CELL BIOLOGY & DEVELOPMENT
Volume 1, Number 1, June 2017 E-ISSN: 2580-4499
Pages: 36-40 DOI: 10.13057/cellbioldev/v010107

Effects of encapsulation matrix on physical properties and germination viability of calcium-alginate encapsulated *plbs* of *Grammatophyllum scriptum*

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Manuscript received: 26 November 2016. Revision accepted: 28 June 2017.

Abstract. *Pitoyo A, Anggarwulan E, Ariza I. 2017. Effects of encapsulation matrix on physical properties and germination viability of calcium-alginate encapsulated plbs of* Grammatophyllum scriptum. *Cell Biol Dev 1: 36-40. Grammatophyllum* is a tropical epiphytic orchid commonly found in the moist areas of South-East Asia. Like most orchid species, the genus comprises species with a very small, micro-size seed mass and lack endosperm. These plants commonly need an in vitro culture for mass propagation and seed germination. Their undeveloped embryos developed globular mass cells, a protocorm after germination. Occasionally, a structure similar to protocorm arises from tissue other than an embryo; thereby, the term protocorm-like body (*plb*) was introduced. Here, we develop synthetic seed hydrogel beads encapsulated *in G. scriptum* plbs, possibly germinating the seed and growing their embedded tissue. The objective of the research was to study the effects of the proportion of G scriptum Bl encapsulation matrix. Synthetic seed made by complexing sodium alginate with $CaCl_2$ on physical properties and germination of protocorm-like bodies (*plbs*) embedded inside the hydrogel. The experiment was designed by a single factor-completely randomized design with the treatments of several combinations of Na-alginate/$CaCl_2$ratios.The result showed that $CaCl_2$ in all concentrations except 25 mM formed spherical hydrogel beads in all levels of Na-alginate. Alginate in concentrations of 2% and 3% gave the optimum result represented by a maximum germination index of 100%. The formation of the new plbs varied among different explants, even in a single explant. The germination time of each synthetic seed varied from 2 weeks to eight weeks after encapsulation. In conclusion, physical properties have no significant barrier for developing *plbs* to emergencies through penetration encapsulation matrix.

Keywords: CaCl2, *Grammatophyllum scriptum*, Na-alginate, synseed

INTRODUCTION

Orchidaceae is one of the diverse and widespread families of flowering plants comprising more than 24,000 species and categorized in 800 genera (Fay and Chase 2009). They occupy a wide range of ecological habitats from tropic to temperate climatic regions but exclude sea water and extremely cold environments (Tan et al. 1998). The amazing flower morphology has put them in a dilemma where their benefit faced conservation issues. Numerous orchid species are economically well-known plants in floriculture industries. Unfortunately, a collection of their native species for illegal trading and habitat destruction has made them in a thread situation (Kull et al. 2006).

The genus *Grammatophyllum* is a large or giant, tropical epiphytic orchid commonly found in the moist areas of South-East Asia. Its members have two distinctive pseudobulb types: some species with very large, long stem-like structures and others with short conical ones. *G. scriptum* is a species with sort conical pseudobulbs with two or three oblanceolate leaves placed near the apex. Its relative species, G. speciosum and G. papuanum are orchids with long stem-like pseudobulb and linear, acute leaves spread in two rows along the length. The inflorescence is about 2 meters, bearing the first apical half inserting closely full flowers and the bottom half occasionally placing some distorted flowers with a wider position. The flowers are 10 cm or more broad. Previous reports indicated that *G. scriptum* was found in many areas in Indonesia, such as Lamedai Nature Reserve, Kolaka, and Southeast Sulawesi (Lestari and Santoso 2011). However, despite the members of *G. scriptum* easily found in cultivation areas or nurseries, their position in the wild has now been classified as rare.

Effective propagation and ex-situ conservation are the key factors that must be seriously managed to save orchids in nature (Fay 1994; Sarasan et al. 2006). However, their reproductive nature was dependent on their association with other organisms. This phenomenon has been considered the consequence of their flower structure influencing their pollination biology. On the other hand, the seed produced from successful fertilization is lacking in the endosperm, so there must be a co-relation with mycorrhiza to acquire a nutrient from environmental surroundings for the development of small immature embryos.

Plant tissue culture has been familiar in orchids' mass propagation because of the low preference of the seeds to germinate and only a small number of new individuals formed through conventional vegetative propagation. Symbiotic dependency with fungal mycorrhiza for germination of their micro-size and lacked endosperm seeds were ignored by culturing them in the aseptic rich-

nutrition medium. Numerous successful attempts in orchid mass propagation via tissue culture have been recorded in some reviews. The medium in axenic condition discovers a symbiotic germination technique of Cymbidium orchid seeds and subsequent successful attempts (Yam and Arditti 2009). That *in vitro* technique was also visible for fixation of elite genotype by multiplying somatic tissue and generating their derivative through organogenesis as well as embryogenesis to become plantlets. Some combination with cryopreservation technique *in vitro* culture over an efficient tool for germplasm conservation for future benefits (Engelmann 2010).

Products of tissue culture such as plantlets, somatic embryos, callus, or protocorm-like bodies (*plbs*) could not easily introduce to the greenhouse or field because of the different environments inside and outside the bottle. The situation remains problematic for consumers with insufficient background in tissue culture. Thus, we have developed a synthetic seed, a *plb* encapsulated calcium-alginate hydrogel, to help the plantlet survive in the field or greenhouse successfully. Moreover, *Plbs* might be representatives of somatic embryos in Orchidaceae. Thus successful attempt would highlight the possibility of direct transfer of somatic embryos of orchids into the plantation.

MATERIALS AND METHODS

Plant material

Sterile plantlets derived from symbiotic germination of *G. scriptum* seeds were used as materials for *plbs* production. First, leaf segments, young shoots, and primary *plb* were isolated and subcultured in the *plb* induction media. Next, fractionated *plb* aggregates obtained from the induction medium would be used as an 'artificial embryo' for synseed (synthetic seed).

Plbs induction medium

The basal MS (Murashige and Skoog) medium plus a vitamin from Phyto Technology Lab. contained 3% (w/v) sucrose and was used to induce *plbs* formation. The medium formulation would be called basal-MS medium in the next discussions. The pH of the medium was adjusted to 5.6-5.8 using 0.1 N HCl and 0.1 N NaOH prior solidified by agar 8 g and sterilized at 121 C at 1 atm for 20 min in an autoclave.

Optimization of encapsulation matrix

Na-alginate (PhytoTechnology Lab.) was used with calcium chloride dihydrate for cross-linked hydrogel formation. Alginate solutions were prepared in various levels by dissolving sodium alginate (2%, 3%, 4%, and 5%) w/v in basal MS plus vitamin and sucrose 3% solution. Calcium chloride solution in various level (25 mM, 50 mM, 75 mM, and 100 mM) were prepared in distilled water.

Encapsulation procedure

Plbs aggregates formed in induction cultures were separated, blot dried, and embedded in the sodium alginate solution. Pipetting dropped each alginate-layering plb into $CaCl_2.2H_2O$ solution; each drop containing a single plb was incubated in a $CaCl_2.2H_2O$ solution for 30 minutes. The solid hydrogel beads formed by the complexation of the two encapsulation matrices were recovered by decanting the $CaCl_2.2H_2O$ solution and washing with sterilized de-ionized water. Beads were placed in a glass bottle (5 beads per bottle) with moist cotton, sealed with aluminum foil, and stored at 25°C for 15 days for evaluation

Data collection and analysis

Germination percentage (%) and the time required for germination were recorded and evaluated for 5 encapsulated *plbs* each treatment. Statistical analysis was made with a completely randomized design (CRD; for a single factor) and factorial CRD (for more than one factor, gelling agents data). Means were evaluated at P # 0: 05 level of significance using Duncan's New Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

plb formation

We used all parts of the plantlet to compare regeneration capabilities of different sources of explant on the induction of *plbs* formation. The result suggests that young shoots and previous *plb*, except leaf segments, have been successfully regenerated as new *plbs* in basal MS medium lacking plant growth regulator (PGR) (Table 1). However, *plb* formed by previous *plb* explants are more numerous than young shoot-derived *plbs*. The number of the new *plb* formed by the previous *plb* explant was around five compared to 1-2 *plbs* from the shoot explant. The limitation of shoot explant-producing *plbs* confirmed similar results from previous studies in the same species, even with the supplement of PGR (Lysnandar 2012).

Furthermore, we found that the development stage of the new *plbs* cannot be synchronized (Figure 1). The formation of the new *plbs* varied among different explants, even in a single explant. The first *plb* starts visibly 2 weeks after subculture and continues developing new plbs in the next days. Thus, four weeks after subculture, there got at least three types of *plbs* based on their development stages, i.e., globular *plb*,*plb* with new shoot meristem, and *plb* with developing shoot. The latter explicitly indicates the capacity of *plb* to regenerate into plantlet in basal MS medium without exogenously plant hormone. The new *plb* also apparently varied in size, ranging from less than 1mm to larger than 4 mm. There would be *plb* with globular stage and 2-3 mm in diameter as candidates for encapsulation to become the synthetic seed.

Table 1. Formation of new *plbs* from different explants

Sources of explant	Number of *plbs*	Time of first emerged (as*)
Young shoot segments	< 2	4 weeks
Leaf segments	-	-
Primary *plbs*	> 5	2 weeks

Note: *as: after subculture

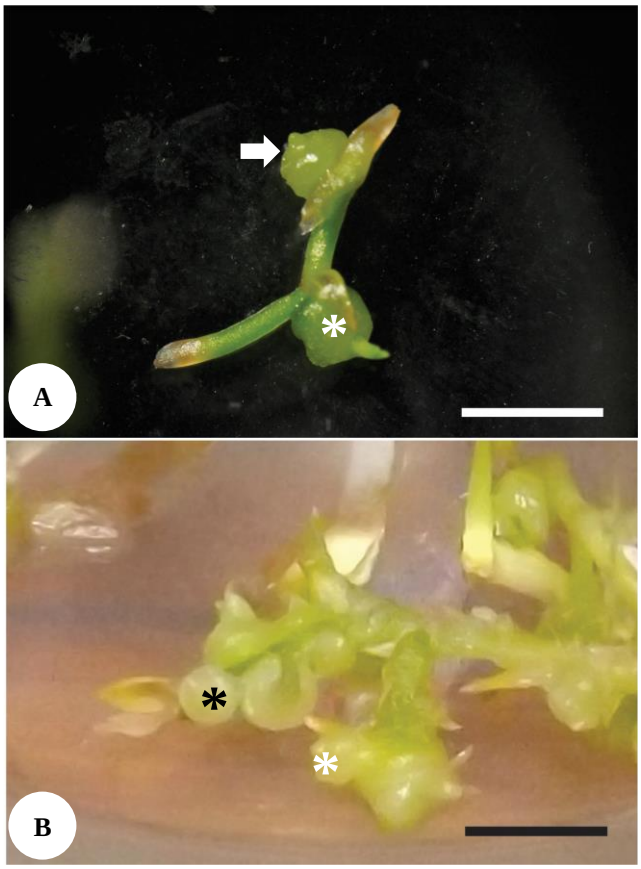

Figure 1. Formation of new *plbs* derived from varied explants cultured in MS basal medium. A. Shoot-derived *plbs*; B. *plb*-derived *plb*. White asterisk: *plb* with young developing shoot; Black asterisk: *plb* in the globular state; white arrow: *plb* with emergence shoot meristem. Bar = 1 cm.

Table 2. Physical properties of a calcium-alginate hydrogel of synthetic seed

Alginate	$CaCl_2.2H_2O$	Gel properties	Color	Shape
2%	25	+	Transparent	Unshape
	50	++	Less transparent	Spheric
	75	+++	Milky white	Spheric
	100	++++	Milky white	Spheric
3%	25	+	Transparent	Spheric
	50	++	Less transparent	Spheric
	75	+++	Milky white	Spheric
	100	++++	Milky white	Spheric
4%	25	+	Transparent	Spheric
	50	++	Less transparent	Spheric
	75	++++	Milky white	Spheric
	100	++++	Milky white	Spheric
5%	25	+	Transparent	Spheric
	50	+++	Less transparent	Spheric
	75	++++	Milky white	Spheric
	100	++++	Milky white	Spheric

Assessment of encapsulation matrix on hydrogel properties and development of encapsulated *plb*

The developed synthetic seed was represented by the structure of solid hydrogel encapsulated *plb* of *G. scriptum.* Alginate-based hydrogel is used because of several characteristics of alginic acid, which are (i) ability to form gels in the presence of divalent (or multivalent) cations, particularly calcium ions; (ii) biocompatible properties; the matrix has well known in medical and life science researchers; and (iii) ability to fix other materials in the gel (Kakita and Kamishima 2008). This matrix also provides the rigidity of the hydrogel bead, thus enhancing better protection of the embedded *plb* from mechanical damage (Saiprasad 2001). The hydrogel coating-*plbs* were formed by cross-linking calcium ions with alginate ions by an ion exchange mechanism. In this study, the complexation of alginate anion from sodium-alginate solution with divalent calcium cation from $CaCl_2.2H_2O$ solution in the different contraction levels provides various degrees of physical properties of hydrogel capsules. Based on Figure 3, the concentration of calcium chloride below 25 mM results in an unspherish structure of the hydrogel. Concomitantly, all levels of sodium alginate solution in combination with appropriate calcium chloride (at least 50 mM) successfully formed solid globular hydrogel calcium alginate.

Since the hydrogel's physical barrier is considered to influence the coating plbs significantly, the stiffness of the hydrogel qualitatively also be tested by pushing the hydrogel with fingers and scored as described in Table 2. The qualitative result found that the hydrogel's stiffness would increase, coinciding with an increase in alginate concentration.

Germination of synthetic seed *G. scriptum*

Germination of the *plb* encapsulated beads and the synthetic seed of *G. scriptum* (Figure 2). were represented by the emergence of shoot or root penetrating the calcium-alginate capsule (Machii, 1992). Several plb grew and developed shoot two weeks after encapsulation, which emerged preceded root penetrating the hydrogel layer. Root was formed later after eight weeks of encapsulation. The hydrogel layer is rich with micro and macronutrients as well as vitamins and sucrose, and this layer represents artificial endosperm which aids the embryo during growth and development. Our results suggested the capacity of *plb* as a superior explant for synthetic seed embryos. Some authors (Lee et al. 2013; Teixeira da Silva and Tanaka 2006) explained that *plb* is a representative of somatic embryos among members of Orchidaceae.

Germination percentages of all treatments from various degrees of the ratio encapsulation matrix were summarized in Figure 3. The chart shows variations in germination percentage of 8 weeks old synthetic seed of *G. scriptum.* The sodium-alginate solution in a concentration of 2% and 3% (w/v) reached maximum capacity to germinate in all levels of calcium chloride ranging from 25 mM to 100 mM. However, germinations were reduced in treatment of 4% and 5% sodium-alginate solution in combination with

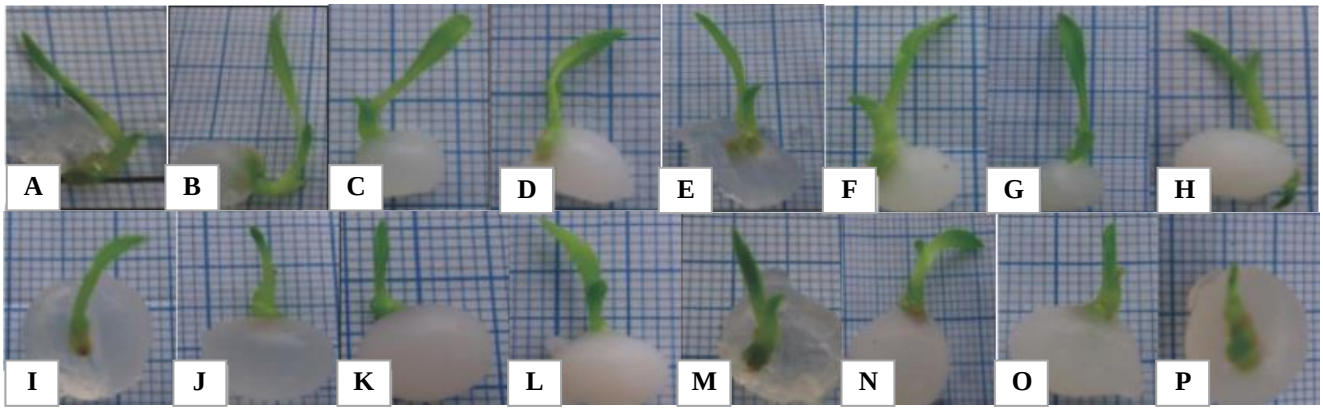

Figure 2. Germination of synthetic seed *G.scriptum*. All combinations of encapsulation matrix gave positive germination. The combination are: A. Alg 2% Cl 25; B. Alg 2% Cl 50; C. Alg 2% Cl 75; D. Alg 2% Cl 100; E. Alg 3% Cl 25; F. Alg 3% Cl 50; G. Alg 3% Cl 75; H. Alg 3% Cl 100; I. Alg 4% Cl 25; J. Alg 4% Cl 50; K. Alg 4% Cl 75; L. Alg 4% Cl 100; M. Alg 5% Cl 25; N. Alg 5% Cl 50; O. Alg 5% Cl 75; P. Alg 5% Cl 100.

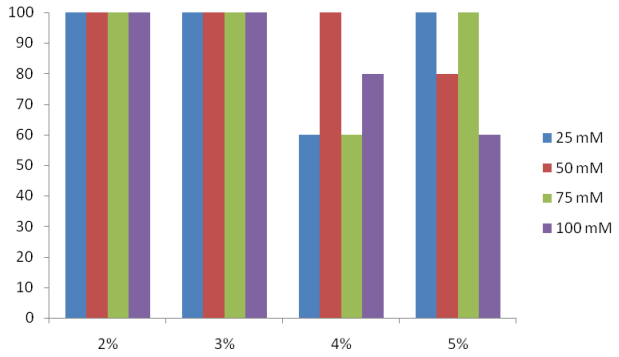

Figure 3. Germination percentage of 8 weeks old synthetic seed with variation in the ratio of encapsulation matrix. The horizontal axis is representative of the concentration of the sodium-alginate solution. Bar legends are representative of $CaCl_2.2H_2O$ concentration.

Calcium chloride in several level concentrations. These results suggest that sodium-alginate in a concentration of 2-3% was recommended for synthetic seed formation. Previous reports supported our finding that the concentration superior for developing synthetic seeds of *three orchid genera* (Saiprasad and Polisetty 2003) was 3% sodium-alginate in combination with 75 or 100 mM $CaCl_2$.

Furthermore, there found that the germination time of each synthetic seed varied from 2 weeks until 8 weeks after encapsulation. This phenomenon was predicted due to variation in the developmental state despite our use of the plbs, which is in uniform size on the *plb*. Previous reviews (Sharma et al. 2013) emphasize the importance of synchronizing high-quality explants to produce synthetic seeds for industrial applications. Somatic embryos represented by plbs in Orchidaceae apparently remained problematic in synchronous their developmental state to achieve the industrial application of the synthetic seed.

In conclusion, our research indicated that the ratio of encapsulation matrix gave various physical properties of hydrogel beads calcium-alginate. Still, they have no significant barrier for developing *plbs* to emergencies through penetration encapsulation matrix.

REFERENCES

Engelmann F. 2010. Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell Dev Biol-Plant 47 (1): 5-16.

Fay MF. 1994. In what situations is in vitro culture appropriate to plant conservations? Biodiv Conserv 3 (2):176-183.

Fay MF, Chase MW. 2009. Orchid biology: from Linnaeus via Darwin to the 21st century. Ann Bot 104 (3): 359-364.

Kakita H. Kamishima H. 2008. Some properties of alginate gels derived from algal sodium alginate. J App Phycol 20 (5): 543-549.

Kull T, Kindlmann P, Hutchings MJ, Primack RB. 2006. Conservation biology of orchids: introduction to the special issue. Biol Conserv 129 (1): 1-3.

Lee YI, Hsu ST, Yeung EC. 2013. Orchid protocorm-like bodies are somatic embryos. Amer J Bot 100 (11): 2121-2131.

Lestari DA, Santoso W. 2011. Inventory and habitat study of orchids species in Lamedai Nature Reserve, Kolaka, Southeast Sulawesi. Biodiversitas 12 (1): 28-33.

Nge KL, New M, Chandrkrachang S, Stevens WF. 2006. Chitosan as a growth stimulator in orchid tissue culture. Plant Sci 170 (6): 1185-1190.

Saiprasad GVS. 2001. Artificial seeds and their applications. Resonance 6 (5): 39-47.

Saiprasad GVS, Polisetty R. 2003. Propagation of three orchid genera using encapsulated protocorm-like bodies. In Vitro Cell Dev Biol-Plant 39 (1): 42-48.

Sarasan V, Cripps R, Ramsay MM, Atherton C, Mc Michen M, Prendergast G, RowntreeJK. 2006. Conservation In vitro of threatened plants-Progress in the past decade. In Vitro Cell Dev Biol-Plant 42 (3): 206-214.

Sharma S, Shahzad A, Teixeira da Silva J. 2013. Synseed technology-a complete synthesis. Biotech Adv 31 (2): 186-207.

Tan TK, Loon WS, Khor E, Loh CS. 1998. Infection of *Spathoglottis plicata* (Orchidaceae) seeds by mycorrhizal fungus. Plant Cell Rep 18 (1-2): 14-19.

Teixeira da Silva J, Tanaka M. 2006. Multiple regeneration pathways via thin cell layers in hybrid *Cymbidium* (Orchidaceae). J Plant Growth Reg 25 (3): 203-210.

Yam TW, Arditti J. 2009. History of orchid propagation: a mirror of the history of biotechnology. Plant Biotech Rep 3 (1): 1-56.