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PRIMACY OF LIQUID MEDIUM TECHNIQUE ON PROTOCORM LIKE BODIES PROPAGATION OF *Phalaenopsis* SP ORCHIDS IN TISSUE CULTURE

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Abstract

Tissue culture have been used for plant propagation generally, and the medium has been important role in its growth. Vegetative propagation on Phalaenopsis sp orchids can be through the protocorm like bodies (PLB). Medium of affect on propagation of PLB was carried out on medium type, kind of basal medium and concentrations ratio of naphtaleneacetic acid (NAA) and benzylamino purine (BAP). The experiment used Completely Randomized Factorial Design with 3 replications and continued with the Duncan Multiple Range Test (DMRT) if there were significant differences. The results showed that the best callus formed in a combination of solid medium type and Murashige & Skoog (MS) basal medium was 100%. The most number of PLB produced from a combination of liquid medium types and a concentration ratio of NAA 1 mgL-1 and BAP 5 mgL-1, the most number of plantlet produced from a combination of MS basal medium and the concentrations ratio of NAA 0.1 mgL-1 and BAP 0.1 mgL-1, the number of PLB germination and PLB with leaves were influenced by each single factor.

Keywords : liquid medium, Phalaenopsis, Naphtaleneacetic Acid, Benzylamino Purine, Protocorm Like Bodies (PLB) and Regeneration

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1. Introduction

Phalaenopsis sp. has 64 species and Philippines has the highest diversity (21 species), then Borneo (16 species). Indonesia has 25 species and 10 of them are endemic [1]. Orchids is a popular ornamental plant, and it has high demand market. Florist prefer mass production of seedling plant through protocorm from seed, but it can produce orchids in vary genotype caused it's segregation. Production of seedling plants in identic genotype can do through *protocorm like bodies* (PLB) or embryo somatic and the orchids will have same quality. Technique of liquid medium through bioreactor and temporary immersed system were best alternative of mass production of PLB [2].

The development of somatic embryos is influenced by medium. Solid medium more widely used than liquid medium, probably solid medium has the optimal of oxygen. Liquid medium can increase embryonic callus, globular and heart phase of embryos [3]. Also, liquid medium results better in multiply shoots and roots, and increase fresh and dry weight than medium different concentration added of gelling agent of *Boswellia serrata* Roxb [4]. Kind of basal medium such as Vacin & Went (VW) and Murashige & Skoog (MS) are also widely used in clonal propagation studies of some orchids [5]. The VW and half strength of MS basal medium were used in research of *cattleya* orchids ploidi [6], MS was used to regeneration of *Phalaenopsis* sp through somatic embryos [7], and New Dogashima Medium was used in *Phalaenopsis* regeneration through PLB [8]. The basal medium provides nutrients and vitamin which are very necessary for the growth and development of explants. Thiamine increases the roots of the *Taxus brevifolia* Peattie wood plant to 61.5% while without thiamine only 30% [9],

Plant growth regulators (PGRs) often added to medium and it can determine the direction of plant development. The use of 0.5 mgL^{-1} NAA, 5 mgL^{-1} BAP and 0.5 mgL^{-1} IAA can produce the most roots in

Phalaenopsis sp [10], and concentration ratio of NAA 1 mg L^{-1} and BAP 15 mg L^{-1} can produce PLB from explants of plantlet leaves and increase PLB of *Phalaenopsis* orchids in bioreactors [2]. The novelty of research was liquid medium efficient to production of PLB, where liquid medium effective in mass production of embryo formed. And, development of embryo to establish shoot and root organ was affected by minimum exogen hormones and endogen hormones.

2. Materials and Methods

Explan and culture

Eksplan using *protocorm like bodies* trimmed base and apical tip within a size of 1-2 mm. Firstly, explants were planted in callus induction medium (CIM) for 2 weeks in dark conditions and 2 weeks later transferred to 16 hours light and 8 hours dark with a temperature of 24-25^{o}C, and continued to regeneration. The liquid medium type was shake using orbital shaker within 75 rpm. Secondly, the callus was transferred to regeneration medium. After 1 month then sub culture was carried out and culture maintained until the 90 days [11].

Medium.

1. Callus induction medium (CIM) was combination of medium type, basal medium and concentration ratio of NAA and BAP. Medium add sucrose 3% for MS basal medium and 2% for VW basal medium [12], gelrite 0.3 %, and organic supplement of coconut water 15%. Coconut water can help the development of embryos and induce cell division [13]. The container of liquid medium uses Erlenmeyer volume 50 mL and the solid medium uses petridish diameter 10 cm, which these glasswares product by Pyrex. Liquid medium was only for the process of propagation, then for the development embryo were transferred to solid medium [3].
2. The regeneration medium using solid type, Vacin & Went (VW) and Murahige & Skoog (MS) basal medium, and coconut water 15%, gelrite 0.3 %, charcoal 0.1% without the NAA and BAP. The CIM and regeneration medium was adjusted to pH 5.8, then autoclaved at 121^{o}C for 20 minutes [9].

Data analysis

Analysis data uses Completely Randomized Factorial Design within 3 replication, continued with Duncan Multiple Range Test if there were different significant (0.05). Application uses Excell program. Completely Randomized Factorial Designed used to knew the factor that affected from treatment, where treatment was given more than one factor, that were medium type, kind of basal medium, and ratio concentration of NAA and BAP.. Medium type namely solid and liquid, the kind of basal medium namely VW and MS, and concentration ratio of NAA and BAP namely NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}, NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}, NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}, NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}, NAA 1 mg L^{-1} and BAP 5 mg L^{-1}, NAA 1 mg L^{-1} and BAP 10 mg L^{-1}, finally there were 24 combinations.

3. Result and Discussion

3.1 The Explan Ability to Callus Formed

The best ability of explants to form callus was 100%, it obtained from combination of solid medium types and MS basal medium, the data shown in Table 1. The solid better than liquid medium in callus initiation, it was affected by the availability of oxygen, where the solid medium has abundat oxygen. The production of *Daucus* embryos decreased significantly if dissolved oxygen was reduced, where 600 somatic embryos per ml at 100% dissolved oxygen levels and 170 somatic embryos per ml at 10% dissolved oxygen levels [3]. Oxygen is important for plant growth, where oxygen needed for respiration. Respiration was enzymatic process that changes carbohydrates to produce pyruvic acid then pyruvic acid undergoes the oxidation process to produce acetyl co-A. Acetyl co-A was an important as raw material for intermediumte organic acids. Electron release was a consequence of the oxidation process and electrons were utilized in the electron transport circuit which produces ATP as growth energy [14]. Murashige & Skoog basal medium support to induce callus caused there was thiamine which has function in the respiration process of the Krebs cycle. The difference of callus initiation and PLB formed on solid and liquid medium types shown in Figures 1.

Table 1. Effect of combination between medium type and basal medium on callus formed.

Medium	Callus formed (%)
Solid-VW	98.33 ± 0.43 ab
Solid-MS	100 ± 0 a
Liquid-VW	99.17 ± 0.34 a
Liquid-MS	95.00 ± 0.75 b

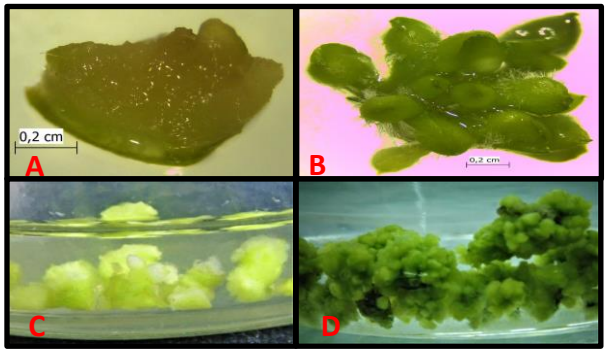

Figure 1. Growth in different medium type. Callus initiation and PLB formed from solid medium (A-B), and liquid medium (C-D).

3.2 Amount of protocorm like bodies (PLB).

The result of combination of liquid medium types and concentration ratio of NAA 1 mg L^{-1} and BAP 5 mg L^{-1} got the most number of PLB that was 45.5 PLB, the data shown in Table 2. Probably there a lot of cells of explants absorb nutrients and PGRs that dissolved in liquid medium, become more cells of explant that respond to form of PLB. The liquid outside the cell has a higher water potential than inside the cell, so the liquid flows from outside into the cells, the direction of flow originates from the high to low water potential [15]. Concentration ratio of NAA 1 mg L^{-1} and BAP 5 mg L^{-1} supported to produced most PLB because the influenced of BAP (cytokinin), which concentration of BAP higher than NAA (auxin). Cytokines influence cell division to be more active, and cells become more numerous. The large number of cells has the potential to form larger quantity of embryonic calluses and PLBs. The BAP concentration of 5 mg L^{-1} was the optimum concentration, where this concentration was most suitable also in the regeneration of *Phalaenopsis* from explants of leaves and roots forming embryonic callus, PLB and planlet of *Phalaenopsis* of 'Join Angle X Sogo Musadian' [9] and also BAP 5 mg L^{-1} in liquid MS basal medium produced the most plantlets in pineapple [16]. The PLB formed in regeneration medium was shown in Figures 2.

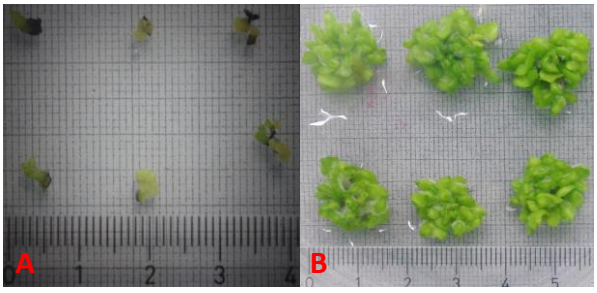

Figure 2. Condition of embryo in regeneration medium, 35 days (A) and 60 days (B).

Tabel 2. Effect of combination between medium type and concentration ratio of NAA and BAP on PLB numbers.

Medium	**PLB**
Solid- NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}	34.50 ± 2.47 ab
Solid- NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}	40.33 ± 6.13 ab
Solid- NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}	37.83 ± 0.35 ab
Solid- NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}	37.50 ± 5.54 ab
Solid- NAA 1 mg L^{-1} and BAP 5 mg L^{-1}	36.33 ± 2.36 ab
Solid- NAA 1 mg L^{-1} and BAP 1 mg L^{-1}	36.17 ± 2.47 ab
Liquid- NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}	37.67 ± 1.89 ab
Liquid- NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}	34.17 ± 2.47 ab
Liquid- NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}	36.67 ± 0.47 ab
Liquid- NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}	22.50 ± 4.60 c
Liquid- NAA 1 mg L^{-1} and BAP 5 mg L^{-1}	45.50 ± 0.83 a
Liquid- NAA 1 mg L^{-1} and BAP 10 mg L^{-1}	29.33 ± 1.18 bc

3.3 Protocorm like bodies germinations.

The PLB germination was PLB that has apical shoots and primordia leaves, as in Figure 3A. Protocorm like bodies germination was influenced by a single factor among medium type, kind of basal medium and concentrations ratio of NAA and BAP. The solid medium type produces PLB germination more quantity than liquid, it was 4.56 PLB, MS basal medium produces PLB germination more quantity than VW, it was 4.75 PLB, and the concentration ratio of NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1} produces the most PLB germination at 6.92 PLB. The data shown in Table 3.

Tabel 3 Effect of single factor on PLB germination.

Medium	PLB Germ.
Medium Type	
Solid	4.56 ± 0.29 a
Liquid	2.72 ± 0.19 b
Basal Medium	
VW	2.36 ± 0.16 b
MS	4.75 ± 0.28 a
Conc. ratio of NAA dan BAP	
NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}	6.92 ± 1.05 a
NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}	5.58 ± 0.77 ab
NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}	2.42 ± 0.42 c
NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}	2.83 ± 0.32 bc
NAA 1 mg L^{-1} and BAP 5 mg L^{-1}	2.25 ± 0.29 bc
NAA 1 mg L^{-1} and BAP 10 mg L^{-1}	0.83 ± 0.22 c

Protocorm like bodies germination was an important phase in the process of somatic embryogenesis because it will be continued to form of plantlet. Concentrations ratio of NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1} produced the most PLB germination because the concentrations given were an optimal amount as exogenous hormones and shoots formed was determined by endogenous hormones. The exogenous hormone of thidiazuron (TDZ) 0.3 mg L^{-1} was not

suitable for inducing bud of vanilla planifolia (*Orchidaceae*) and shoot differentiation occurred at TDZ removal [17]. Auxin can reduce the synthesis of cytokinins, the addition of auxins to accumulate in cells can suppress STM (SHOOT MERISTEMLESS) gene expression. Cytokinins function in embryonic-SAM initiation and flowering meristems, cytokinin levels were reduced by the presence of excess auxin [18]. Solid medium types produces more PLB germination than liquid medium types, possibly because the solid medium suitable for PLB to germinate. The development of embryos to form organs in regeneration was more suitable in solid medium or liquid medium with the method of temporary immerse system (TIS) [3].

The MS basal medium produces PLB germination more than VW basal medium because MS basal medium contains calcium. Inorganic compounds has an important role in cells, calcium plays a role in cell elongation and division, cell wall formation and stability, maintaining cell membrane structure and permeability [19]. And, the availability of N-organic from organic compounds such as glycine in MS basal medium was likely to play a role in PLB germination, where the use of N-organic from casein hydrolysate, peptone, and tryptone-peptone produced more shoot multiplication than controls [20]. Also, MS has sucrose 30000 mg L^{-1} which it more quantity than VW [11]. Sucrose was contributed to embryo development, PLB appeared on medium within the addition of sucrose, medium without sucrose did not produce PLB and optimum sucrose at concentration of 30 gL^{-1}[21].

3.4 Protocorm like bodies with leaves

Establishment of PLB with leaves were influenced by factor of basal medium and also concentrations ratio of NAA and BAP. The MS basal medium produced more PLB with leaves than VW, it was 2.56 PLB, and concentrations ratio of NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1} produced the most PLB leaves, it was 3.88 PLB. Data was shown in Table 4. The PLB's leaves were the continued growth of leaf primordia and the leaves come out of the first sequence followed by the second and subsequent leaves sequentially as a monocotyl plant mechanism [22].

The MS basal medium produced PLB with leaves more quantity than VW, this was the similar to variable of the number of PLB germinating. The reason was the primordia leaf develop to complete leaf. The lack of boron in VW basal medium results in disruption of normal cell lengthening in the meristem because it can inhibit DNA and RNA synthesis, cell division in the bud meristem was also inhibited. Most evidence of boron deficiency was impaired in the vascular and the entry of silicon (Si) into the cell wall. Zinc (Zn) plays a role in the formation of enzymes that were essential in their function, and was thought to play a role in the formation of chlorophyll and prevent damage. Copper (Cu) plays a role in the formation of enzymes, especially in the oxidation-reduction process, such as cytochrom oxidase. Molybdenum (Mo) was involved in the activity of the nitrate reductase enzyme, which converts nitrate ions into nitrites [14].

Tabel 4 Effect of single factor on PLB with leaves.

Medium	PLB leaves
Basal Medium	
VW	1.14 ± 0.10 b
MS	2.56 ± 0.19 a
Conc.Ratio of NAA and BAP	
NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}	3.83 ± 0.82 a
NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}	2.25 ± 0.44 ab
NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}	1.00 ± 0.20 ab
NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}	1.67 ± 0.48 ab
NAA 1 mg L^{-1} and BAP 5 mg L^{-1}	2.08 ± 0.19 b
NAA 1 mg L^{-1} and BAP 10 mg L^{-1}	0.25 ± 0.04 b

3.5 Plantlets

Planlet was the final development of somatic embryos in the small plants formed, with leaf and root organs. The most planlets formed in treatment of combination of MS basal medium with concentration ratio of NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}. Data shown in Table 5.

Table 5. Effect of combination basal medium and concentrations ratio of NAA and BAP on plantlets.

Medium	Planlet
VW- NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}	0.00 ± 0 c
VW- NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}	0.00 ± 0 c
VW- NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}	0.00 ± 0 c
VW- NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}	1.00 ± 0.71 ab
VW- NAA 1 mg L^{-1} and BAP 5 mg L^{-1}	0.17 ± 0.12 bc
VW- NAA 1 mg L^{-1} and BAP 10 mg L^{-1}	0.17 ± 0.12 bc
MS- NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}	1.50 ± 0.12 a
MS- NAA 0.1 mg L^{-1} and BAP 5 mg L^{-1}	0.17 ± 0.12 bc
MS- NAA 0.1 mg L^{-1} and BAP 10 mg L^{-1}	0.33 ± 0.24 bc
MS- NAA 1 mg L^{-1} and BAP 0.1 mg L^{-1}	0.00 ± 0 c
MS- NAA 1 mg L^{-1} and BAP 5 mg L^{-1}	0.17 ± 0.12 bc
MS- NAA 1 mg L^{-1} and BAP 10 mg L^{-1}	0.00 ± 0 c

Figure 3. Further development of somatic embryo *Phalaenopsis* sp. A. Protocorm like bodies germination, B. Protocorm like bodies with leaf, C. Planlet.

Roots of planlet were adventive roots and the most of roots grow in MS basal medium with concentrations ratio of NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}. The reason was MS basal medium contains useful compounds for root induction including mio inositol and thiamine. Mio-inositol can improve plant growth and morphogenesis. Thiamine together with cytokines play a role in root induction and callus growth, and nicotinic acid is needed in plants for synthesis amino acids and support carbohydrate metabolism [23]. The combination of IAA, NAA, IBA with CuSO4 1 µmol L^{-1} can increase 20x sorghum roots compared to control in MS basal medium in vitro [24]. Concentration ratio of NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1} support the root form because concentration was optimal and endogenous hormones can induce roots without experiencing obstacles. Estabilishmen of root was directly determined by endogenous hormones, but exogenous auxins was indirectly influenced root formation by that saturate cells to prevent cells from maturing and then form callus [25]. Auxin has a very important role, and there were interactions between auxin and cytokinins in the root meristem development. Interactin of auxin and cytokinin occur in the root transition zone. ARR1 was a signal regulating protein from cytokinins, if there was cytokinin application from the outside then the protein binds to the SHY2 promoter. SHY2 was part of the auxin in the tissue transport (vascular), so that there was no expression occur in SHY2. As a result, there is no regulation in the vascular due to loss of SHY2 transcription function and irregularities in the form of enlarged meristems or vice versa [18]. Higher cytokinin concentrations are likely to result in the mechanism mentioned above so that it causes disruption of plant root formation.

CONCLUSION

The type of medium between liquid and solid influences in propagation of PLB, which combination of the liquid medium and concentration ratio of NAA 1 mg L^{-1} and BAP 5 mg L^{-1} produced most of PLB, it was 45.50 PLB. The kind of basal medium such as MS can provide good nutrition for the development of somatic embryos. The optimum concentration ratio to result planlet in somatic embryogenesis of *Phalaenopsis* sp was NAA 0.1 mg L^{-1} and BAP 0.1 mg L^{-1}.

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