose concentrations on PLBs growth rate

Low growth rate percentage was observed in semi-solid media (Figure 2a) and liquid media (Figure 2b) devoid of sucrose (0 g.L^{-1}). The addition of 10 g.L^{-1} sucrose into semi-solid and liquid culture system increases the growth rate percentages to the maximum growth rate of 12.8% (Figure 2a) and 8.1% (Figure 2b) respectively. Increasing sucrose concentration to 20 and 30 g.L^{-1} decreases the growth rate percentage of PLBs in liquid culture system (Figure 2b). The growth rate percentage of PLBs were significantly higher [t(142)=9.24, p=0.00] by two folds in semi-solid compared to liquid media (Figure 2c). The PLBs in MS media free of sucrose supply shows primordial leaf formation instead of forming PLBs (Figure 3a). PLBs sizes were larger in semi-solid media (Figure 3b-d) compared to PLBs in liquid media supplemented with sucrose.

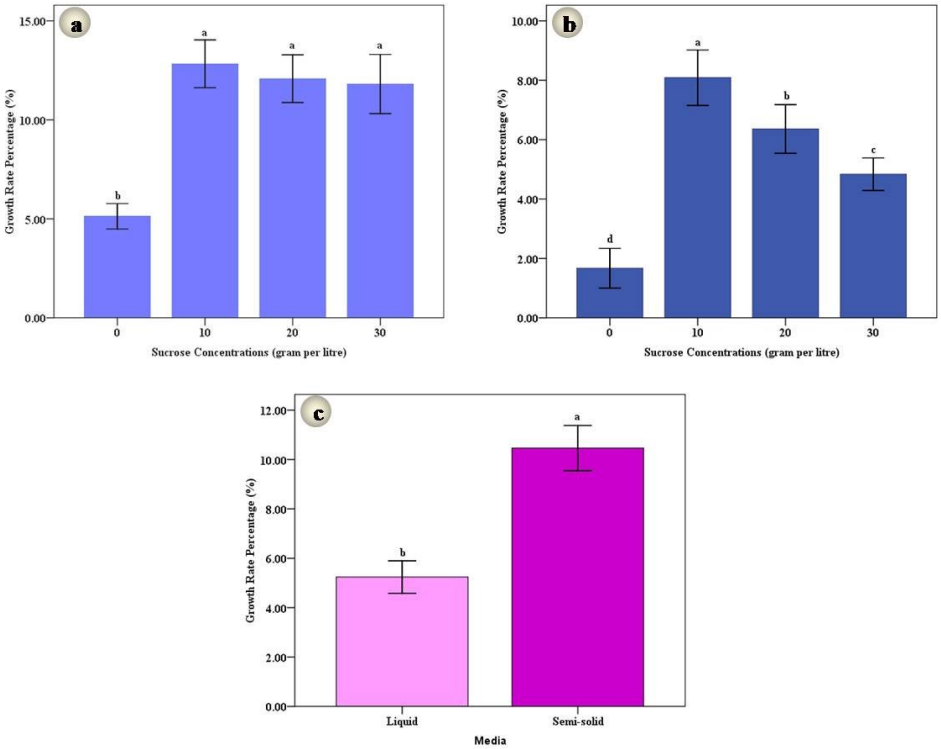

Figure 2. Growth rate percentage (Mean % ± SD) of PLBs of Dendrobium sonia-28 in different sucrose concentrations. a=in semi-solid culture. b=in liquid culture. c=in different sucrose treatment either in or liquid media (t=9.24. df=142. p=0.00). Means with different letter are significantly different (p<0.05).

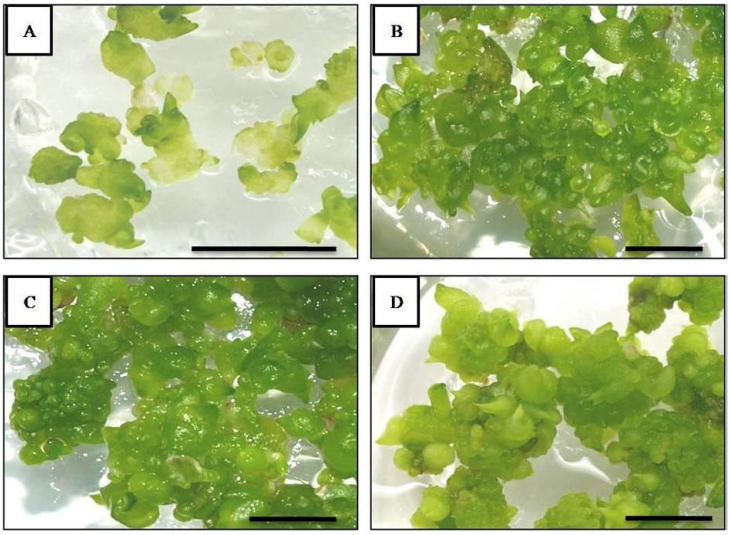

Figure 3. PLBs of *Dendrobium* sonia-28 cultivated in half strength MS media supplemented with different sucrose concentrations in semi-solid media. A=0g.L^{-1} sucrose; B=10 g.L^{-1} sucrose; C=20g.L^{-1} sucrose; D=30g.L^{-1} sucrose. Bars=5 mm.

The same result of 10 g L^{-1} sucrose was also observed in PLBs formation in *Phalaenopsis* Snow Parade (Tokuhara and Mii, 2003). On the other hand, 10 g.L^{-1} of sucrose was extremely inefficient for production of PLBs of *Dendrobium huoshanense* compared to 35 g L^{-1} sucrose (Zha et al., 2007). Deficiency of sucrose supply to *in vitro* orchid is detrimental for cell growth rate. Sucrose is specifically needed in plant embryo to increase cell division by cell encouraging cell expansion and reserve accumulation (Borisjuk et al., 2003). However, increasing sucrose over the threshold concentration could lead to excessive carbohydrate accumulation and hinder photosynthesis which eventually impairs cell growth of rose plant (Cappellades et al., 1991).

The efficiency of semi-solid media was also reported in embryo induction in *Avena sativa* compared to liquid media (Slusarkiewicz-Jarzina and Ponitka, 2007). Amendment of agar in media significantly affects the physical or biochemical changes of *in vitro* plants in response to water stress (Gopal et al., 2008). Symptom like hyperhydricity is commonly observed in plant with excess availability of water and nutrient (Ibrahim et al., 2005). Apart from that, the gelling agent in semi-solid media provide matrix of preventing the movement or spreading of phenolic compound which is detrimental to plant growth released by stressed plant tissue (Senaratna, 1992; Cervelli and Senaratna, 1995).

Effects of BAP and NAA concentrations on PLBs growth rate

High growth rate percentage (14.45%) was observed in media devoid of PGRs (Figure 4). Addition of either BAP or NAA reduces PLBs growth rate percentage with the lowest growth rate percentage (10.91%) was observed in the 13.32 µM BAP. PLBs growth rate percentage were improved in the media supplemented with the combinations of BAP and NAA compared to the usage of individual PGRs. Figure 5 illustrated the morphogenesis development of PLBs in BAP and NAA treatment. Shoot and primordial leaf formations were observed mainly in media supplemented with BAP and NAA combinations (Figure 5g).

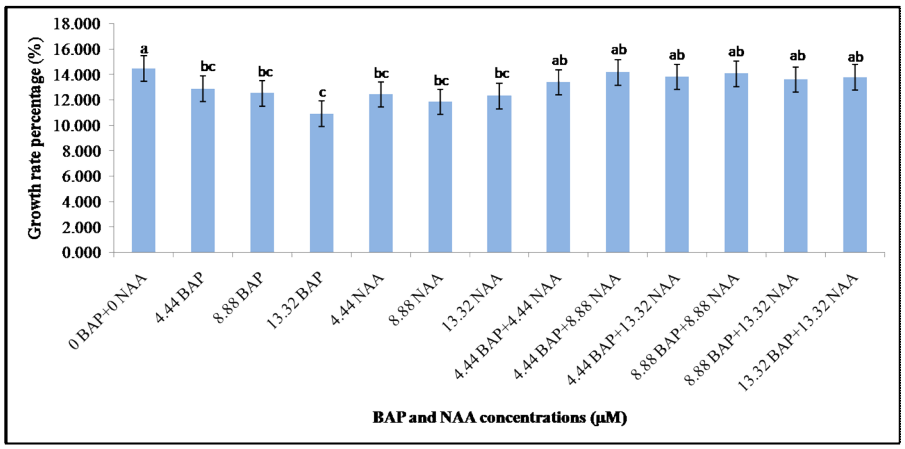

Figure 4. Growth rate percentage (Mean %±SD) of PLBs of *Dendrobium* sonia-28 in different BAP and NAA concentrations. Means with different letter are significantly different (p<0.05).

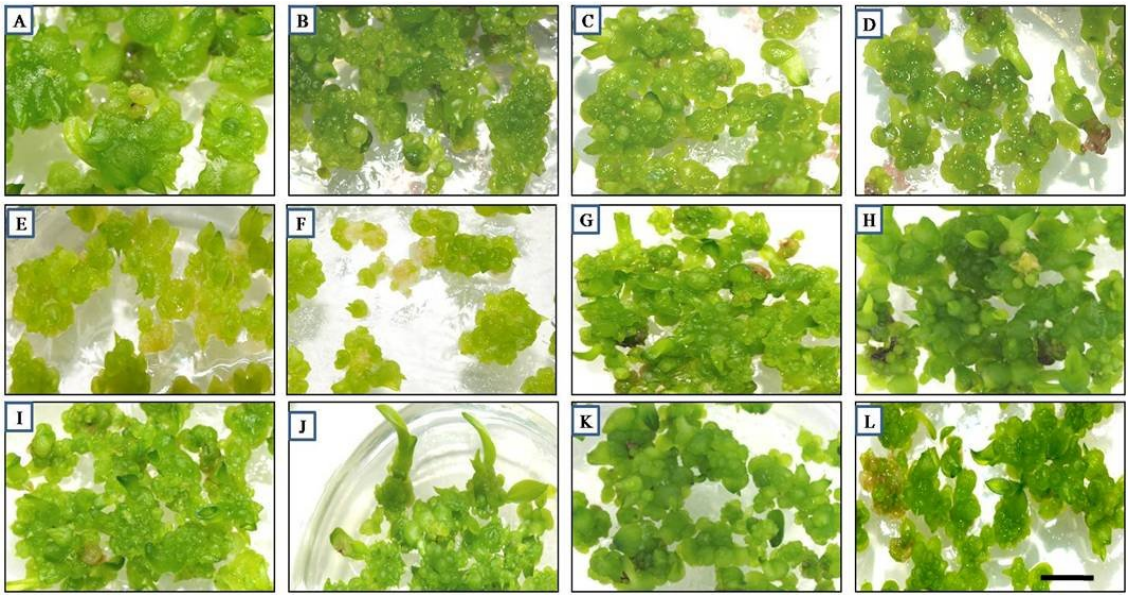

Figure 5. PLBs of *Dendrobium* sonia-28 cultivated on half MS media with different BAP and NAA concentrations. A=0 µM BAP, 0 µM NAA; B=4.44 µM BAP, 0 µM NAA; C=8.88 µM BAP, 0 µM NAA; D=13.32 µM BAP, 0 µM NAA; E=0 µM BAP, 4.44µM NAA; F = 0 µM BAP, 13.32 µM NAA; G = 4.44 µM BAP, 4.44 µM NAA; H=4.44 µM BAP, 8.88 µM NAA; I=4.44 µM BAP, 13.32 µM NAA; J=8.88 µM BAP, 8.88 µM NAA; K = 8.88 µM BAP, 13.32 µM NAA; and L=13.32 µM BAP, 13.32 µM NAA. Bar=5 mm.

PGRs free medium is adequate to induce the highest number of PLBs of *Dendrobium fimbriatum* (Roy and Banerjee, 2003). However, many have reported that PLBs formation were inhibited in the PGRs-free medium as observed *Cymbidium* (Pant and Pradhan, 2010), and *Phalaenopsis* (Niknejad et al., 2011) in which low concentrations ranging from 0.5 to 5 mg.L^{-1} were required for PLBs multiplications. A number of PGRs were reported to be least efficient when used individually compared to when applied in combination with other PGRs. Significant increase in PLBs proliferation were observed in the combination of 1 mg.L^{-1} BAP and 1 mg.L^{-1} NAA for *Dendrobium* hybrid (Khatun et al., 2010) and 1 gm.L^{-1} BAP with

2 mg.L^{-1} NAA for *Hygrochilus* orchid (Shadang et al., 2007).

Conclusion

In a conclusion, half strength of Murashige and Skoog (MS) semi-solid media supplemented with 10 g.L^{-1} sucrose, combinations of 4.44 or 8.88 µM BAP and 8.88 µM NAA produce higher PLBs proliferation rate in *Dendrobium* sonia-28 orchid.

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