Micropropagation of *Phalaenopsis* 'R11 x R10' Through Somatic Embryogenesis Method

Febriya Antensari, Totik Sri Mariani, Adhityo Wicaksono

School of Life Sciences and Technology, Institut Teknologi Bandung
Jalan Ganesha 10 40132 Bandung, Indonesia
E-mail: ve.bee04 {at} gmail.com

ABSTRACT--- ***Phalaenopsis 'R11 x R10' is one of hybrid orchids with is a potential commercial orchids as cut flowers and potted plants. Therefore, it is important to increase Phalaenopsis 'R11 x R10' production both in quantities and quality. Unfortunately it was difficult to propagate vegetative because Phalaenopsis is monopodial orchid which can not propagate by using keiki. Besides, the availability of good quality orchid seeds is limited due to genetic variability in traditional breeding. Micropropagation method has been providing good quality clones of orchid hybrid in large quantities with minimum genetic variability. In this research two carbohydrate variation (sucrose and maltose) with different concentrations (1%, 3%, and 6%) were tested on PLB proliferation as one of somatic embryogenesis method for improving it. The objective of this study has to evaluate the effect of sucrose and maltose in proliferation stage. The effect of 1%, 3%, and 6% sucrose, 1%, 3%, and 6% maltose, and 3% sucrose as control on proliferation, germination, and plantlet conversion of the PLB. After ten weeks observation, highest proliferation was shown on 3% sucrose as control (71.2 PLBs per PLB). Based on statistical analysis one-way ANOVA with post hoc test tukey test (p<0,05), the result shown significantly different response to sugar concentration and variation. Observation PLB germination was performed after incubating PLB proliferation germination for ten weeks. After eight weeks observation on conversion medium, highest PLB conversion (7 plantlets per ten PLBs) was achieved in PLB with 6% maltose treatment. It is concluded that 6% maltose is the optimum concentration in promoting plantlet conversion.***

Keywords--- Somatic embryo, Protocorm *like bodies*, Maltose, Sucrose, *Phalaenopsis*

1. INTRODUCTION

Orchids are the largest family of flowering plants and consist of more than 800 genera and 25,000 species, of which many are grown commercially worldwide as cut flowers and potted plants (Li *et al.* 2005). *Phalaenopsis* hybrid has been become common issue of commercial purpose lately. *Phalaenopsis* hybrid is a monopodial orchid which has difficulty in propagating vegetatively. Besides, the characteristics of seedling from traditional breeding are not uniform, long reproductive cycle and propagation through tissue culture has been desired (Li *et al.* 2005).

Several tissue culture techniques for micropropagation *Phalaenopsis* hybrid have been developed, including the culture of flower stalks with axillary buds, meristems, flower stalks explants, internodal segments of flower stalk (Arditti and Ernst, 1993), floral stalk derived leaves (Park *et al.* 2002), and root tips (Arditti and Ernst, 1993). Unfortunately, all techniques which are mentioned above have been inadequate for commercial scale due to protocorm like bodies (PLBs) so far were not optimal and have low percentage of vigour plantlet conversion (Jheng *et al*. 2006; Kosir *et al.* 2004). However protocol developed by Li *et al.* (2005) and Jheng *et al.* (2006) using osmotic treatment with high carbohydrate concentration of *Oncidium* enhanced *Oncidium* multiplication. In this paper effect of carbohydrate sources as sucrose and maltose are compared to enhance PLB proliferation and plantlet conversion of *Phalaenopsis* hybrid.

2. MATERIAL AND METHODS

2.1. Plant Material

Phalaenopsis hybrid named *Phalaenopsis* 'R11 x R10' was used as source material. This materials were cultured on MS medium (Murrashige and Skoog) for eight months and have been subcultured several times.

2.2. PLBs Induction

Phalaenopsis 'R11 x R10' leaves and root tips were cut and stem explants were cultured on half-strength MS (1/2 MS) medium supplemented with 44.4 and 88.8 μM BAP, 5.37 μM NAA, 15% coconut water (v/v), 0.01% active

charcoal (w/v) and 0.03% *Polyvinylpyrrolidone* (PVP) (w/v). The pH of medium was adjusted to 5.6 - 5.8, and solidified with 0.025% phytagel agar (w/v). One explant was cultured in a bottle of glass containing 15 ml medium. Explants were incubated at 25^0C and 16 h photoperiods. PLBs induction was observed after 12 weeks. Somatic embryos at PLBs induction phase were observed using light microscope.

2.3. Sucrose and Maltose Treatment on Proliferation of PLBs

Mature PLBs were separated and laterally cut. Two kinds of carbohydrate sources, sucrose and maltose (1, 3, and 6 % (w/v)) were individually added to PLBs proliferation medium. The basic medium used in all experiments consisted of half-strength MS (1/2 MS) supplemented with 88.8 µM BAP, 5.37 µM NAA, 15% coconut water (v/v) and 0.03% PVP (w/v). pH of medium was adjusted to 5.6 - 5.8, and solidified with 0.025% phytagel agar (w/v). Explants incubated at 25^0C and 16 h photoperiods. Two PLBs used in each treatment and the experiment was repeated 5 times. The number of proliferated PLBs was determined after eight weeks treatment without subcultured.

2.4. Germination and Conversion of PLBs

PLBs were subcultured on half-strength MS (1/2 MS) medium supplemented with 3 % sucrose (w/v), 10 % banana homogenate (w/v), 0.01% active charcoal (w/v) and solidified with 0,43% phytagel (w/v) for ten weeks for PLB germination medium. After these all germinated, PLBs were subcultured on half-strength MS (1/2 MS) medium supplemented with 5µM BAP, 3 % sucrose (w/v), 10% banana homogenate (w/v), 0.01% active charcoal (w/v) and solidified with 0,43% phytagel (w/v) for eight weeks for PLB conversion. The culture room was maintained at 27^oC. Ten PLBs were cultured on bottle glass, which contain 30 ml medium and the experiment was repeated 3 times.

2.5. Statistical Analysis

All experiments were arranged in random design. The data were statistically analyzed using one-way ANNOVA with *post hoc test Tukey HSD test* by SPSS

3. RESULT AND DISCUSSION

3.1. Somatic Embryos Induction and Development into PLBs

After three weeks being cultured, *Phalaenopsis* 'R11 xR10' explants formed shoots (Fig. 3.1 A) due to higher ratio of exogenous cytokinin rather than exogenous auxin on PLB induction medium. Somatic embryos were induced directly on basal segment of stem (Fig. 3.1 B) after five weeks without callus formation. Somatic embryos characters were white in color and granular. Somatic embryos grown and developed into PLBs after 12 weeks of cultured (Fig. 3.1 C) which had characteristic of increasing somatic embryos volume, white color of somatic embryos turning into greenish, and formed shoot and root apex polarity.

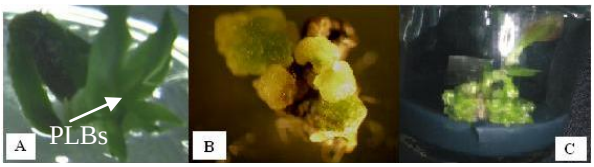

FIGURE 3.1 Somatic embryo development 'R11 x R 10' explants formed shoots (A), Somatic Embryos (SE) (B), Somatic embryos developed into PLBs (C).

Somatic embryogenesis induction on *Phalaenopsis* 'R11 x R10 showed basal segments of stem competence which dedifferentiated into embryogenic cells. It was caused by the presence of auxin exogenous NAA that was generally essential for embryo initiation (Jheng *et al.* 2006). Besides, George *et al.* (2008) reported that cytokinin exogenous BAP had activity to promote cell division.

So far, somatic embryogenesis of *Phalaenopsis* hybrid generally using leaf segments (Klo *et al.* 2005) or floral stalks which are derived from leaves as material source (Park *et al.* 2002). However, these methods have limitation and hard to replicate due to several leaves explants become necrotic so no embryo formation, embryo formation strongly affected by explants position and orientation, and needed longer culturing time. Therefore, stem segments more desirable material source for somatic embryogenesis of *Phalaenopsis* hybrid.

3.2. Effect of Sucrose and Maltose on PLBs Proliferation

After ten weeks incubated on proliferation medium, primary PLBs formed secondary PLBs.

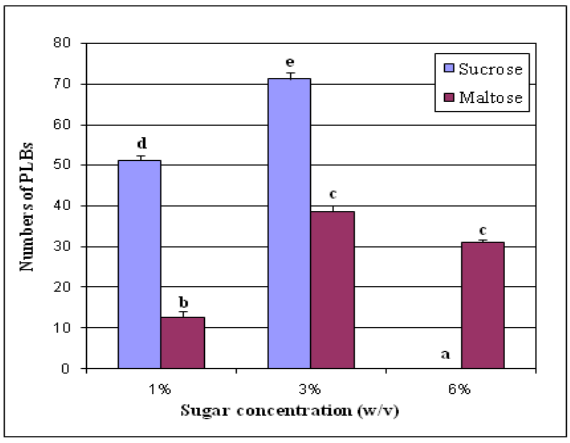

FIGURE 3.2 Numbers of PLBs *Phalaenopsis* 'R11 x R10' proliferation after ten weeks of culture. Sucrose 3% was control. Values are the average value of five replicates. The variably letters are significantly giving difference at P<0.05 with Tukey's HSD test.

Increasing sugar concentration lead two types of PLBs proliferation responses according to the type of sugar used (sucrose and maltose). When PLBs were maintained in a medium containing sucrose, the PLBs proliferation response was increasing to the sucrose; except for 6% sucrose (Fig. 3.2). The best response was observed with 3% sucrose. In this condition, average value of PLBs was 71.2 (Fig 3.2). For the 1% sucrose only 51.2 of the PLBs were formed, and was never observed for the highest sucrose treatment (Fig 3.2).

Different response was observed with increasing maltose concentration. When the 1% maltose was used, the amount of PLB increased and getting higher when 3% maltose was used (Fig 3.2). The average of PLBs increased steadily during means increasing maltose and reached 31.8 PLBs for 6% maltose (Fig 3.2). The average value of PLBs was 38.6 for 3% maltose (Fig 3.2).

PLBs proliferation on sucrose showed higher value than maltose, except for 6% sucrose (Fig 3.2). It is accepted that osmotic treatment such as increasing sugar concentration induced PLBs formation (Li *et al.* 2005). Sucrose on medium culture hydrolized faster into monosaccharide than maltose, so that the osmotic potential of sucrose in sugar component medium such as sucrose, glucose, and fructose will be lower than the total osmotic potential of maltose (George, 2008). In this condition, plasmolyzing stress affected on PLBs would be higher on sucrose than maltose. At 6% sucrose, PLBs have lead to cessation of growth and death due to imbalance water movement. Biahoua and Bonneau (1999) reported that higher sucrose concentration more than 5% was inhibitory embryos formation even lead embryos to necrosis (Stasolla and Yeung, 2003). Sucrose break down into fructose and glucose act through an osmotic effect, not as a carbohydrate source as reported Biahoua and Bonneau (1999).

From the experiment resulted that PLBs proliferation on *Phalaenopsis* 'R11 x R 10' are influenced by the sugar medium and carbohydrate in types and concentrations

Germination and Conversion of PLBs

Incubating PLBs on germination and conversion medium showed two responses, first PLBs only formed shoot (Fig. 3.3 A) and second, PLBs formed plantlet (Fig 3.3 B).

FIGURE 3.3 Germination and conversion of PLBs. Shoot germination (A), Plantlet conversion (B) (abbreviation: s = shoot, r = root).

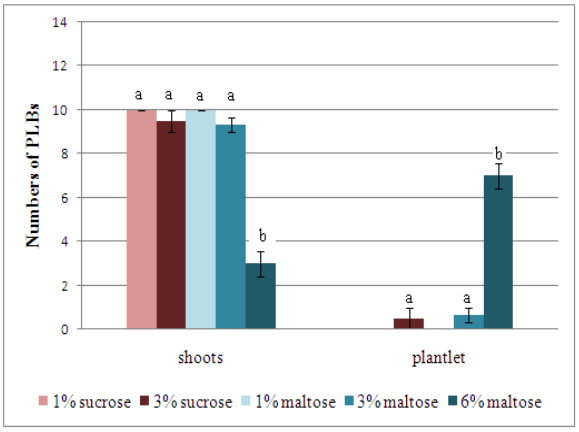

FIGURE 3.4 Numbers of PLBs *Phalaenopsis* 'R11 x R10' germination and conversion. Sucrose 3% was control. Values are the average value of three replicates. The variably letters are significantly giving difference at P<0.05 with Tukey's HSD test

In all sucrose and maltose treatments, PLBs germinated into shoot (Fig. 3.4). For PLBs germination into shoot, control and all treatments were not significantly different, except at 6% maltose which have shoot formation average values as 3 shoots (Fig. 3.4). PLBs maintained on 6% maltose showing the best plantlet conversion. In this condition, average value of plantlet conversion was significantly different with 3%. Plantlet conversion average value of 3% sucrose had 0.5 plantlet, while at 6% maltose had 7 plantlets (Fig 3.4). The plantlet conversion average values of control and 3% maltose showed steady (Fig. 3.4). In 3% maltose, average value of plantlet conversion was 0.5 plantlet (Fig. 3.4).

Stasolla and Yeung (2003) reported that embryos successfully germinate and converted into plantlets unless they achieve 'morphological' and 'physiological' maturity. Somatic embryos lack of physiological maturity due to not having desiccation period before germinate. It is accepted that improvement of embryos quality through manipulation of culture condition by using the increasing of sugar concentration. Jheng *et al.* (2006) reported that when substituted for sucrose, maltose was increasing efficiency of embryos to plant conversion. it might be because of maltose hydrolized into monosaccharide slower than sucrose. In this condition, maltose facilitated the creation of even more natural osmotic environment. In our study, the result of 6% maltose (Fig. 3.4) showed a significant effect on plantlet conversion. This result implies that the maltose can be more validly activate physiological maturity of PLBs than sucrose.

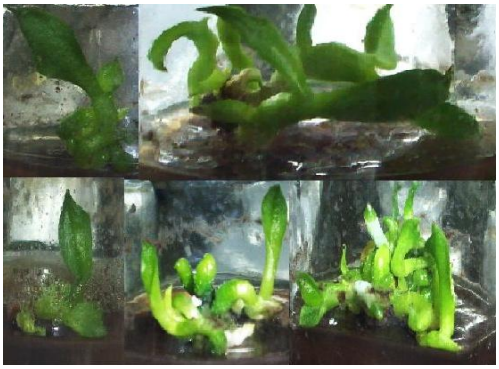

FIGURE 3.5 Shoots and roots development on conversion medium after eight weeks of culture. 1% sucrose (A), 3% sucrose (B), 1% maltose (C), 3% maltose (D), 6% maltose (E) (abbreviation: s = shoot, r = root).

All shoots on sucrose and maltose treatment developed, while root development only observed at 6% maltose treatment on conversion medium (Fig 3.5).

The similarity of shoot forming pattern by germination and PLB conversion resulted that one of the cause of root forming's failure in this experiment is incomplete PLB's maturation. This result concluded shoots and roots development at conversion medium affected by successfully on PLBs maturity. PLB's maturation success as explained in the paragraph above, is affected by the type and sugar concentration

4. CONCLUSION

These result concluded that proliferation of *Phalaenopsis* 'R11 x R10' showed the best response at 3% sucrose. The average value of PLBs proliferation was 71.2. The best response of plantlet conversion was on 6% maltose. In this condition, the average value of PLBs was 7 and showed shoots along with roots development on conversion medium.

5. REFERENCES

1. Arditti, J.and R, Ernst. 1993. *Micropropagation of Orchids*. John Wiley and Sons, Inc. : Canada
2. Biahoua, A and L. Bonneau. 1999. Control of in vitro somatic embryogenesis of spindle tree (*Euonymus europaeus* L) by the sugar type and the osmotic potensial of culture medium. *Plant Cell Reports* 19: 185-190
3. George, E. F., M.A. Hall and G.J De Klerk. 2008. *Plant Propagation by Tissue Culture 3rd Edition Volume I.* The Background. *Springer : Netherlands*
4. Ishii, Y., T. Takamura, M. Goi and M. Tanaka. 1998. Callus Induction and Somatic Embryogenesis of *Phalaenopsis*. *Plant Cell Reports* 17: 446-450
5. Jheng, F.Y., Y.Y. Do, Y.W. Liauh, J.P. Chung and P.L. Huang. 2006. Enhancement of Growth and Regeneration Efficiency from Embryogenic callus cultures of *Oncidium* 'Gower Ramsey' by Adjusting Carbohydrate Sources. *Plant Science* 170: 1133-1140
6. Li, S.H., C.S. Kuoh, Y.H. Chen, H.H. Chen. and W. H. Chen. 2005. Osmotic Sucrose Enhancement of Single-Cell Embryogenesis and transformation Efficiency in *Oncidium*. *Plant Cell, Tissue and Organ Culture* 81: 183-192
7. Park, S.Y., H.N. Murthy, and K.Y. Paek. 2002. Rapid Propagation of *Phalaenopsis* from Floral Stalk-Derived Leaves. *In vitro Cell, Development, Biology-Plant* 38: 168-172
8. Stasolla, C. and E.C. Yeung, E. C. 2003. Recent Advances in Conifer Somatic Embryogenesis: Improving Somatic Embryo Quality. *Plant cell. Tissue and Organ Culture* 74: 15-35
9. Trigiano, R.N. and D.J. Gray. 2005. *Plant Development and Biotechonolgy*. CRC Press LLC : Florida