Cryopreservation of Protocorms of *Grammatophyllum speciosum* Blume by Vitrification and Encapsulation/Vitrification.

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ชื่อวิทยานิพนธ์ การเก็บรักษาโพรโทคอร์มของกล้วยไม้เพชรหึง (*Grammatophyllum speciosum* Blume) โดยการแช่แข็งใน ไนโตรเจนเหลวด้วยวิธี Vitrification และ Encapsulation/Vitrification

ผู้เขียน นางสาวมนทกานต์ พิมเสน

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บทคัดย่อ

อาหารเพาะเลี้ยงเนื้อเยื่อที่มีประสิทธิภาพในการชักนำโพรโทคอร์มไลค์บอดี้จากโพรโทคอร์มของกล้วยไม้เพชรหึง (*Grammatophyllum speciosum* Blume) คือ อาหารสูตร Murashige and Skoog (MS) ดัดแปลงโดยเติมน้ำมะพร้าว 15 เปอร์เซ็นต์ น้ำตาลซูโครส 2 เปอร์เซ็นต์ และทำให้แข็งโดยใช้วุ้น 8 กรัมต่อลิตร (อาหารสูตรชักนำโพรโทคอร์ม) อัตราการเกิดโพรโทคอร์มไลค์บอดี้สูงสุดคือ 84.00 เปอร์เซ็นต์ และสร้างโพรโทคอร์มไลค์บอดี้ 3.1 ชิ้นต่อชิ้นส่วนพืช โพรโทคอร์มของกล้วยไม้เพชรหึงสามารถเพาะเลี้ยงในอาหารเหลวสูตร MS ที่มีน้ำตาลแมนนิทอลหรือซอบิทอล 2-8 เปอร์เซ็นต์ได้เป็นเวลา 4 สัปดาห์ และกลับมาเจริญเติบโตได้ 100เปอร์เซ็นต์หลังย้ายไปเพาะเลี้ยงบนอาหารสูตรชักนำโพรโทคอร์ม สำหรับการทำเมล็ดเทียม โพรโทคอร์มที่เคลือบด้วยโซเดียมแอลจิเนตความเข้มข้น 3 เปอร์เซ็นต์ ทำให้แข็งตัวด้วยสารละลายแคลเซียมคลอไรด์ความเข้มข้น 75 มิลลิโมลาร์ เป็นเวลา 30 นาที เกิดการฟอร์มตัวเป็นเม็ดดีที่สุด เมล็ดเทียมสามารถเก็บไว้ที่อุณหภูมิ 25 องศาเซลเซียส ในที่มืด ได้เป็นเวลา 16 สัปดาห์ และสามารถพัฒนาเป็นต้นได้หลังย้ายไปเพาะเลี้ยงบนอาหารสูตรชักนำเป็นเวลา 3-4 สัปดาห์

โพรโทคอร์มของกล้วยไม้เพชรหึงที่เก็บรักษาโดยการแช่แข็งในไนโตรเจนเหลวโดยวิธี vitrification 2 ขั้นตอน, vitrification และ encapsulation ตามด้วย vitrification และตรวจสอบความมีชีวิตด้วย Evan's blue พบโพรโทคอร์มมีความมีชีวิต 85 เปอร์เซ็นต์หลังเก็บรักษาไว้ในไนโตรเจนเหลวด้วยวิธี vitrification 2 ขั้นตอน โพรโทคอร์มถูกนำมาดึงน้ำออกด้วย ½ PVS2 (กลีเซอรอลความเข้มข้น 15 เปอร์เซ็นต์, ethylene glycol ความเข้มข้น 7.5 เปอร์เซ็นต์, dimethyl-sulfoxide (DMSO) ความเข้มข้น 7.5 เปอร์เซ็นต์) เป็นเวลา 60 นาที ตามด้วย PVS2 (กลีเซอรอลความเข้มข้น 30 เปอร์เซ็นต์, ethylene glycol ความเข้มข้น 15 เปอร์เซ็นต์, dimethyl-sulfoxide (DMSO) ความเข้มข้น 15 เปอร์เซ็นต์) 40 นาที ก่อนนำไปเก็บในไนโตรเจนเหลวเป็นเวลา 1 ชั่วโมง สำหรับวิธี vitrification เมื่อนำโพรโทคอร์มมาป้องกันแรงดันออสโมติกโดยการแช่ในสารละลาย Loading solution (LS, น้ำตาลซูโครสความเข้มข้น 1

โมลาร์ และ กลีเซอรอลความเข้มข้น 2 โมลาร์) เป็นเวลา 20 นาที และดึงน้ำออกด้วยสารละลาย PVS2 เป็นเวลา 60-80 นาที ก่อนเก็บในไนโตรเจนเหลว หลังจากนำมาอุ่นที่อุณหภูมิ 40±2 องศาเซลเซียสเป็นเวลา 2 นาที อัตราความมีชีวิตคือ 60.00 เปอร์เซ็นต์ นำโพรโทคอร์มแช่ในสารละลาย Unloading solution (น้ำตาลซูโครสความเข้มข้น 1 โมลาร์) 20 นาทีก่อนนำไปทดสอบความมีชีวิต สำหรับวิธี encapsulation ตามด้วย vitrification นำเมล็ดเทียมมาปรับสภาพด้วยอาหารเหลวสูตร MS ดัดแปลงที่มีน้ำตาลซูโครสเข้มข้น 0.5 โมลาร์ เป็นเวลา 2 วัน และป้องกันแรงดันออสโมติกโดยการแช่ในสารละลาย LS เป็นเวลา 20 นาที ก่อนดึงน้ำออกด้วยสารละลาย PVS2 เป็นเวลา 100 นาที และนำไปแช่ในไนโตรเจนเหลว หลังจากอุ่นแล้ว นำเมล็ดเทียมแช่ในสารละลาย Unloading solution 20 นาที ให้อัตราการมีชีวิต 66.67 เปอร์เซ็นต์ อย่างไรก็ตามโพรโทคอร์มไม่สามารถพัฒนาไปเป็นต้นได้

Thesis Title Cryopreservation of Protocorms of *Grammatophyllum speciosum* Blume by Vitrification and Encapsulation /Vitrification.

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ABSTRACT

The efficient medium for protocorm-like body (PLB) induction from protocorms of *Grammatophyllum speciosum* Blume was Murashige and Skoog (MS) medium modified by supplemented with 15 % (v/v) coconut water, 2 % (w/v) sucrose, and solidified with 8 g/l agar (PLB induction medium). The highest PLB formation rate was 84.00 % and produced 3.1 PLBs per explant. Protocorms of *G. speciosum* could be cultured in MS liquid medium containing 2-8 % mannitol or sorbitol for 4 weeks and gave 100 % regeneration after transfer to PLB induction medium. For encapsulation, protocorms were encapsulated with 3 % (w/v) sodium alginate complexing with 75 mM calcium chloride solution for 30 min gave the best formation of beads. The encapsulated beads could be stored at 25°C in dark condition for 16 weeks and regenerated into plantlets after transfer to PLB induction medium for 3-4 weeks.

The protocorms of *G. speciosum* were cryopreserved by 2-step vitrification, vitrification, and encapsulation/vitrification technique and the viability percentage was assessed by Evan's blue test. Protocorms gave 85 % viability after cryopreserved by 2-step vitrification technique. Protocorms were dehydrated with ½ PVS2 (15 % (v/v) glycerol, 7.5 % (v/v) ethylene glycol, 7.5 % (v/v) dimethyl-sulfoxide (DMSO)) for 60 min followed by PVS2 (30 % (v/v) glycerol, 15 % (v/v) ethylene glycol, 15 % (v/v) DMSO) for 40 min before plunging into liquid nitrogen (LN) for 1 hour. For vitrification technique, the viability rate was 60.00 % when protocorms were osmoprotected with loading solution (LS, 1 M sucrose and 2 M glycerol) for 20 min and dehydrated with PVS2 for 60-80 min before plunging into LN. After rewarming at 40±2°C for 2 min, the protocorms were treated with unloading solution (1 M sucrose) for 20 min before viability test. For

encapsulation/vitrification technique, the encapsulated protocorms were precultured in modified MS liquid medium supplemented with 0.5 M sucrose for 2 days and osmoprotected with LS for 20 min before dehydrating with PVS2 for 100 min and plunging into LN. The cryopreserved encapsulated protocorms gave 66.67 % viability after treating with unloading solution for 20 min after rewarming. However, regeneration of protocorm was not obtained.

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LIST OF ABBREVIATIONS AND SYMBOLS

2, 4-D	=	2, 4-Dichlorophenoxyacetic acid
BA	=	6-Benzyladenine
CW	=	Coconut water
D	=	day
DMSO	=	dimethyl-sulfoxide
h	=	hour
IAA	=	Indole-acetic acid
IBA	=	Indolebutyric acid
KC	=	Knudson C medium
LN	=	Liquid Nitrogen
LS	=	Loading Solution
NAA	=	α-Naphthaleneacetic acid
min	=	Minute
MS	=	Murashige and Skoog medium
PGRs	=	Plant growth regulators
PLBs	=	Protocorm-like bodies
PVS2	=	Plant Vitrification Solution II
TDZ	=	Thidiazuron
VW	=	Vacin and Went medium

CHAPTER 1
INTRODUCTION

1.1 Introduction

Thailand is in the Southeast Asia with suitable climate for the growth of tropical orchids hence we can find many orchid species in the nature both epiphytic orchids and terrestrial orchids. Orchids about 1,100 species are distributed all over the country (Thammasiri, 2007). Orchids are popular worldwide as a potted plants or cut flowers due to their colorful and elegant flowers in each species.

Grammatophyllum speciosum Blume or Tiger orchid is the only species of the genus *Grammatophyllum* that is native to Thailand. It has a large size, long trunk and beautiful flowers that attract viewers. It can be found growing on the branches of large tree. Nowadays, *G. speciosum* is rarely found in the nature because they were taken from the forest for trade. The forest encroachment and climate change interact with habitat loss and fragmentation. From this reason, most of orchids in the world are listed under Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES). CITES has declared all members of the family Orchidaceae formally protected. There are strict controls on trade and import and export of all orchids. *G. speciosum* was including in Appendix II. Appendix II plants are considered endangered and as such, the international trade is regulated by CITES and trade in the taxa would require a permit or certificate.

G. speciosum has slow growth rate in nature and has become endangered. Therefore, micropropagation and conservation are needed. Cryopreservation using liquid nitrogen (LN) is an important technique for long-term storage. The potential advantages of cryopreservation over conventional techniques are an absence of complicated temperature and humidity controls, free from damage by pest and diseases and indefinite longevity with little or no genetic damage (Lanly, 1993) Parts of *G. speciosum* plants will be used for cryopreservation such as seeds, protocorms, shoot tips and protocorm-like bodies (PLBs). Protocorms were the explants selected

for cryopreservation because they are very small which can easily develop into whole plants without intermediate callus formation.

1.2 Literature Review

Orchids are monocotyledon in the family Orchidaceae. Orchidaceae is the biggest family in class Angiosperm consists of about 25,000 species of orchid (Thammasiri, 2007). The great majority are to be found in the tropics, mostly Asia, South America and Central America. Orchids are one of the most important ornamental plants in Thailand. Tropical orchids were found in Thailand about 1,100 species. They exhibit an incredible range of diversity in size, shape and color of their flowers.

1.2.1 Types of orchid sort by habitat

1.2.1.1 Epiphytic orchids

Epiphytic orchids are mostly found in the forest. These orchids were naturally grown on the other tree. They receive nutrient from organic decay, such as leaf decay and dead insects. The outer layer orchid root has velamen that is the multi-layered epidermis of aerial orchid root. Its function is providing mechanical protection, reducing water loss and possibly specialized for water absorption (Lawrence, 2000). Root of epiphytic orchid has chlorophyll hence it can photosynthesize for its own nutrient.

Epiphytic orchids have two different types of growth structure (Figure 1) one is to grow from the vegetative apex (monopodial), such as *Vanda*, *Ascocentrum*, *Rhyncostylis*, *Phalaenopsis*, etc. The other is to grow out horizontally (sympodial), sending pseudobulbs up from the rhizome. The new shoots will collect

and replenish the old ones as a source of nutrient. Orchids that grow in this trend are, for instance, *Dendrobium, Oncidium, Cattleya* etc (Thammasiri, 2007).

1.2.1.2 Terrestrial orchid

Terrestrial orchids are orchids that grow on the ground. Most of them have rhizome. Terrestrial orchids are dormant in summer and produce leave and flowers in rain, such as *Habenaria* and *Pecteilis*. In *Paphiopedilum* species, they grow in crack of rock and have green leaves all through the year (Thammasiri, 2004).

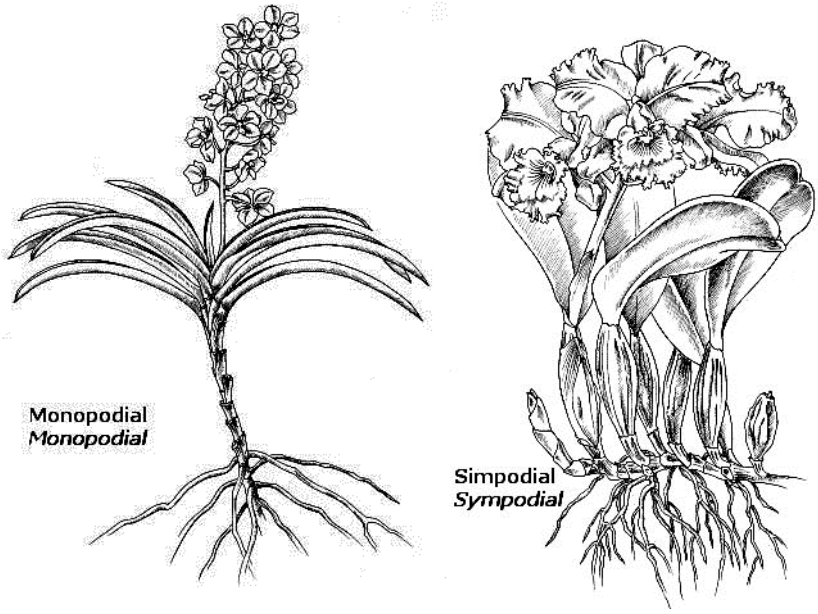


Figure 1 Two different types of orchid growth structure

(Sources: http://www.jbrj.gov.br/saibamais/orquideas/crescimentomonopodial.htm and http://www.jbrj.gov.br/saibamais/orquideas/tamanhos.htm (accessed January 17, 2013))

Grammatophyllum is from Greek, *gramma* refers to letter and *phyllon* refers to leaf and the conspicuous marking on the floral segments. The genus *Grammatophyllum* contains twelve species and *G. speciosum* is the only species of the genus *Grammatophyllum* that is native to Thailand. It also known as the Tiger

orchid or Giant orchid and is considered to be the largest member of the orchid family. *G. speciosum* (Figure 2) is the largest epiphytic orchid distributed from Southeast Asia, Indonesia, New Guinea to the Solomon Islands. The cane-like growth can reach up to 2 m in height and is usually found growing on very large tree (Figure 3) with 2-6 cm in diameter. It has thick roots form basket-like structure. Leaf is in parallel venation 30-70 cm long and 3-5 cm wide with waxy green color and have alternate arrangement. Its flower stalk can reach to 2 m and contain more than 30 flowers. The single flower is around 10 cm in diameter. It's yellow with dark red spot spread around the petal and it blooms in July to October and can remain in bloom for up to 2 months. When the fruit rips, it takes about 5-7 months and retains millions of powder-like seeds. In nature, new seedlings are formed when they have nutrient from mycorrhiza.

Figure 2 *Grammatophyllum speciosum*

Figure 3 *Grammatophyllum speciosum* growing on the large tree in nature.

(Sources: http://arkitrek.com/http:/arkitrek.com/little-large-of-the-orchid-world/ (accessed January 17, 2013))

Scientific classification of *Grammatophyllum speciosum* Blume.

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Asparagales

Family: Orchidaceae

Subfamily: Epidendroideae

Tribe: Cymbidieae

SubTribe: Cyrtopodiinae

Genus: Grammatophyllum

Species: *Grammatophyllum speciosum*

1.2.2 Conservation of orchids

Conservation of plant genetic resources has 2 basic approaches, they are *in situ* and *ex situ* conservation. *In situ* conservation, conserved plants in ecosystem and natural habitats. It can maintain and recovery of viable population of species in their natural surrounding. *Ex situ* conservation, conserved plants out of their habitats. *Ex situ* conservation involves a higher degree of protection and greater isolation of germplasm than *in situ* conservation (Lanly, 1993). *In situ* conservation includes forest and national park while *ex situ* conservation includes seed storage, pollen storage, *in vitro* storage, gene bank, greenhouse and botanical gardens.

Many orchid species in the world risk to extinction because of global warming, flood, drought stresses, deforestation and wild orchid trade. Orchids are well known as important ornamental plants in Thailand, especially wild orchids. Thai orchid species are famous for their beauty and distinct features and are in high demand all over the world. When orchids were taken from the wild and traded to buyers. The buyers don't know how to look after wild orchids and orchids often die thereafter. These causes to increase wild orchid trade and some orchid species have become extinct.

In orchids, they can be maintained in their natural (*in situ* conservation) and out of their habitats (*ex situ* conservation). *In situ* conservation is the best way to conserve genetic diversity of orchid germplasm but it is very difficult to maintain for a long period and it risks being lost from pest, disease, biotic and abiotic stress. In addition, field storage is costly due to labor and land uses. Germplasm conservation using tissue culture may take a long time and must be subcultured several times. The maintenance of orchid *in vitro* is expensive, laborious, accumulation of somaclonal variation and losing materials by contamination. To avoid this, cryopreservation was used to preserve their genetic diversity.

1.2.3 Tissue culture of orchids

Orchid seeds are very small and tiny and almost powder-like and produced in large number. Number of seeds per fruit may contain 1,300-4,000,000 seeds. Orchid seeds are vary in shape, size and color. Most of orchid seeds are 0.09-

0.27 mm wide and 0.25-1.2 mm long. Seed weight extends from 0.0003-0.0014 mg (0.3-1.4 µg). When seed pods break, seeds can float in the air for long periods. The seed contains a small embryo, lack endosperm to enable seed to germinate; hence, orchid seeds require a symbiotic relationship with mycorrhizal fungi.

Germination rates of orchid seeds are very low under normal condition. Traditional asexual propagation of orchids is extremely slow. Tissue culture has been adopted as a useful technique for orchid micropropagation. Tissue culture methods for the regeneration of orchids using various plant parts of orchid are used in culture media. In micropropagation, the explants contain the meristematic cells, such as shoot tip, root tip, or bud which were used to culture in synthetic medium under aseptic condition and controlled environment. Meristematic cells are active cells and have ability to divide to promote organogenesis. But each plant species have different hormone in their own cells.

1.2.4 Orchid culture media

The suitable media for orchid micropropagation have many formulas, such as Knuson C (1946), Murashige and Skoog (MS) (1962) and Vacin and Went (VW) (1949). Culture media are different in their macronutrients, micronutrients and other minerals. In general, the growth and development of *in vitro* grown plants depend on culture medium. Some organic substances, potato homogenate were added in seed culture medium in order to promote multiple shoot formation in *Cypripedium flavum* (Yan *et al*., 2006). Seed germination frequency of threatened *Paphiopedilum villosum* var. "*Densissimum*" was highest in coconut water treatment (Long *et al*., 2010). The growth of *Phalaenopsis* was obtained with a culture medium containing banana homogenate (Hinnen *et al*., 1989). New *Phalaenopsis* medium supplemented with potato extract, corn extract and papaya extract were used to induce plantlets from PLBs of *Doritaenopsis* orchid and the results showed that corn extract gave the highest rate of plantlet regeneration (Rahman *et al*., 2004).

Not only organic substance that were added into culture medium, plant growth regulators are commonly used for multiplication in orchid culture. There are two main groups of plant growth regulators, namely cytokinins and auxins.

Cytokinins, such as BA (6-Benzyladenine) and TDZ (Thidiazuron) are normally used to induce shoot proliferation by breaking dormancy in lateral buds or adventitious meristems. Auxins, such as 2, 4-D (2, 4-Dichlorophenoxyacetic acid), NAA (α-Naphthaleneacetic acid), IAA (Indoleacetic acid), and IBA (Indolebutyric acid) are used to induce root and callus formation.

1.2.5 Carbon sources

The sources of carbon are very important component in culture medium for growth and development of tissue. Carbon sources were added to culture medium because the light energy deficiency and low CO_2 concentration present in *in vitro* condition. Sugars were used as a carbon source and as an osmotic regulator in culture medium. There are various types of sugars used as carbon source in *in vitro* culture, such as sucrose, glucose, maltose, fructose, lactose, and sorbitol. Sucrose at concentration of 20 and 30 g/l are the most commonly used in orchid tissue culture studies. However, in *Dendrobium nobile*, sucrose at 60 g/l in modified MS medium gave greater increase in plant height and high seedling multiplication without the addition of plant hormones (Faria *et al*, 2004). In the study of *Oncidium* Grower Ramsey and Sweet Sugar, five kinds of carbon sources namely, cellobiose, fructose, glucose, maltose and sucrose were used for induction of direct embryo formation. The highest amount of embryos was obtained at 30 g/l sucrose for Grower Ramsey ant at 20 g/l of glucose for Sweet Sugar (Hong et al, 2008a). The influences of various carbohydrate sources were used to estimate on growth and development of shoot tip-derived suspension cell of *Phalaenopsis* and *Doritaenopsis* orchids. Among the carbohydrate tested on *Doritaenopsis* cultured on agar medium, glucose at 58.4 mM gave the highest efficiency of PLB formation. For suspension cultures of *Phalaenopsis*, PLB formation was the most efficiently induced by sucrose at 29.2 mM for *P.* Snow Parade and 14.6 mM glucose for *P.* Wedding Promenade.(Tokuhara and Mii, 2003)

1.2.6 Micropropagation of *Grammatophyllum speciosum* Blume

Grammatophyllum speciosum has a sympodial growth and produces 1-2 offshoots per year. Traditionally, they are propagated by cutting a new offshoot but the propagated rate is very slow. Orchid can be rapidly propagated by *in vitro* seed culture or PLB induction and multiplication by shoot tip or bud culture. The excision of shoot tip causes the loss of plant source. Thus, seed culture is good for clonal propagation because it does not damage the donor plants, there are many seeds in one capsule and easy to sterilize.

There are a few studied in micropropagation of *Grammatophyllum speciosum*. Such as, the study of PLB induction from shoot tips from *in vitro* seedling of *G. speciosum*, the highest frequency of PLBs (93 %) was observed on explants incubated on ½ MS liquid medium containing 2 % (w/v) sucrose without any plant growth regulators (PGRs). The tests of different carbon sources revealed that maltose promoted 7-fold increase of PLBs. The addition of 15 mg/l of chitosan promoted a 7-fold increase in PLB growth (Sopalun *et al*., 2010). Sterilized seeds were grown in VW medium supplemented with various organic substances and different concentrations of BA and 2, 4-D. The results indicated that the difference of banana pulp, pineapple pulp, tomato pulp and Jew's Ear mushroom extract could promote the increment of protocorms in culture medium containing BA and 2, 4-D treatments (Montri *et al*., 2009). They also examined the factors influencing *in vitro* germination of *G. speciosum* by investigating the effects of basic inorganic salts, activated charcoal, light, dark and growth regulators (BA and TDZ). The results showed that Knuson C medium without activated charcoal gave the highest seed germination with no effect on light treatment. In addition BA, TDZ, light and dark treatments had no effect on seed germination and activated charcoal showed negative effect on seed germination of *G. speciosum* (Khampa *et al*., 2010).

In the study of other species, *Grammatophyllum scriptum* Lindl., it was found that the best medium for seed germinating was KC basal medium supplemented with 30 % (v/v) mature coconut water (CW) and the best medium for growing and developing of seedlings to fully plantlets was ½ MS medium supplemented with 40 % (v/v) CW (Abbas *et al*., 2011).

1.2.7 Encapsulation

Encapsulation materials and methods for production of artificial seeds were introduced by Murashige in 1997 (Patel *et al*., 2000). The production of artificial seeds is especially useful for plants which do not produce viable seeds, heterozygosis of seeds, very small seed size, presence of reduced endosperm and the requirement of seed with mycorrhizal fungi association for germination, such as orchids and also in some seedless varieties of crop plants like grapes, watermelon, etc. Artificial seeds can be produced by encapsulating a plant propagule in a matrix which will allow it to grow into a plant. Plant propagule may consist of spores, seeds, shoot buds, or somatic embryos.

Artificial seeds are small size and provide advantages in storage and easy to transport. It has potential for short-term storage without losing viability and maintains the nature clonal of plant and can grow directly to the greenhouse or field. This artificial seed production technology is high volume, low cost production technology. The concept of artificial seed is shown in Figure 4.

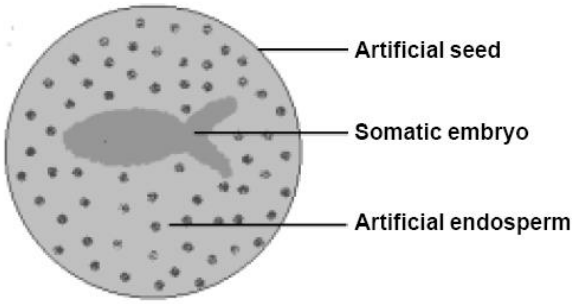


Figure 4 Artificial seed concept (Saiprasad, 2001).

In the production of artificial seeds, an artificial endosperm created within the encapsulation matrix. The encapsulation matrix is a hydrogel. Somatic embryo or other plant propagule acts like a zygotic embryo were mixed in gel then dropped into an appropriate electrolyte to form a bead.

1.2.7.1 Types of gelling agents used for encapsulation

Gelling agents that use for encapsulation made from natural extracts, such as agar, carageenan and alginate extract from seaweed, tragacanth extract from plant, guar from seed gum or dextran, gelling, and xanthan gum extract from microorganism. Among those, alginate hydrogel is frequently selected as a matrix for artificial seed because of its moderate viscosity and low spinnability of solution, low toxicity for plant propagules and quick gellation, low cost and bio-compatibility characteristics. For storage, alginate was chosen because it enhances capsule formation and also the rigidity of alginate beads provides the protection.

1.2.7.2 Principle and conditions for encapsulation with alginate matrix.

The major principles involved in the alginate encapsulation process is that the sodium alginate droplets containing explants when drop into the $CaCl_2$ solution ($CaCl_2.2H_2O$) alginate matrix formed round and firm beads due to ion exchange between Na^+ in sodium alginate with Ca^{2+} in $CaCl_2$ solution. The hardness or rigidity of the capsule mainly depends on the number of sodium ion exchanged with calcium ions. Hence, the concentration of the two encapsulation agents and the complexing time should be optimized for the formation of the capsule with optimum bead hardness and rigidity for the production of viable artificial seeds.

1.2.7.3 Artificial endosperm

The explants that used as a zygotic embryo lack seed coat and endosperm that provides protection and nutrition to growing. To augment these deficiencies, the addition of nutrients and growth regulators to the encapsulation matrix is desired, which serves as an artificial endosperm. It gave the increase in efficiency of germination and viability of artificial seeds and can store for a long period of time without losing viability.

Development of artificial seed production is currently considered an effective, alternative method of propagation for several commercially

important agronomic and horticultural crops, such as seedless grape, seedless watermelon, seedless jackfruit, seedless cucumber, corn, cotton, soybean, hybrid tomato, hybrid cereals, forage legume, pine, potato, banana, etc. (Saiprasad and Polisetty, 2003).

1.2.7.4 Encapsulation of orchids

Artificial seeds of orchids are produced by encapsulation of seeds, shoot buds, lateral buds, protocorms or PLBs in an alginate matrix. If encapsulated explants can be stored for a long duration, it will greatly enhance the efficiency of micropropagation by this technique. Until now artificial seed production of orchid has been achieved in only a few species. For example, 3 orchid genera were propagated by using encapsulated PLBs. The leaf primordial stage of PLBs was encapsulated using, 2-5 % sodium alginate at and 25-100 mM of $CaCl_2$ solution. The best encapsulation response was observed with 3 % sodium alginate upon complex with 75 mM $CaCl_2$ solution. Encapsulated PLBs of *Dendrobium*, *Oncidium* and *Cattleya* were stored at 4°C for 75, 60 and 30 days, respectively (Saiprasad and Polisetty, 2003). In the study of the production of artificial seeds of *Coelogyne breviscapa* through encapsulation of PLBs, 60-day-old PLBs established from embryo cultures were encapsulated in 3 % sodium alginate matrix and 50 mM $CaCl_2$ solution. The encapsulated PLBs stored at 4°C for 60 days showed no reduction in viability (Mohanraj *et al*., 2009).

1.2.8 Cryopreservation

1.2.8.1 Principles of cryopreservation

Cryopreservation commonly refers to the use of ultra-low temperatures (-80°C to -196°C) for preserving biological materials. The potential advantages of cryopreservation are an absence of complicated temperature and humidity control, the freedom from damage by pets and diseases and indefinite

longevity with little or no genetic damage (Lanly, 1993). When require, it can be recover and grow to regenerate a whole plant. A variety of plant materials can be used including calluses, shoot meristems, root meristems, seeds, pollens, embryos, protocorms, or PLBs.

1.2.8.2 Cryopreservation procedures

a) Plant materials

In cryogenic protocol, the physiological state of cells and tissues to be cryopreserved must be tolerant for dehydration and producing vigorous recovery growth (Sakai and Engelmann, 2007). The explants that are appropriate to cryopreserve are those at an optimal development state and contain meristematic tissues, such as embryos, shoot tips, root tips, protocorms and PLBs. Because microorgan containing meristematic tissues can differentiate into shoots and roots. The meristematic cells are modest size and have a large nucleus, numerous membranes and small vacuoles. They contain less water than the adult plant cells (Chetverikova, 2008). Before cryopreservation some procedure need to prepare for protection. Those are preculture, loading treatment, and dehydration.

b) Preculture

The cells and tissues that use to cryopreserve must be in a physiologically optimum status for acquisition of dehydration tolerance and to produce vigorous recovery of growth. Preculture of plant materials in media containing high concentration of sugars enhanced the tolerance of explants against the treatment with PVS (Plant Vitrification Solution) (Sakai *et al*., 1990) and improved regrowth after cryopreservation.

For successful cryopreservation, it is essential to avoid intracellular ice formation during the stage of freezing. Soluble sugars have been implicated to protect membranes through water replacement and to protect the

cytoplasm by transiting them into glassy state (Bian *et al*., 2002). To avoid an ice crystal formation, preculture with sugars were needed. Sucrose was used in preculture treatment for many plant species since sucrose has many advantages as cryoprotectant e.g. the tolerance to temperatures below the freezing point is related to the sugar content of cell. The cytoplasm of cell with high sucrose can easily vitrify and supercool (Burke, 1987).

Conditions of sugar preculture of tissue on medium containing 0.1 up to 1.0 M during 1-5 days are often applied to improve dehydration tolerance. In *Doritaenopsis*, the highest viability at 64 % after cryopreservation by vitrification was obtained when the cells were precultured in New Dogashima liquid medium supplemented with 0.1 M sucrose and 1.0 mg/l abscisic acid for 1 week (Tsukazaki *et al*, 2000). PLBs of *Dendrobium* Sonia-28 were successfully cryopreserved by vitrification technique (Hooi *et al*., 2010). The most suitable preculture sucrose concentration is 0.6 M. Cryopreservation of *Dendrobium candidum* PLBs by encapsulation/dehydration using 0.75 M sucrose in preculture treatment and the survival rate was above 85 % (Yin and Hong, 2009). Shoot tips of *Dendrobium* Walter Oumae cryopreserved by encapsulation/dehydration gave the highest survival rate at 16.18 mg living cells/100 mg total cells and 13.33 % regrowth when encapsulated shoot tips were precultured on 0.3 M sucrose agar medium for 2 days (Lursvijidjarus and Thammasiri, 2004).

In other plant species, sucrose was used in preculture treatment, of shoot tips of 'Troyer' citrange by encapsulation/vitrification, the optimal survival was obtained when precultured with 1 M sucrose for 2 to 5 days (Wang *et al*., 2002). Shoot tips of *Rosa* were successfully cryopreserved by a combined droplet vitrification method. Shoot tips precultured in MS medium containing 0.1 - 1.0 M sucrose for 24 and 48 h, respectively and the results showed that preculture in sucrose enhanced the tolerance of shoot tips also against the treatment with the PVS2 (Halmagyi and Pinker, 2006).

The precultured explants with sugar gave a higher viability after immersion in liquid nitrogen (LN). Preculture is used to increase tolerance of explants to dehydration and subsequent freezing in LN. The type and concentration of

sugar commonly used in the preculture medium appears to be important in cryopreservation.

c) Loading treatment (Osmoprotection)

For many species, preculture with sucrose appears to be insufficient to produce a high level of survival by vitrification. The explants that use to cryopreserve by vitrification thus have to be treated (loaded) with cryoprotective solution (loading solution) (Sakai and Engelmann, 2007). Loading solution is a mixture of glycerol and sucrose. It affects inducing tolerance to dehydration by vitrification. Loading treatment was applied in many studies, such as cryopreservation of axillary shoot tips of grape (*Vitis*) by a two-step vitrification protocol. Grape shoot tips were precultured on solidified medium supplemented with 0.3 M sucrose for 3 days and then treated with loading solution (a mixture of 2M glycerol plus 0.4 M sucrose) for 20 min at 25°C before dehydrated with PVS2 and plunged into LN. The recovery rate of shoot tips was to approximately 60 % (Matsumoto and Sakai, 2003).

In Orchid species, cells of a suspension culture of *Doritaenopsis* were placed in loading solution (a mixture of 2 M glycerol and 0.4 M sucrose) for 15 min and then dehydrated with a vitrification solution gave the highest viability after plunged into LN (Tsukazaki *et al*., 2000). PLBs of *Dendrobium candidum*, osmoprotected with a mixture of 2 M glycerol and 1 M sucrose for 80 min before encapsulating and dehydrating gave survival rate of encapsulated-vitrified PLBs was above 85 % (Yin and Hong, 2009). Thus, the loading solution treatment following preculture with sucrose enriched medium is a very promising step for successful cryopreservation.

d) Dehydration

Water is the major component of all living cells as a nature's biological solvent and must be available for the chemical processes of life to occur. Cellular metabolism stops when all water in the system is turned to ice. The ice crystal can be harmful to plant cells and needs to be avoided. For successful

cryopreservation, dehydration procedure is needed to reduce water content in plant cells to avoid ice formation when store in LN. For dehydration, the following techniques are applied:

d.1) Air drying

Samples are dehydrated by the sterile air-flow from a lamina air-flow cabinet or desiccated on silica gel to optimal humidity. For these methods, there are not any control of temperature and air humidity of the environment.

d.2) Slow freezing

It is popularly believed that water freezes at 0°C, but this is rarely the case. In the absence of templates that allow the coming together of H_2O molecules, water super-cools to freezing points below zero. The lowest possible super-cooling temperature in most biological systems is the point of homogeneous ice nucleation around or at -40°C (Reed, 2008).

Slow freezing is based on osmotic regulation of cell contents and freeze-induced dehydration. The samples are cooled at 0.5 to 2°C/min to an intermediate temperature around -35°C or -40°C. Ice nucleation initiated at about -9°C. The samples are pretreated in cryoprotectant solution before cooling. At the freezing point of the cryoprotectant solution, ice nucleation is initiated and ice formed in the cryoprotectant solution and intercellular spaces. The cytoplasm remains unfrozen due to solute concentration and the cell wall protects the cell membrane from damaging ice crystal. As the temperature is further decreased to -35°C or -40°C, the extracellular solution becomes increasingly icy and the intracellular solutes become highly concentrated. The plant cells lose water to the exterior ice and the cytoplasm is further concentrated. The intracellular freezable water is safely reduced before samples are plunged into LN. If the cells are optimally dehydrated, the cytoplasm will vitrifies on contact with LN. If samples are under dehydrated, leaving freezable water in the cytoplasm, ice will form. If over

dehydrated, the cells may die from desiccation (Reed and Uchendu, 2008). Slow freezing step is very efficient for storing plant cells in LN. The cells were completely vitrified without crystallization and stored without injury.

d.3) Vitrification

Vitrification refers to the solidification of liquid without crystallization and finally solidifies into a glassy state. Vitrification is an effective freeze-avoidance mechanism. Vitrification of water in biological systems is dependent on increase cell viscosity. Increased viscosity inhibits the water to form ice. As a glass is exceedingly viscous and stops all chemical reaction that requires molecular diffusion. The vitrified state is metastable, meaning that it can relatively easily revert back to liquid and/or devitrifies to form ice (Reed, 2008).

Vitrification can be achieved by direct immersion in LN without air drying or slow freezing step, which is replaced by exposure of explants to an extremely concentrated of cryoprotectant solution.

d.3.1) Cryoprotectants

Cryoprotectants are chemicals that dissolve in water and have lower melting point than water. Common examples are glycerol, ethylene glycol (EG), propylene glycol and dimethylsulfoxide (DMSO). Cryoprotectants reduce the injury of cells during freezing and thawing. Cryoprotectants are separated into two classes, namely penetrating cryoprotectants and non-penetrating cryoprotectants.

a) Penetrating cryoprotectants

Penetrating cryoprotectants are small molecules that easily to move across cell membranes. The role of penetrating cryoprotectants is to reduce ice growth and reduce cell dehydration during freezing. Penetrating cryoprotectants, such as DMSO, EG and glycerol are the major

ingredients of vitrification solution. In vitrification, the role of penetrating cryoprotectants is to completely prevent ice formation (Wowk, 2007).

b) Non-penetrating cryoprotectants

Non- penetrating cryoprotectants are large molecule polymers, such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). They inhibit ice growth by the same mechanisms as penetrating cryoprotectants but do not enter cells. Non-penetrating cryoprotectants are usually less toxic than penetrating cryoprotectants at the same concentration. They reduce the amount of penetrating cryoprotectants needed by mimicking outside the cell. The cryoprotective affect to protein inside the cell (Wowk, 2007).

c) Vitrification solution

The vitrification solutions consist of a concentrated mixture of penetrating and non-penetrating cryoprotectants. The most commonly applied solution, named "PVS2" (Plant Vitrification Solution 2) is composed of 30 % (v/v) glycerol, 15 % (v/v) ethylene glycol, 15 % (v/v) DMSO and 0.4 M sucrose. PVS2 easily supercools below -100°C upon rapid cooling and solidifies into a metastable glass at about -115°C. Upon subsequent slow warming, differential scanning calorimetry records the vitrified PVS2 at displaying a glass transition (Tg) at about -115°C, with an exothermic devitrification (crystallization) (Td) at about -75°C and an endothermic melting (Tm) at about -36°C (Sakai *et al*., 2008).

The optimization of time and temperature of exposure to the PVS2 solution is the most important for producing a high level of regeneration after vitrification and prevent injury by chemical toxicity or excessive osmotic stress during treatment with the PVS2 solution. Thus, the exposure time in PVS2 solution may be associated with the size and structure of the explants. In general, the small explants are highly sensitive to PVS2 treatment. On the other hand, the larger explants require a much longer time for dehydration.

e) Freezing and rewarming

The cryoprotected explants required high cooling and warming rates. The explants are immersed directly in LN for cooling and stored at least for 1 h. The crystallization during the rewarming process can be prevented by rapid plunged the sample in a water bath thermoslated at 40°C (warming rate about 250°C/min) for 1-2 min (Sakai and Engelmann, 2007). After rewarming, the highly concentrated vitrification solution was removed to prevent the explants from toxic effect. The explants are rinsed with a high concentration of osmoregulating agent and usually use 1.2 M sucrose and finally plated on an appropriate medium.

f) Recovery and viability testing

Recovery of the explants is the goal of all cryopreserved storage. It is varying important to optimize the recovery medium for explants. Recovery of the explants requires the optimal environmental condition and regrowth media. In the cryopreservation experiment, regrowth is often achieved only after a long period of time, especially if a procedure is still in a sub-optimal state. In this case application of viability tests are useful. Three different methods are usually used for viability test of plant cells.

f.1) Tetrazolium (TTC) test

Cryopreserved explants are incubated in a colorless solution of TTC (2, 3, 5-Triphenyltetrazoliumchloride) in the dark at 25±2°C for 24 h. The living plant cells should show red color because dehydrogenase enzyme in living plant cells reduced the colorless triphenyltetrazoliumchloride to triphenylformazan (reduced TTC) in mitochondria (Maneerattanarungroj *et al*., 2007).

f.2) Fluorescien diacetate (FDA) test

Fluorescien diacetate is claved by cell wall esterase to derivative showing a green fluorescence. FDA-stained plant cells are observed under the fluorescent microscope. Both fluorescent and non-fluorescent celld can be seen. The green fluorescence indicates living celld (Dobbernack *et al*., 2008).

f.3) Evan's blue test

Evan's blue is a dye which penetrates into cells only after membrane damage. For viability testing with Evan's blue, cells are incubated in the dye for some time. Evan's blue stains the dead cell wall by showing the dark blue color. The explants which have higher absorbance value indicating the higher rate of mortality and lower absorbance value indicating higher viability rate (Hooi *et al*., 2010).

1.2.9 Cryopreservation technique

Cryopreservation is becoming a very important tool for long-term storage of plant genetic resources for future generation. Cryopreservation requires a minimum of space and maintenance. New cryopreservation techniques were developed base on vitrification procedure.

1.2.9.1 Vitrification technique

In vitrification procedure, the explants must be dehydrated with a vitrification solution to avoid lethal injury from immersion in LN. Crystallization during the rewarming process can be prevented if warming occurs rapidly. The complete vitrification method for unencapsulated tissues involves the following steps (Sakai and Engelmann, 2007).

A. Preculture of explants on solidified medium with 0.3 M sucrose for a specified duration at 25°C or 0°C.

B. Osmoprotection (loading treatment). Precultured explants are placed in a 2 ml cryotube and osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20-30 min at 25°C. An osmotic loading treatment increases the osmolarity of cell and minimizes osmotic damage caused by vitrification solution.

C. Dehydration with a vitrification solution. After removing the osmoprotection solution, vitrification solution is added and gentle mixed and held at 25°C or 0°C for different period of time.

D. Rapid cooling. The explants are suspended in 0.5 ml of vitrification solution in cryotube and then directly plunged into LN and held at -196°C for a minimum of 1 h.

E. Rapid warming (rewarming). Cryotubes are rapidly transferred to sterile distilled water in a water bath at 40°C. The cryotubes are vigorously shaken during rewarming for 1.5 min.

F. Unloading. Immediately after warming, the vitrification is drained from the cryotubes and replaced with 2 ml of basal medium with 1.2 M sucrose and held for 20 min.

G. Plating. The explants are transferred onto a double sterile filter paper disc over a Petri dish filled with culture medium. After 1 day, the explants are transferred to fresh filter paper in Petri dish containing the medium.

1.2.9.2 Encapsulation/dehydration technique

In the encapsulation/dehydration technique, explants are encapsulated in alginate beads that are osmoprotected in medium enriched with high concentration of sucrose before being dehydrated by air-drying in a laminar air-flow cabinet or with silica gel, and then plunged into LN. In this technique, the extraction of water results in progressive osmotic dehydration, and additional loss of water is obtained by evaporation and the subsequent increase of sucrose concentration in the beads. Thus, the sucrose molarity in the beads increases markedly during the drying process and reaches or exceeds the saturation point of the sucrose solution, resulting in glass transition during cooling to -196°C. The dehydration technique allows much more flexibility for handing large amounts of materials because the time schedule for

all steps is much broader than with vitrification and this technique does not use any other cryoprotectants other than sucrose (Sakai, 2004).

1.2.9.3 Encapsulation/vitrification technique

Encapsulation/vitrification technique is a combination of encapsulation and vitrification technique. The explants or precultured explants are encapsulated in alginate bead and place in loading solution for 20-30 min. They were dehydrated with vitrification solution before plunging into LN. For rewarming and unloading treatment, they were the same as vitrification technique. Washed encapsulated/vitrified explants were transferred onto solidified medium for regrowth. The encapsulation/vitrification method is easy to manage and eliminates the time requires for air desiccation (Sakai and Engelmann, 2007).

1.2.9.4 Droplet-vitrification technique

In droplet-vitrification technique, the explants are loaded and treated with vitrification solution by a drop of 5-10 µl of vitrification solution on a piece of aluminum foil, then immersed in LN. For rewarming, the aluminum foil are plunged in liquid medium containing 1.2 M sucrose and after 20 min of unloading, the explants are retrieved and placed on recovery medium. The main interest of this technique is the possibility of achieving very high cooling/warming rate due to the very small volume of cryoprotective medium in which the explants are placed (Sakai and Engelmann, 2007)

1.2.10 Cryopreservation of orchids

1.2.10.1 Cryopreservation of orchids by vitrification

Vitrification is a simple, fast and effective method for cryopreservation. In cryopreservation of orchid, the vitrification solution that was

commonly used is PVS2. The explants that used to cryopreserve by vitrification have various types, such as seeds, protocorms and cell suspension.

Thammasiri and Soamkul (2007) studied cryopreservation of *Vanda coerulea* Griff. Ex Lindl. seeds by vitrification, they used 7-month-old seeds dehydrated in 2 ml cryotubes filled with PVS2 that prepared in modified VW medium at 25±2°C for 70 min before plunged into LN. Then rewarming in a water bath at 40°C for 2 min, the PVS2 were replaced with unloading solution (1.2 M sucrose in VW liquid medium) at 25±2°C for 20 min prior transfer to VW agar medium. About 67 % were able to develop into normal seedling, while there was no survival after cryopreservation without PVS2 treatment.

Immature seeds of *Bletilla striata* were cryopreserved by vitrification. When immature seeds collected 3 and 4 months after pollination were precultured for 3 days on New Dogashima medium (ND medium) supplemented with 0.3 M sucrose and cryoprotected with loading solution (2 M sucrose and 0.4 M sucrose in ND medium) at 25°C for 15 min following removal loading solution, the seeds were dehydrated with PVS2 prepared in ND medium supplemented with 0.4 M sucrose at 0°C for 120 min after plunging into LN, the cryotubes were rewarmed in water bath at 38°C. The PVS2 solution was drained and replaced with liquid ND medium containing 1.2 M sucrose and held for 10 min then replaced with fresh liquid ND medium supplemented with 0.4 M sucrose. Finally, the seeds were sown on solidified ND medium supplemented with 88 mM sucrose and culture at 25°C. The survival rate after preservation, as assessed by staining with TTC was 92 % and 81 %, respectively (Hirano *et al*., 2005).

The study in *Cymbidium* species, the mature seeds were cryopreserved by vitrification. Seeds were treated with loading solution for 15 min at 25°C and then dehydrated with PVS2 at 0°C for various periods. The seeds were directly immersed in LN for 30 min. Then, the cryotubes were rewarming in a water bath at 38°C for 2 min. After PVS2 was drained from the cryotubes, the seeds were treated with 1.2 M sucrose for 10 min. This solution was then drained off and the seeds were treated with 0.4 M sucrose for 10 min before sawing on solidified ND medium supplemented with 58mM sucrose and incubated at 25°C. The survival rate of seeds was estimated by TTC test. And the results showed that the optimum period

of exposure to vitrification solution were found to be 60 min for *C. goeringii* and *C. macrorhizon* seeds and 30 min for *C. finlaysonianum* seeds and the germination rates of cryopreserved seeds were 32 % for *C. goeringii*, 82 % for *C. macrorhizon* and 76 % for *C. finlaysonianum* (Hirano *et al*., 2011).

Ishikawa *et al* (1997) studied in *Bletilla striata*, they cryopreserved zygotic embryo by vitrification. 10-day-old embryo of *Bletilla striata* were precultured on ND medium supplemented with 0.3 M sucrose for 3 days before treated with loading solution for 15 min and finally dehydrated with PVS2 for 180 min at 0℃ prior to immersions into LN for 30 min. After rapid warming, the embryos were washed with ND liquid medium supplemented with 1.2 M sucrose for 20 min and then plated on ND solid medium. Successfully vitrified and warmed embryos developed into normal plantlets. The rate of plant regeneration amounted to about 60 %.

Tsukazaki *et al* (2000) in *Doritaenopsis* cv. New Toyohashi, they use cells of a suspension culture to cryopreserved by vitrification. The cells were placed in loading solution for 15 min at room temperature and then dehydrated with PVS2 for 1-3 h (there was no significant difference in viability) on ice and plunged into LN. The high viability was 64 % by TTC test was obtained when the cells were precultured in ND liquid medium with 0.1 M sucrose and 1.0 mg/l abscisic acid for 1 week at 25℃ in the light.

1.2.10.2 Cryopreservation of orchids by encapsulation/dehydration

In vitro shoot tips of *Dendrobium* Walter Oumae were used to cryopreserved by encapsulation/dehydration technique. Shoot tips were suspended in calcium-free modified VW medium supplemented with 3 % (w/v) sodium alginate plus 0.4 M sucrose. The shoot tips with suspension were dropped into 0.1 M $CaCl_2$ solution for 40 min to form beads. The survival ratio and regrowth measured by TTC assay and regrowth culture test, respectively. The highest survival ratio was 16.18 mg living cells/100 mg total cells and regrowth rate was about 13.33 % resulted from encapsulated shoot tips that were precultured on 0.3 M sucrose agar medium for 2 days and dehydrated by incubation in the sterile air flow of a laminar air-flow cabinet

for 6-8 h before storing in LN. The regrowth of shoot tips developed directly into complete plantlets without protocorm formation and had normal morphology (Lurswijidjarus and Thammasiri, 2004).

In rare Thai orchid, *Cleisostoma areitinum* (Rchb. F.) Garay. protocorms were cryopreserved by encapsulation/dehydration. Protocorms were precultured on ND semi-solid medium with 0.25 M sucrose in the dark for 1 week then transferred to ND liquid medium containing 0.75 M sucrose for 2 days. The precultured protocorms were dehydrated with silica gel in a laminar air-flow cabinet for 5 h prior to immersion in LN for 1 h. The survival rate of cryopreserved protocorms was 49 % while percentage of TTC assay was 77 % (Maneerattanarungroj *et al*., 2007).

Protocorms of *Vanda coerulea* were successfully cryopreserved by encapsulation/dehydration. 70-day-old protocorms were encapsulated in an alginate matrix composed of 2 % sodium alginate, 2 M glycerol plus 0.4 M sucrose (loading solution) and precultured in modified VW liquid medium supplemented with 0.7 M sucrose at 25±2°C for 20 h before dehydrating in a sterile air-flow in a laminar air-flow cabinet and then plunging into LN for 1 day. After rewarming at 40°C for 2 min, cryopreserved beads were cultured on modified VW agar medium for regrowth. The highest regrowth was 40 % observed with cryopreserved beads that dehydrated for 8 h (Jitsopakul *et al*., 2008). In cryopreservation of *Grammatophyllum speciosum* by encapsulation/dehydration, similar procedure to the study of *V. coerulea* was performed. The encapsulated protocorms precultured in ½ MS liquid medium containing 0.4 M sucrose for 2 days, followed by soaking in loading solution prepared with ½ MS liquid medium for 20 min and dehydrated in laminar air-flow cabinet for 7 h gave 24 % protocorm regrowth (Sopalun *et al*., 2010).

Two desiccation methods were used in the study of *Phalaenopsis bellina* (Rchb. F.). The encapsulated PLBs were precultured in ½ MS medium supplemented with 0.75 M sucrose for 3 days. Then, desiccation by using silica gel and larminar air-flow were applied before plunging into LN. After storage in LN the encapsulated PLBs were rewarmed. Two weeks after rewarming, PLB viability was determined by TTC reduction and regrowth was assessed. Encapsulated

PLBs desiccated using silica gel for 5 h resulting in the highest post rewarming viability in term of TTC reduction at 46.6 % of control PLBs and 30 % regrowth (Khoddamzadeh *et al*., 2011).

1.2.10.3 Cryopreservation of orchids by encapsulation/vitrification

The shoot tips of *Vanda coerulea* were successfully cryopreserved by encapsulation/vitrification technique and developed into normal plantlets. The rate of plant regeneration was about 60 % when encapsulated shoot tips were precultured on VW agar medium supplemented with 0.3 M sucrose for 3 days and treated with loading solution for 20 min and dehydrated with PVS2 for 3 h before plunging into LN for 1 day (Soamkul and Thammasiri, 2005).

In *Dendrobium candidum*, PLBs were precultured in MS liquid medium containing 0.2 mg/l NAA and 0.5 mg/l BA enriched with 0.75 M sucrose and grown under continuous light at 25±1°C for 5 days. Precultured PLBs were osmoprotected with a mixture of 2 M glycerol and 1 M sucrose for 80 min at 25°C before encapsulated with 2 % sodium alginate. Then, they were dehydrated with a PVS2 in 0.5 M sucrose for 150 min at 0°C before plunging into LN for 1 h. Cryopreserved PLBs were rapidly rewarmed in a water bath at 40°C for 3 min and then washed with MS liquid medium containing 1.2 M sucrose. The survival rate was above 85 %. The cryopreserved PLBs developed normal shoots and roots without any morphological abnormalities (Yin and Hong, 2009).

In the study of *Grammatophyllum speciosum*, the cryopreservation by encapsulation/vitrification gave 14 % survival rate when encapsulated protocorms were precultured in ½ MS liquid medium containing 0.4 M sucrose for 2 days followed by soaking in loading solution (2 M glycerol and 0.4 M sucrose in ½ MS liquid medium) for 20 min and dehydrated with PVS2 for 60 min before stored in LN (Sopalun *et al.*, 2010).

1.2.10.4 Cryopreservation of orchids by droplet-vitrification

Droplet-vitrification was studied in *Bletilla striata*. Seeds and

protocorms in various stages were cryopreserved. The results showed the highest germination percentage of mature seeds, 3-day germination seeds and survival of cryopreserved 6-day-old protocorms was 93 %, 91 % and 84 %, respectively when precultured the explants in liquid ND medium supplemented with 0.3 M sucrose for 3 h on a shaker (110 rpm) and dehydrated with loading solution (2 M glycerol and 0.4 M sucrose in liquid medium) for 15 min and exposed to PVS2 for 60 min at 25°C before plunged into LN. For cryopreservation of 9-day-old protocorms, the highest survival rate at 66 % was achieved after precultured with 0.5 M sucrose for 3 h, dehydrated with loading solution for 15 min, exposured to PVS2 for 40 min (Jitsopakul *et al*., 2008).

In cryopreservation of *Grammatophyllum speciosum* by droplet-vitrification, the protocorms were precultured on filter paper soaked in MS medium containing 0.4 M sucrose at 25±2°C for 2 days, followed by soaking in loading solution (2 M glycerol and 0.4 M sucrose in ½ MS liquid medium) for 20 min and dehydrated with PVS2 for 30 min showed high protocorms regrowth at 38% (Sopalun *et al*., 2010).

1.3 Objectives of the research

1.3.1 To investigate the effects of basal media and sugar types on *in vitro* culture of *G. speciosum*.

1.3.2 To findout the encapsulation condition and short-term storage condition for *G. speciosum*.

1.3.3 To investigate the potential of the 2-step vitrification technique to cryopreserve the protocorms of *G. speciosum*.

1.3.4 To investigate the potential of the vitrification technique to cryopreserve the protocorms of *G. speciosum*.

1.3.5 To investigate the potential of the encapsulation/vitrification technique to cryopreserve the protocorms of *G. speciosum*.

CHAPTER 2
MATERIALS AND METHODS

2.1 Seed culture

2.1.1 Plant materials

6-7-month-old seed pods of *G. spe*ciosum from self pollinated plants were collected from the garden in Khuankalong district, Satun province, Thailand.

2.1.2 Culture medium and condition

Modified MS medium containing 3 % (w/v) sucrose, 15 % (v/v) coconut water (CW) and solidified with 8.2 g/l agar. The pH of the medium was adjusted to 5.5 prior to autoclaving at 121°C at 1 kPa for 20 min. The cultures were incubated in standard condition that is, incubated at 25±2°C under 16/8 (day/night) photoperiod provided by white fluorescent tubes (Philips) at an intensity of 1,960 lux.

2.1.3 Disinfestations

Seed pods were washed with running tap water before immersion in 95 % ethanol and flamed for 3 times then they were dissected longitudinally and the seeds were cultured on culture medium. Protocorms from seeds culture were used as explants for this study.

2.2 The effects of basal media and sugar types on *in vitro* culture

2.2.1 Effect of basal media

Protocorms about 2-4 mm in length were cultured on MS and VW media supplemented with 15 % (v/v) coconut water and 2 or 3 % (w/v) sucrose. The pH of the medium was adjusted to 5.5 before adding 8 g/l agar. The culture vessels were incubated at 25±2 ºC with a 16 h photoperiod. The formation of PLBs was determined after 4 weeks of culture and compared between the twodifferent culture media using least significant of variant ($P \leq 0.05$). The better media will be selected and were used for PLBs induction.

2.2.2 Effects of types and concentrations of sugar

Protocorms were cultured on MS liquid media supplemented with 15 % CW and several types of sugars namely sucrose, glucose, sorbitol and mannitol at concentrations of 0, 2, 4, 6 or 8 % (w/v). The cultures were incubated at 25±1 ºC with a 16 h photoperiod for 1, 2, 3 or 4 weeks. Twenty incubated protocorms of each treatment were transferred to the induction medium and incubated in standard conditions. Regenerated protocorms were counted after culture for 4 weeks and evaluated in term of survival percentage.

2.3 The encapsulation production and short-term storage conditions

2.3.1 Encapsulation production

Sodium alginate at concentration of 1, 3, and 5 % (w/v) were prepared with sterile distilled water. Then dropped the sodium alginate matrix into 50, 75, or 100 mM $CaCl_2$ solution for 15, 30, or 45 min to form beads. The beads were washed in sterile distilled water before being transferred to Petri dish. The morphology of beads was observed. The best forming bead conditions were used for encapsulating protocorm in encapsulation/vitrification procedure.

2.3.2 Short-term storage conditions

For short-term storage, 2-4 mm-long protocorms were encapsulated with 1, 3, and 5 % sodium alginate in sterile distilled water and dropped into 50, 75, and 100 mM $CaCl_2$ solution for 30 min to form round beads. The beads were washed with sterile distilled water before being transferred to Petri dishes. Forty beads of encapsulated protocorms were placed in 5 cm-diameter Petri dish and all of them were covered with aluminum foil for dark condition. The covered Petri dishes were incubated in refrigerator at 4°C and at 25°C in culture room.

Twenty beads of encapsulated protocorms from each treatment were transferred to induction medium every 2 weeks for 16 weeks. The suitable conditions for short-term storage of encapsulated protocorms were observed by the plantlet conversion percentage of encapsulated protocorm as the following equation.

$$\text{Plantlet conversion percentage} = \frac{\text{Regenerated encapsulated protocorm}}{\qquad\qquad\qquad\qquad} \text{ X } 100$$

2.4 Cryopreservation protocorms by 2-step vitrification

The 2-4 mm-long protocorms were used as explants. There were 2 types of explant preparation used in this experiment, precultured and non-precultured protocorms. For precultured protocorms, the protocorms were precultured in 0.5 M sucrose for 1 day on the orbital shaker (80 rpm) at 25±2°C under 16/8 (day/night) photoperiod with white fluorescent tubes (Philips) at an intensity of 1,960 lux. Twenty pieces of precultured and non-precultured protocorms were put in 2 ml cryotube. The explants were dehydrated with ½ PVS2 for 0, 20, 40, 60, 80, and 100 min then removed and rapidly replaced with PVS2 and soaked for 0, 20, 40, 60, 80, and 100 min at 25±2°C and stored in LN for 1 d.

After freeze storage, cryotubes were rapidly rewarmed at 40±2°C for 2 min in water bath. The cryopreserved protocorms were test for viability by 0.5 % (w/v) Evan's blue for 2 h and 30 min. Then, washed with distilled water for 3 times. Evan's blue stained the dead protocorm by showing the dark blue color.

2.5 cryopreservation protocorms by vitrification technique

The 2-4 mm-long protocorms were precultured with MS liquid medium supplemented with 15 % (v/v) CW and 0.5 M sucrose for 0, 1, 2, and 3 days on the shaker (80 rpm) at 25±2°C under 16/8 (day/night) photoperiod with white fluorescent tubes (Philips) at an intensity of 1,960 lux. Precultured protocorms were osmoprotected with loading solution consisted of 1 M sucrose and 2 M glycerol in sterile distilled water for 20 min before dehydration. Twenty precultured and osmoprotected protocorms were put in 2 ml cryotube. The explants were dehydrated with PVS2 for 0, 20, 40, 60, 80, and 100 min at 25±2°C and stored in LN for 1 d.

After freeze storage, the cryotubes were rapidly rewarmed at 40±2°C for 2 min in water bath. The PVS2 solution were removed and replaced with unloading solution consisted of 1 M sucrose in sterile distilled water for 20 min at 25±2°C for 20 min. Then, the cryopreserved protocorms were tested for viability by 0.5 % (w/v) Evan's blue for 2 h and 30 min. Then, washed with distilled water for 3 times. Evan's blue stained the dead protocorm by showing the dark blue color.

2.6 Cryopreservation protocorms by encapsulation/vitrification technique

The 2-4 mm-long protocorms from seed culture were encapsulated with 3 % sodium alginate and complexed with 75 mM $CaCl_2$ solution for 30 min to form round beads. The encapsulated protocorms were precultured in 0.5 M sucrose for 0, 1, 2, or 3 d. Encapsulated protocorms were osmoprotected with loading solution that consisted of 1 M sucrose and 2 M glycerol in sterile distilled water for 20 min before dehydration in 2 ml cryotube. The explants were dehydrated with PVS2 for 0, 20, 40, 60, 80, and 100 min at 25±2°C and stored in LN for 1 d.

After freeze storage, the cryotubes were rapidly rewarmed at 40±2°C for 2 min in water bath. The PVS2 solution were removed and replaced with unloading

solution that consisted of 1 M sucrose in sterile distilled water for 20 min at 25±2°C for 20 min. Then, the cryopreserved protocorms were taken out from the beads and tested for viability by 0.5 % (w/v) Evan's blue for 2 h and 30 min. Then, washed with distilled water for 3 times. Evan's blue stained the dead protocorm by showing the dark blue color.

CHAPTER 3
RESULTS

3.1 Seed culture

Seeds were germinated, increased in size, became swollen and turned to green protocorms after cultured on modified MS medium for 5-6 months (Figure 5). The 2-4 mm-long protocorms were used as explants for other experiments in this study.

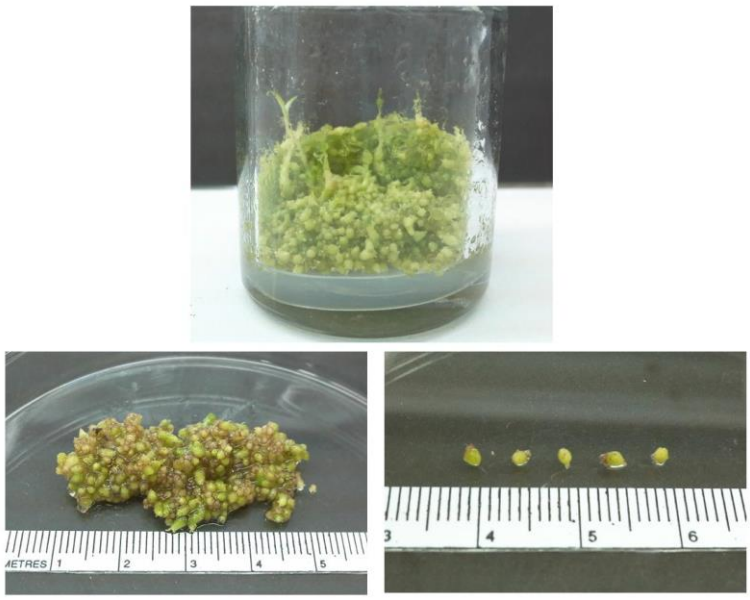


Figure 5 Protocorms after cultured on modified MS medium with out PGR for 5-6 months.

3.2 The effect of basal media and sugar types on *in vitro* culture.

3.2.1 Effect of basal media

After 4 weeks of culture of *G. speciosum* protocorms on solidified MS medium containing 15 % (v/v) coconut water and 2 % (w/v) sucrose most of the protocorms had a high PLBs formation rate at 84 % and number of PLBs at 3.1 PLBs/explant. The lowest formation of PLBs at 1.46 PLBs /explants was found when cultured on VW medium containing 15 % CW and 3 % sucrose (Figure 6). This result showed that MS medium was more suitable for the formation *G. speciosum* PLBs than VW.

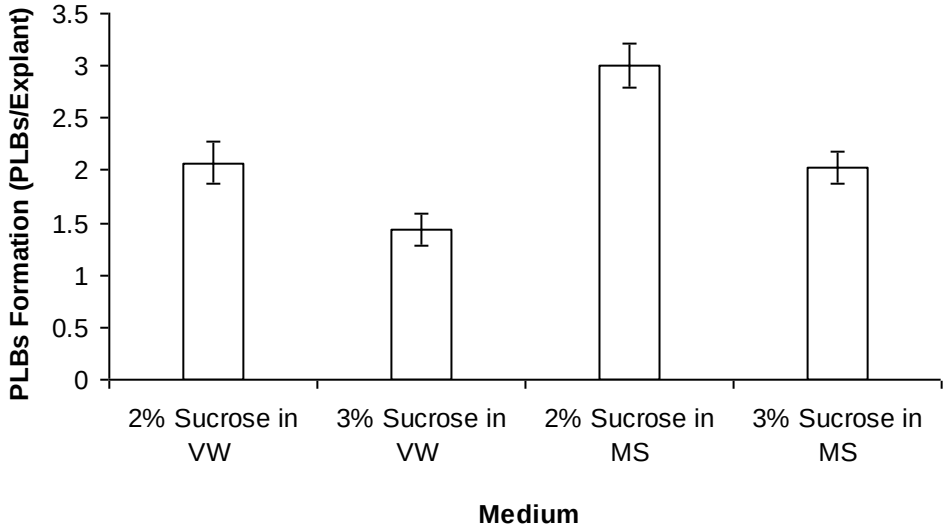
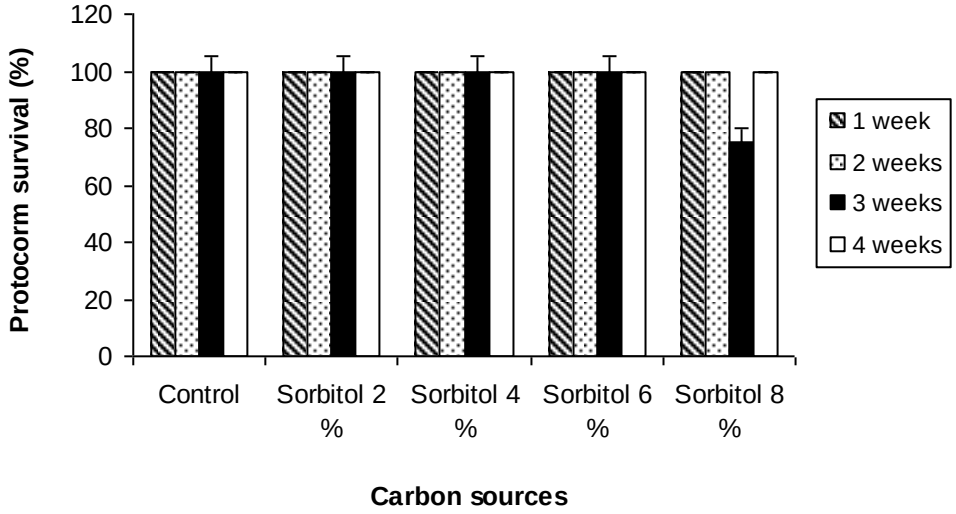


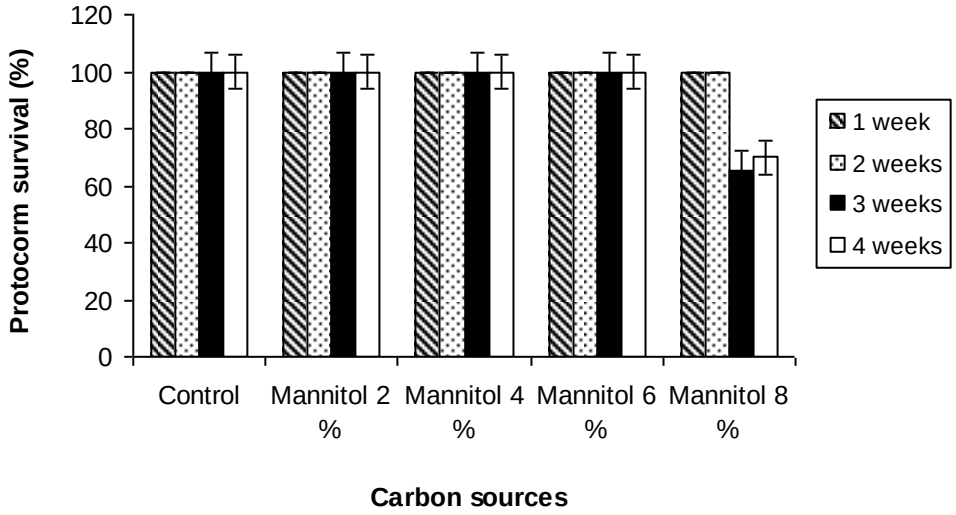
Figure 6 Formation of PLBs after 4 weeks in different basal medium and concentrations of sucrose. All media contained 15 % (v/v) coconut water. In each treatment 100 explants were studied. *Bar* = standard error.

3.3.2 Effect of types and concentrations of sugar.

Figure 7 shows the effect of different types and concentrations of sugar on regeneration of protocorms. MS medium supplemented with either mannitol or sorbitol gave 100 % regeneration of green protocorms and produced PLBs (Figure 7 A and B). Sucrose and glucose had an inhibitory effect on the growth of protocorms

and 8 % glucose gave the lowest survival of protocorms (30 %). Most of the protocorms turned brown and did not regenerate into plantlets. The results showed that different carbon sources and concentrations had a significant effect on the regeneration of plants





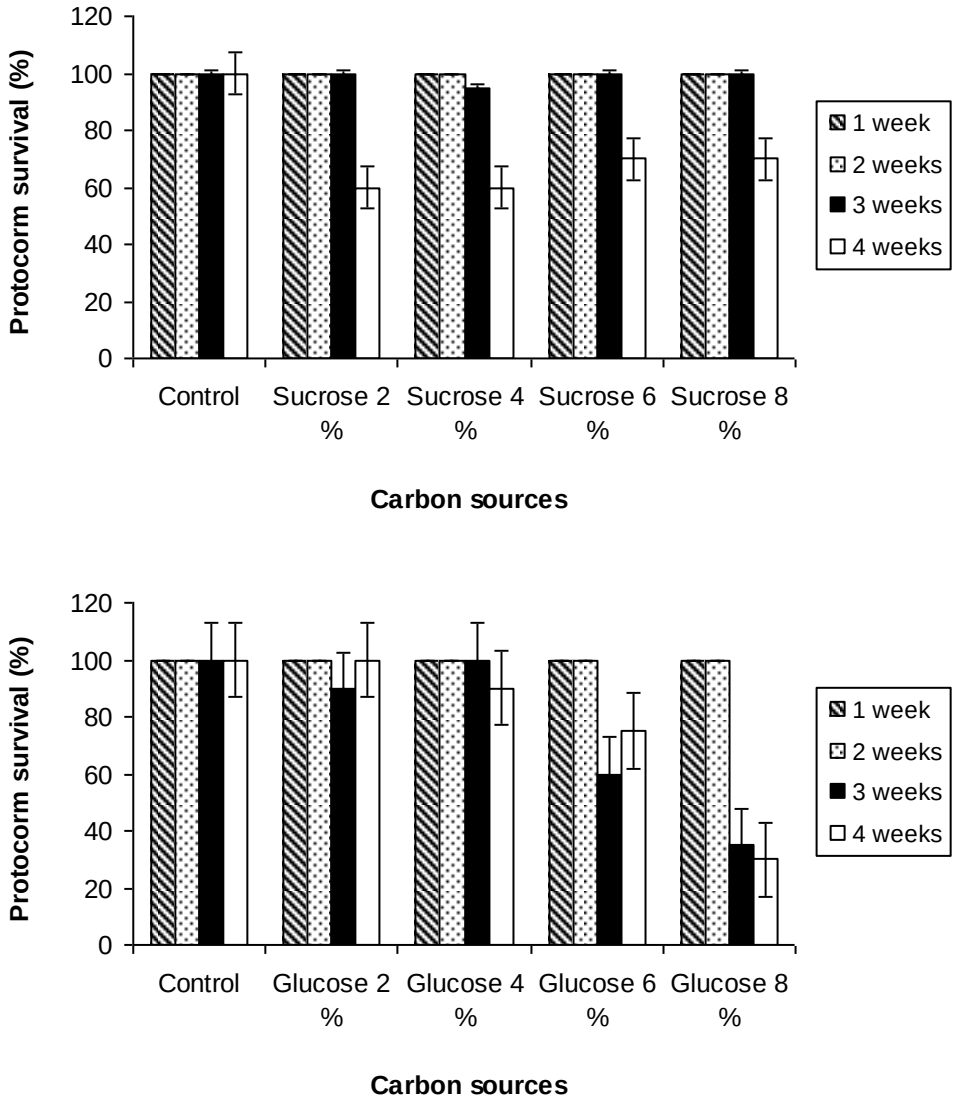


Figure 7 Regeneration of protocorms after 4 weeks in media with different types and concentrations of sugar. In each treatment 20 explants were studied. *Bar* = standard error.

3.3 The encapsulation production and short-term storage conditions

3.3.1 Encapsulation production

Encapsulated beads had various forms depended on the concentrations of sodium alginate, $CaCl_2$ solution, and polymerized time exposure

(Figure 8, 9, 10). 1 % sodium alginate did not form round beads in all conditions. 3 % sodium alginate gave the best round beads and 5% sodium alginate formed round tailed beads. When the encapsulated beads exposed in $CaCl_2$ solution for a long period the beads were hardened and milky in color (Table 1). The appropriate condition to produce the 5 mm in diameter round beads was 3 % sodium alginate and polymerized in 75 mM $CaCl_2$ solution for 30 min (Figure 11).

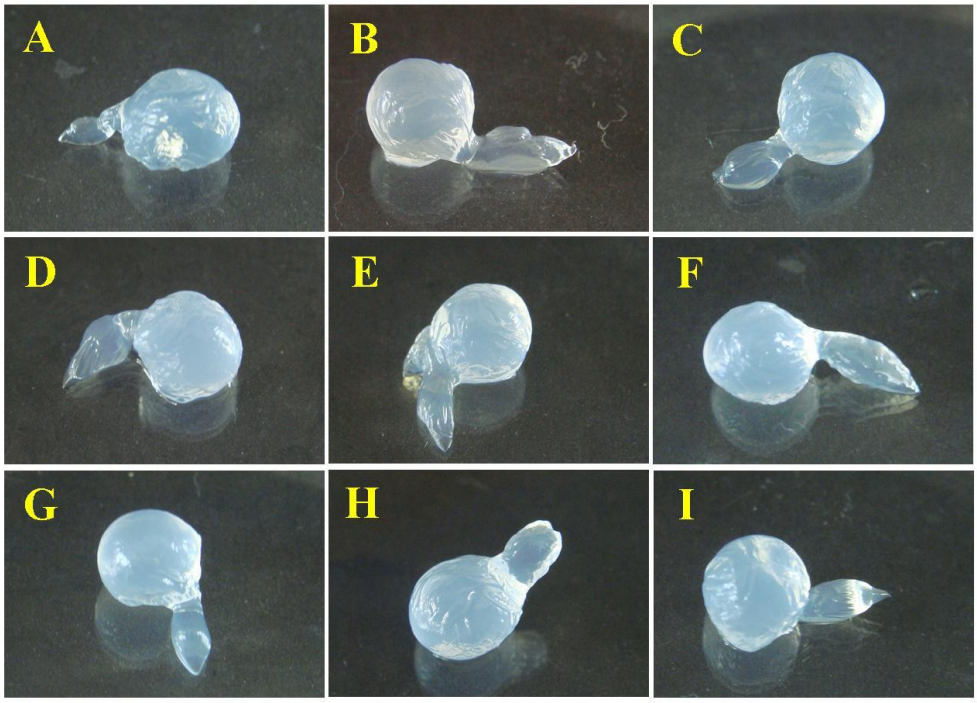


Figure 8 The encapsulated beads of 1 % sodium alginate that polymerized in different concentration of $CaCl_2$.

A) 50 mM $CaCl_2$ for 15 min, B) 50 mM $CaCl_2$ for 30 min,

C) 50 mM $CaCl_2$ for 45 min, D) 75 mM $CaCl_2$ for 15 min,

E) 75 mM $CaCl_2$ for 30 min, F) 75 mM $CaCl_2$ for 75 min,

G) 100 mM $CaCl_2$ for 15 min, H) 100 mM $CaCl_2$ for 30 min,

I) 100 mM $CaCl_2$ for 45 min.

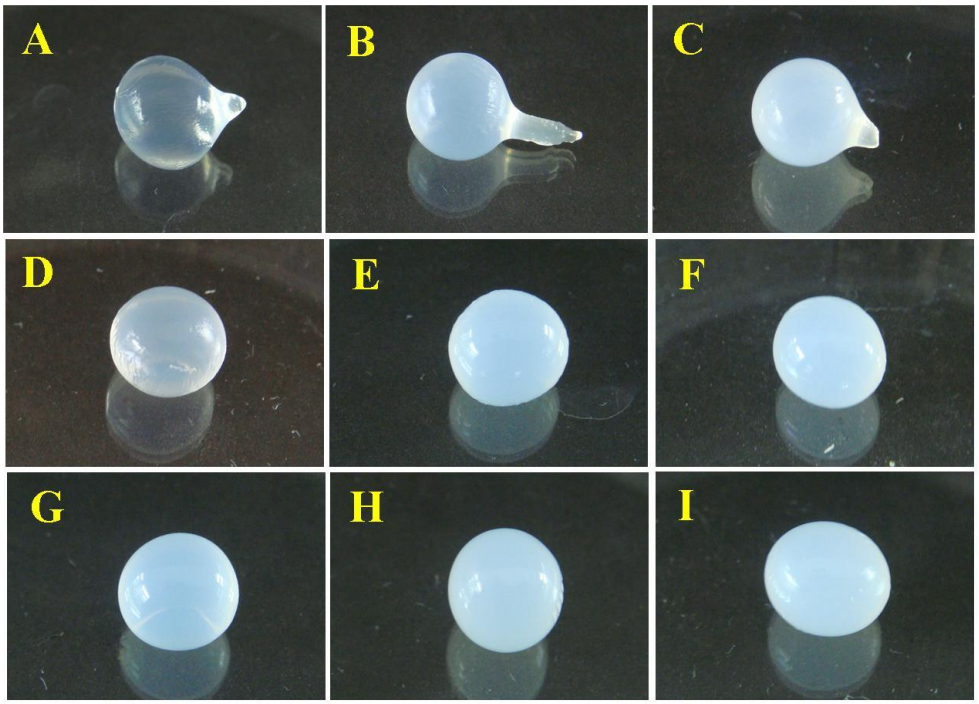


Figure 9 The encapsulated beads of 3 % sodium alginate that polymerized in different concentration of $CaCl_2$.

A) 50 mM $CaCl_2$ for 15 min, B) 50 mM $CaCl_2$ for 30 min,

C) 50 mM $CaCl_2$ for 45 min, D) 75 mM $CaCl_2$ for 15 min,

E) 75 mM $CaCl_2$ for 30 min, F) 75 mM $CaCl_2$ for 75 min,

G) 100 mM $CaCl_2$ for 15 min, H) 100 mM $CaCl_2$ for 30 min,

I) 100 mM $CaCl_2$ for 45 min.

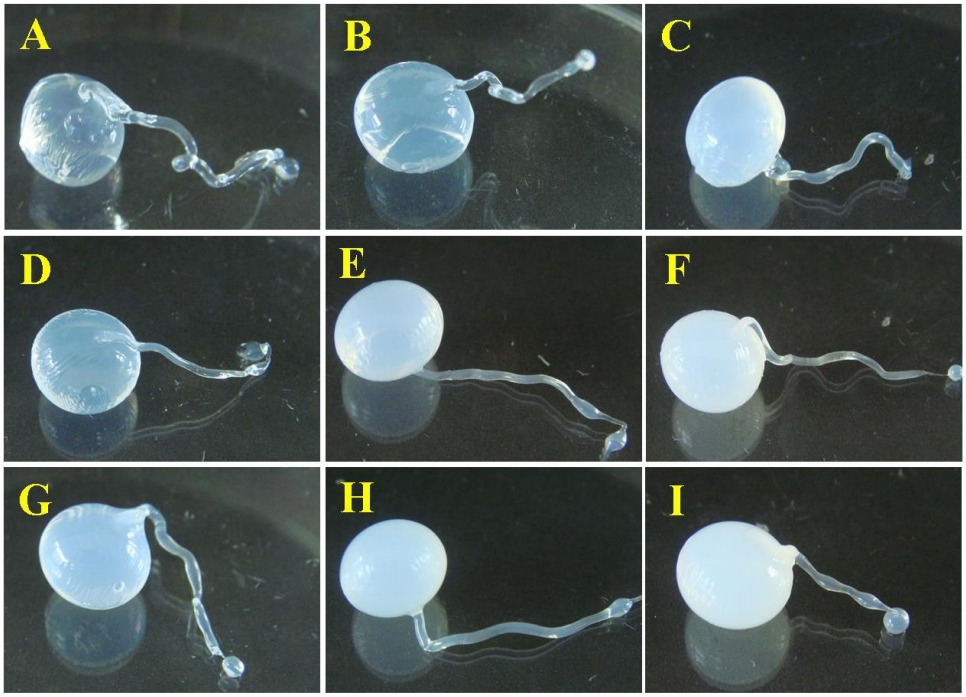


Figure 10 The encapsulated beads of 5 % sodium alginate that polymerized in different concentration of $CaCl_2$.

A) 50 mM $CaCl_2$ for 15 min, B) 50 mM $CaCl_2$ for 30 min,

C) 50 mM $CaCl_2$ for 45 min, D) 75 mM $CaCl_2$ for 15 min,

E) 75 mM $CaCl_2$ for 30 min, F) 75 mM $CaCl_2$ for 75 min,

G) 100 mM $CaCl_2$ for 15 min, H) 100 mM $CaCl_2$ for 30 min,

I) 100 mM $CaCl_2$ for 45 min.

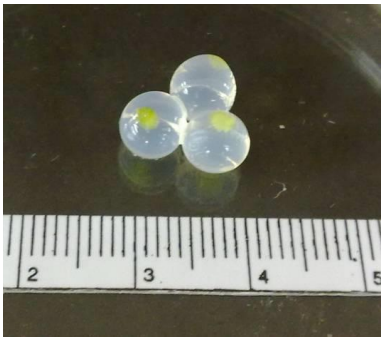


Figure 11 Encapsulated bead sizes of 3 % sodium alginate polymerized with 75 mM $CaCl_2$.

Table 1 Encapsulated beads formed from different concentrations of sodium alginate, $CaCl_2$ solution and polymerized exposure time.

Sodium alginate (%)	$CaCl_2$ solution (mM)	Polymerized exposure time (min)		
		15	30	45
1	50	+	+	+
	75	+	+	+
	100	+	+	+
3	50	++	++	++ ^{m}
	75	+++	+++ ^{m}	+++ ^{m}
	100	+++	+++ ^{m}	+++ ^{m}
5	50	++	++	++ ^{m}
	75	++	++	++ ^{m}
	100	++	++ ^{m}	++ ^{m}

\+ = Do not form round beads.

++ = Nearly round and tailed beads.

+++ = Round beads.

^{m} = Milky beads.

3.3.2 Short-term storage conditions

The protocorms of *G. speciosum* could be conserved by encapsulation with 3 % sodium alginate complexed with 75 or 100 mM $CaCl_2$ solution and stored at 25°C in dark condition (Table 2) and give 100 % survival rate after storage at 16 weeks (Fig 13 B, and C). The storage at low temperature gave ineffective for germination rate of encapsulated protocorm of *G. speciosum*. All encapsulated beads could store at 4°C for 2-8 weeks, however, low survival rate was obtained (Fig 12, 13, and 14). The protocorms encapsulated with 1 % sodium alginate complexed with 50 mM $CaCl_2$ solution gave 55 % survival rate after stored at 25°C in dark condition for 8 weeks (Figure 12A). Encapsulated beads with 5 % sodium alginate complexed with 75 mM $CaCl_2$ solution could stored at 25°C for 10 weeks and an average of 3 encapsulated protocorm (60 %) can regenerated into plantlets (Figure 15 B). And. An average of 1.75 encapsulated protocorm (35 %) was regenerated into plantlets after store at 4°C for 6 weeks.

Table 2 Regeneration of encapsulated protocorms of *G. speciosum* after stored at 4 and 25°C in dark condition for 2-16 weeks (Mean±SD).

Encapsulated condition	Temp. (°C)	Storage time (weeks)							
		2	4	6	8	10	12	14	16
1 % sodium alginate + 50 mM $CaCl_2$	4	1.25±0.96	0.25±0.50	-	-	-	-	-	-
	25	-	-	3.25±0.96	2.75±1.26	-	-	-	-
1 % sodium alginate + 75 mM $CaCl_2$	4	0.75±0.96	-	-	-	-	-	-	-
	25	1.75±0.50	1.25±0.96	-	-	-	-	-	-
1 % sodium alginate + 100 mM $CaCl_2$	4	2.00±0.82	-	-	-	-	-	-	-
	25	4.50±0.58	4.25±0.50	2.50±0.58	1.50±1.29	-	-	-	-
3 % sodium alginate + 50 mM $CaCl_2$	4	5.00±0.00	3.00±0.82	0.25±0.50	0.25±0.50	-	-	-	-
	25	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	4.00±0.82	2.25±1.26	-
3 % sodium alginate + 75 mM $CaCl_2$	4	5.00±0.00	4.25±0.50	0.50±0.58	0.25±0.50	-	-	-	-
	25	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.00±00	5.00±0.00	5.00±0.00	5.00±0.00
3 % sodium alginate + 100 mM $CaCl_2$	4	5.00±0.00	1.00±0.82	-	1.0±0.82	-	-	-	-
	25	5.00±0.00	5.0±0.00	5.00±0.00	3.75±0.50	4.75±0.50	4.75±0.50	5.00±0.00	5.00±0.00
5 % sodium alginate + 50 mM $CaCl_2$	4	3.75±0.96	2.25±1.71	1.00±1.15	-	-	-	-	-
	25	5.0±0.00	5.00±0.00	-	-	-	-	-	-
5 % sodium alginate + 75 mM $CaCl_2$	4	4.25±0.96	3.00±0.82	1.75±1.26	-	-	-	-	-
	25	4.25±0.50	5.00±0.00	3.50±1.29	3.00±0.82	3.00±0.82	-	-	-
5 % sodium alginate + 100 mM $CaCl_2$	4	4.50±0.58	0.50±0.58	-	-	-	-	-	-
	25	2.00±0.82	-	-	-	-	-	-	-

Number of protocorms in each treatment was 5 protocorms.

Mean±SD ($P \leq 0.05$) calculated by Microsoft Office Excel 2003.

- = No regeneration

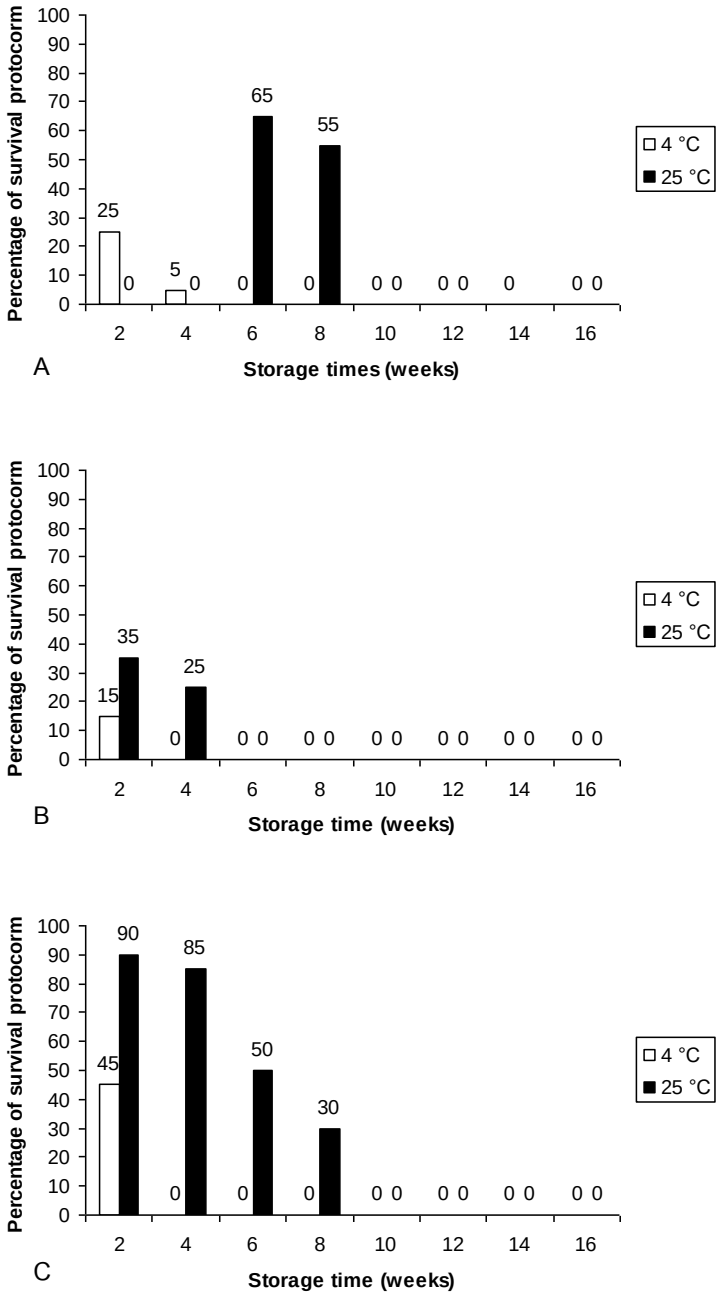


Figure 12 Percentage of survival of 1 % sodium alginate encapsulated protocorms of *G. speciosum* after stored at 4 and 25°C for different storage period. A) 1 % sodium alginate complexed with 50 mM $CaCl_2$, B) 1 % sodium alginate complexed with 75 mM $CaCl_2$, C) 1 % sodium alginate complexed with 100 mM $CaCl_2$.

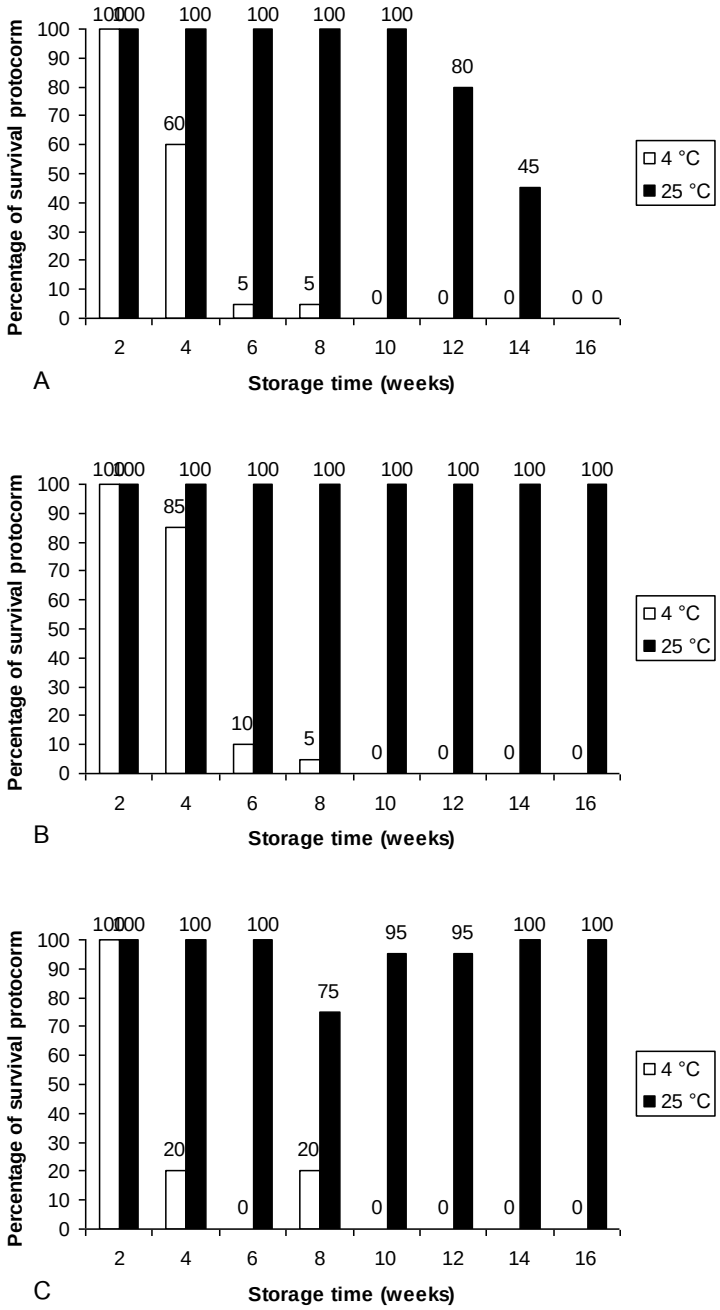


Figure 13 Percentage of survival of 3 % sodium alginate encapsulated protocorms of *G. speciosum* after stored at 4°C and 25°C in different storage period. A) 3 % sodium alginate complexed with 50 mM $CaCl_2$, B) 3 % sodium alginate complexed with 75 mM $CaCl_2$, C) 3 % sodium alginate complexed with 100 mM $CaCl_2$.

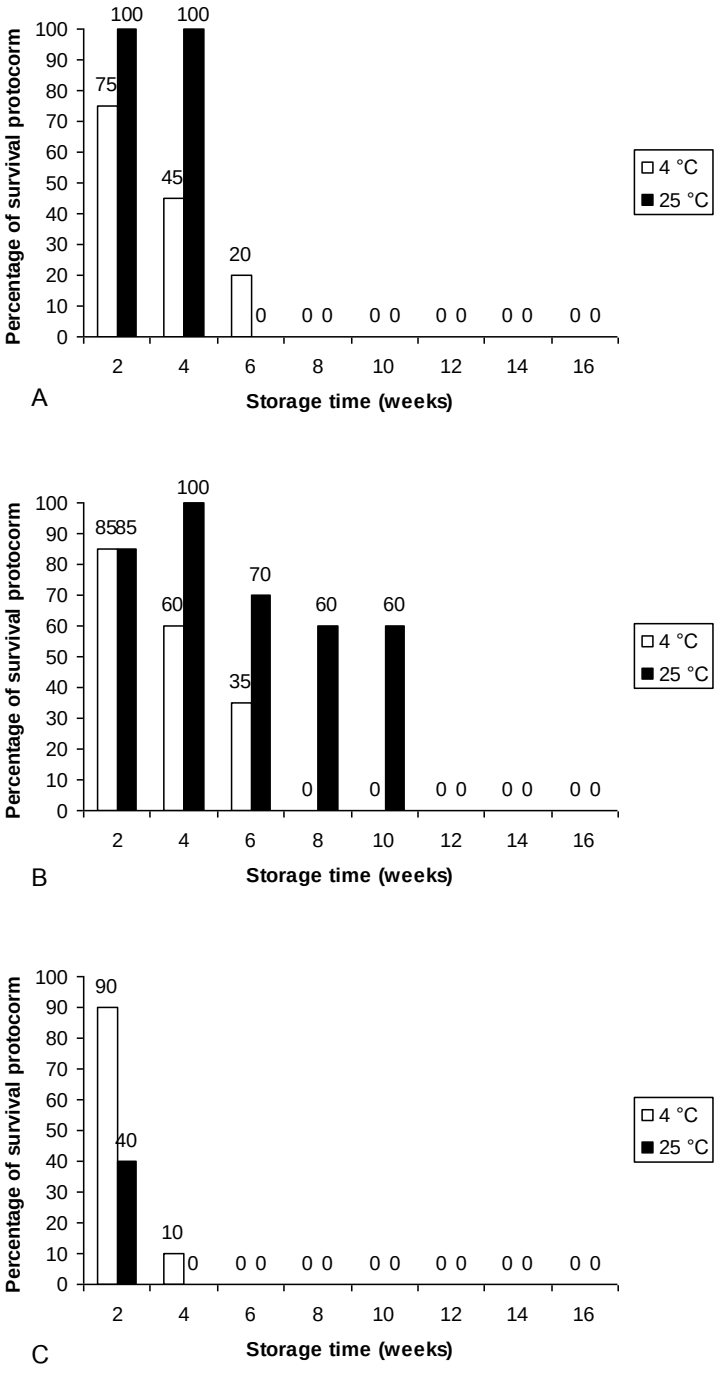


Figure 14 Percentage of survival of 5 % sodium alginate encapsulated protocorms of *G. speciosum* after stored at 4°C and 25°C in different storage period. A) 5 % sodium alginate complexed with 50 mM $CaCl_2$, B) 5 % sodium alginate complexed with 75 mM $CaCl_2$, C) 5 % sodium alginate complexed with 100 mM $CaCl_2$.

Protocorms of *G. speciosum* produced encapsulated by 3 % sodium alginate complexed with 75 mM $CaCl_2$ solution could store at 25°C in dark condition for 16 weeks. Encapsulation can be useful for short time storage or conservation. The encapsulated protocorms were regenerated after transferred to induction medium and incubated in standard condition for 3-4 weeks (Figure 15). Most encapsulated protocorms that stored at 4°C did not regenerated and turned to white after transferred to PLB induction medium (Figure 16 A) while protocorms in beads stored at 25°C were still green in color (Figure 16 B).

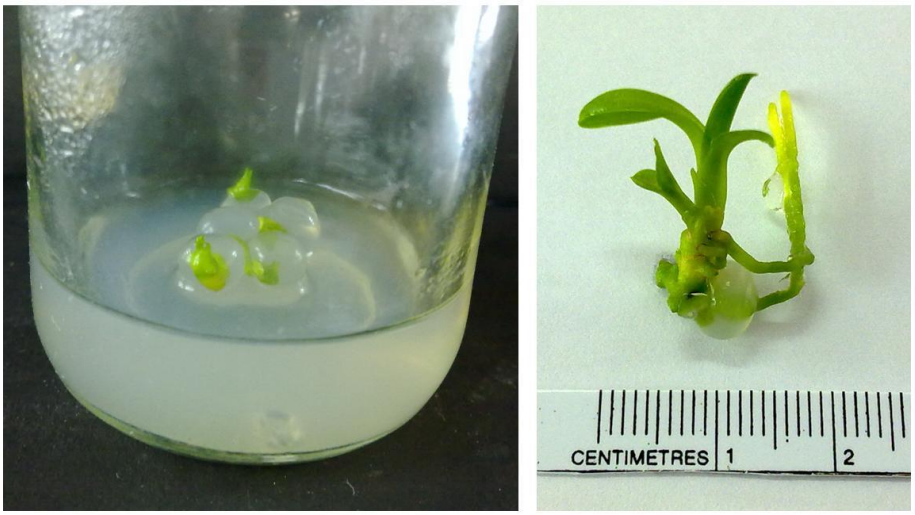


Figure 15 Plantlet from encapsulated protocorms after storage for 16 weeks at 25°C and transferred to PLB induction medium.

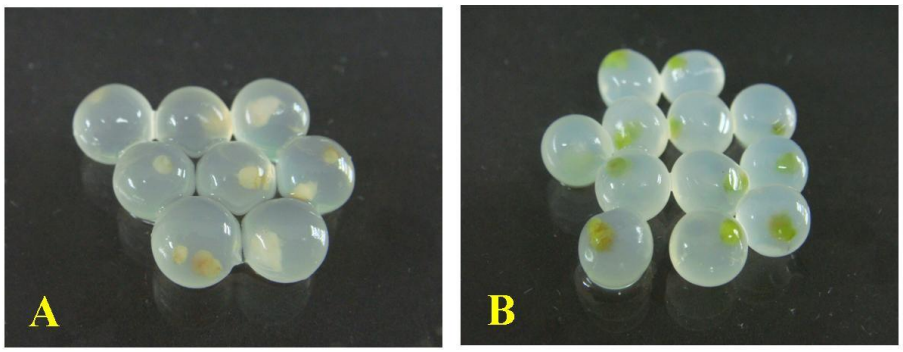


Figure 16 Encapsulated beads after storage for 16 weeks, A) stored at 4°C, B) stored at 25°C.

3.4 The potential of the 2-step vitrification technique to cryopreserved protocorms of *G. speciosum*

When the protocorms were dehydrated with ½ PVS2 for 60 min followed by PVS2 for 0-100 min at 25±2°C they gave the high viability percentage. The highest viability percentage of the protocorms was 85 % (Figure 17) when they were dehydrated with ½ PVS2 for 60 min followed by PVS2 for 40 min. They have viability about 4.25 protocorms out of 5 protocorms (Table 3). The protocorms that dehydrated with PVS2 for 60 min gave 70 % viability and 3.50 protocorms have viability after cryopreservation. The protocorms dehydrated with ½ PVS2 for 40-60 min followed by PVS2 for 40-60 min gave high viability percentage about 70-85 %.

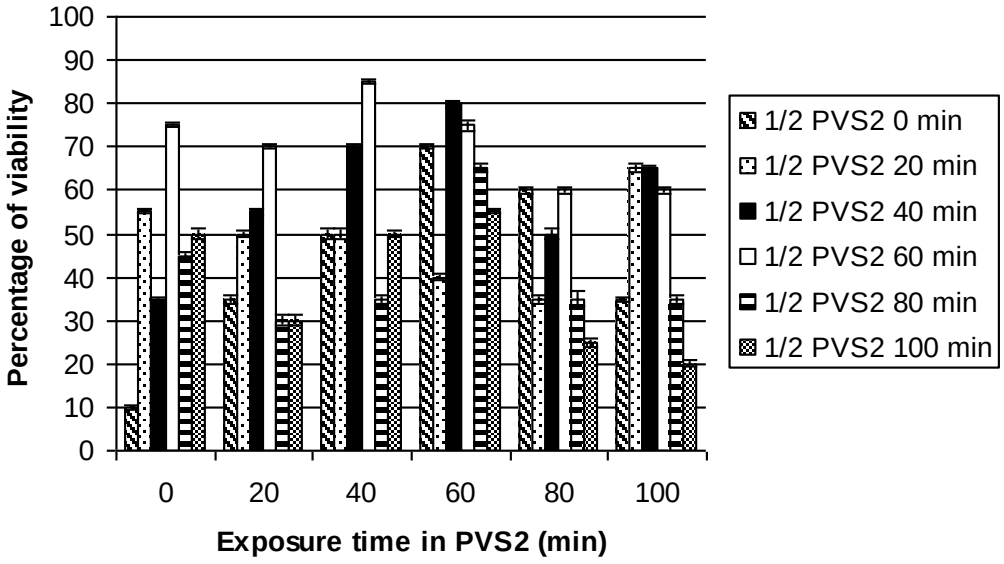


Figure 17 Viability percentage of protocorms after cryopreservation by 2-step vitrification technique.

Table 3 Viability of protocorm after cryopreservation by 2-step vitrification technique (Mean±SD).

Time exposure in ½ PVS2 (min)	Exposure time in PVS2 (min)					
	0	20	40	60	80	100
0	0.50±0.58	1.75±0.96	2.50±1.00	3.50±0.58	3.00±0.82	1.75±0.50
20	2.75±0.50	2.50±0.58	2.50±1.00	2.00±0.82	1.75±0.96	3.25±0.96
40	1.75±0.50	2.75±0.96	3.50±0.58	4.00±0.82	2.50±1.29	3.25±0.50
60	3.75±0.50	3.50±0.58	4.25±0.50	3.75±1.26	3.00±0.82	3.00±0.82
80	2.25±0.96	1.50±1.29	1.75±0.96	3.25±0.96	1.75±1.70	1.75±0.98
100	2.50±1.29	1.50±1.29	2.50±0.58	2.75±0.50	1.25±0.96	1.00±0.82

Mean±SD ($P \leq 0.05$) calculated by Microsoft Office Excel 2003.

When the protocorms were precultured with 0.5 M sucrose for 1 day before cryopreservation, the highest viability percentage was 70 % when the precultured protocorms were dehydrated with ½ PVS2 for 40 min followed by PVS2 for 100 min or dehydrated with ½ PVS2 for 100 min followed by PVS2 for 40 min (Figure 18), the results showed viability about 3.5 out of 5 protocorms (Table 4). The precultured protocorms gave 55 and 60 % of viability after cryopreserved when dehydrated with ½ PVS2 for 20 min followed by PVS2 for 80 and 100 min, respectively. The precultured protocorms that dehydrated with PVS2 for 60, 80, and 1000 min gave the percentage viability at 35, 65 and 60 %, respectively.

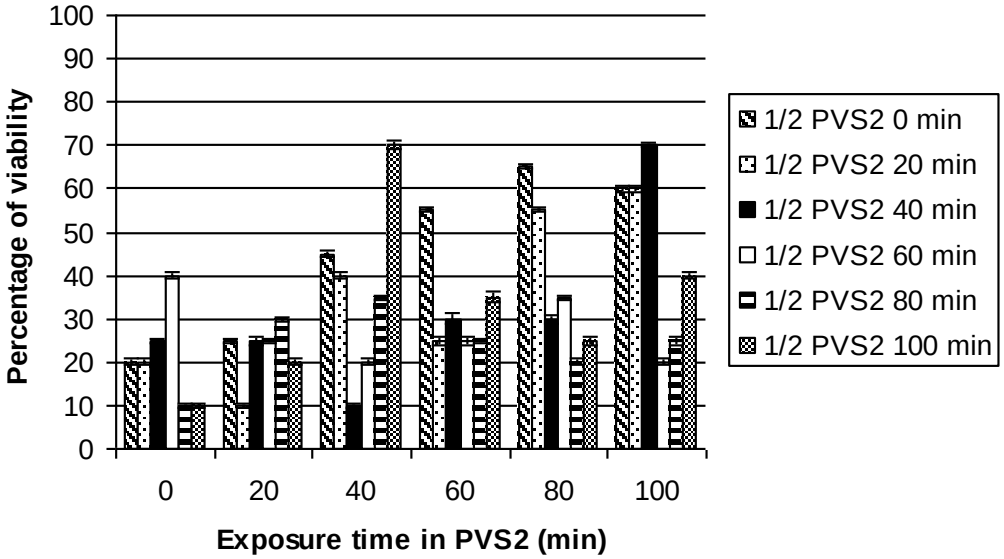


Figure 18 Viability percentage of precultured protocorms after cryopreservation by 2-step vitrification technique.

Table 4 Viability of precultured protocorms after cryopreservation by 2-step vitrification technique (Mean±SD).

Time exposure in ½ PVS2 (min)	Exposure time in PVS2 (min)					
	0	20	40	60	80	100
0	1.00±0.82	1.25±0.50	2.25±0.96	2.75±0.50	3.25±0.50	3.00±0.82
20	1.00±0.82	0.50±0.58	2.00±0.82	1.25±0.96	2.75±0.50	3.00±0.82
40	1.25±0.50	1.25±0.96	0.50±0.58	1.50±1.29	1.50±1.00	3.50±0.58
60	2.00±0.82	1.25±0.50	1.00±0.82	1.25±0.96	1.75±0.50	1.00±0.82
80	0.50±0.58	1.50±0.58	1.75±0.50	1.25±0.50	1.00±0.82	1.25±0.96
100	0.50±0.58	1.00±0.82	3.50±1.00	1.75±1.25	1.25±0.96	2.00±0.82

Mean±SD ($P \leq 0.05$) calculated by Microsoft Office Excel 2003.

3.5 The potential of the vitrification technique to cryopreserve the protocorms of *G. speciosum*.

The non-precultured protocorms gave the highest viability percentage at 60 % when the protocorms were dehydrated with PVS2 for 60-80 min (Figure 19) with an average of 3 protocorms out of 5 protocorms has viability (Table 5). The viability percentage was 55 % when protocorms were precultured with 0.5 M sucrose for 1 day and dehydrated with PVS2 for 60 min. The precultured protocorms for 2 or 3 days followed by dehydration with PVS2 for 60 min gave 35 % and 40 % viability, respectively. In addition, 2-day precultured protocorms without dehydration with PVS2 did not have viability after cryopreservation.

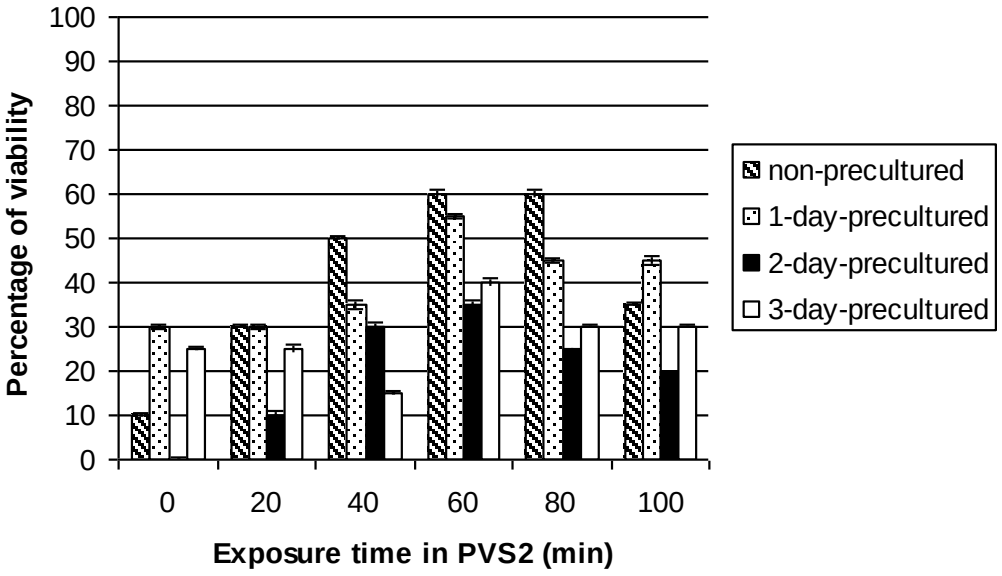


Figure 19 Viability percentage of protocorms after vitrification in PVS2 at various times and cryopreservation for 1 h.

Table 5 Viability of protocorms after vitrification in PVS2 at various times and cryopreservation for 1 h. (Mean±SD).

Preculture in 0.5 M Sucrose (days)	Exposure time in PVS2 (min)					
	0	20	40	60	80	100
0	0.50±0.58	1.50±0.58	2.50±0.58	3.00±0.82	3.00±0.82	1.75±0.50
1	1.50±0.58	1.50±0.58	1.75±0.96	2.75±0.50	2.25±0.50	2.25±0.96
2	0.00±0.00	0.50±0.58	1.50±0.58	1.75±0.96	1.25±0.96	1.00±0.82
3	1.25±0.50	1.25±0.96	0.75±0.50	2.00±0.82	1.50±0.58	1.50±0.58

Mean±SD ($P \leq 0.05$) calculated by Microsoft Office Excel 2003.

3.6 The potential of the encapsulation/vitrification technique to cryopreserve of the protocorms of *G. speciosum*.

The encapsulated protocorms precultured with 0.5 M sucrose for 2 days, followed by dehydration with PVS2 for 100 min before plunging into LN gave the highest viability percentage at 66.67 % (Figure 20) and 4 protocorms from 6 protocorms have viability (Table 6). The encapsulated protocorms precultured for 3 days, followed by dehydration with PVS2 for 40 and 80 min gave 55.56 and 61.11 % viability, respectively. The encapsulated protocorms precultured for 1 day gave 44.44 % viability when dehydrated with PVS2 for 40 and 80 min. The non-precultured encapsulated protocorms have less viability when dehydrated with PVS2 for 60 min. They gave 38.89 % viability.

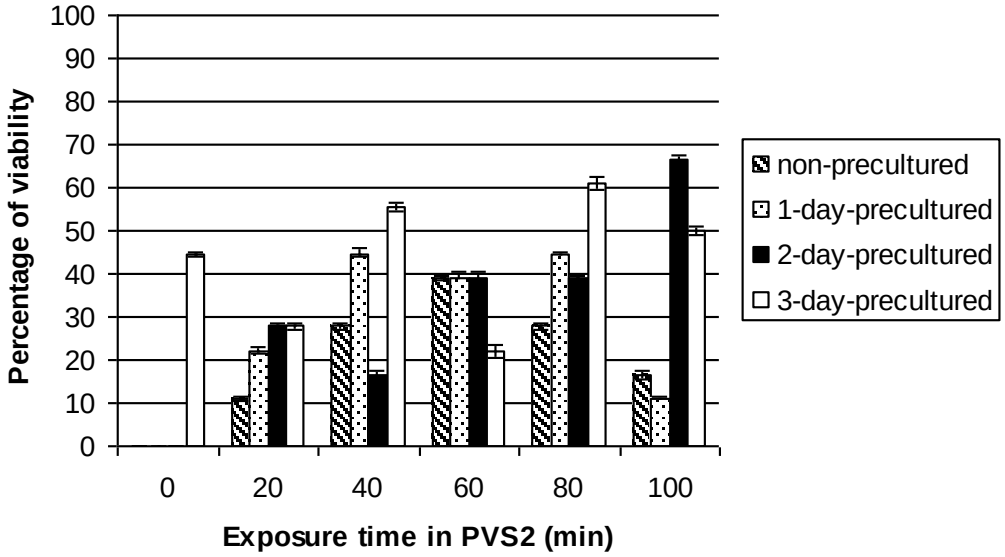


Figure 20 Viability percentages of encapsulated protocorms after cryopreservation by encapsulation/vitrification technique.

Table 6 Viability of encapsulated protocorms after exposure to PVS2 at various times and plunged in LN for 1 h (Mean±SD).

Preculture in 0.5 M Sucrose (days)	Exposure time in PVS2 (min)					
	0	20	40	60	80	100
0	0.00±0.00	0.67±0.58	1.67±0.58	2.33±0.58	1.67±0.58	1.00±1.00
1	0.00±0.00	1.33±0.58	2.67±1.53	2.33±1.53	2.67±0.58	0.67±0.58
2	0.00±0.00	1.67±0.58	1.00±1.00	2.33±1.53	2.33±0.58	4.00±1.00
3	2.67±0.58	1.67±0.58	3.33±1.15	1.33±1.53	3.67±1.54	3.00±1.00

Mean±SD ($P \leq 0.05$) calculated by Microsoft Office Excel 2003.

CHAPTER4
DISCUSSION

4.1 Seed culture

The successful in seed culture depends on many conditions, such as illumination, temperature, basal medium, the addition of plant growth regulators and the seedpod age. The seedpods of 5-9 months after pollination are usually used in orchid seeds culture. In seed culture of *G. speciosum*, seeds from 6-7 months-old seedpod were successfully germinated after culture on MS medium supplemented with 15 % (v/v) CW. In previous study of *G. speciosum*, the seedpods were harvested at 7 months after self-pollinated and the seeds germinated after being sown on KC medium for 1 month (Yam *et al*., 2010). The MS basal medium was suitable for seed culture of orchid. Immature embryo of 9 months after pollination of *Cymbidium aloifolium* was successfully germinated on MS medium supplemented with NAA and BA (Deb and Pongener, 2011). Coconut water can stimulate seed germination. In the study of seed germination of *Coelogyne nervosa*. Seeds were cultured on MS medium supplemented with 30 % (v/v) CW gave the highest response of seed germination (Abraham *et al*., 2012).

4.2 Effects of basal media and sugar types on in vitro culture of *G. speciosum*

4.2.1 Effects of basal media

The success of the propagation method through tissue culture technique depends on numerous factors like plant species, type of explants, plant growth regulators, basal media and sugar. The most often used basal media for micropropagation of orchids are MS, VW, and KC. This study was conducted in MS and VW basal medium supplemented with 2 and 3 % (w/v) sucrose and 15 % (v/v) CW. MS medium supplemented with 2 % (w/v) sucrose gave the highest PLBs

formation compared to the other treatments. There are a few studies on the effect of basal media on orchid micropropagation. MS basal medium was found to be the most effective for orchid micropropagation. MS medium supplemented with 3 % (w/v) sucrose was more effective for *in vitro* germination of endangered orchid *Laelia speciosa* than KC medium, ½ MS medium and MS medium without sucrose (Diaz *et al*., 2009). The effect of basal medium and concentration of sugar to the growth and development of Borneo's endemic orchid, *Vanda dearei* protocorms were studied. Among the 3 basal media, growth of protocorms was the best in ½ MS medium followed by KC and VW, respectively. The medium supplemented with 2 % (w/v) sucrose was the best compared to other treatments (Jawan *et al*., 2010).

There are many studies that employed MS or ½ MS medium as a basal medium such as *Phalaenopsis amabilis* (Chen and Chang, 2006), *Epidendrum radicans* (Chen *et al*., 2002), *Phalaenopsis amabilis* and *P. nebula* (Gow *et al*., 2008), *Rhynchostylis gigantean* (Le *et al*., 1999), *Dendrobium densiflorum* (Luo *et al*., 2008), *Aerides maculosum* (Murthy and Pyati, 2001), *Dendrobium* cv. Chiengmai Pink (Chung *et al*., 2007), *Dendrobium candidum* (Zhao *et al*., 2008), *Oncidium* 'Gower Ramsey' (Chen and Chang, 2004) *Paphiopedilum* Alma Gavaert (Hong *et al*., 2008b)

Optimal growth and development of tissue may vary for different plants according to their nutritional requirements. Plant tissue culture media should generally contain some or all of the following components; macronutrients, micronutrients, vitamins, amino acid, carbon sources, organic compounds, plant growth regulators and solidifying agents. When compare between MS and VW medium, VW medium lacks of micronutrients, vitamins and myo-inositol. From this reason, when VW basal medium was used in micropropagation of *G. speciosum* or other species it gave a lower growth than MS medium.

The essential of micronutrients for plant cell and tissue growth are iron, manganese, zinc, boron, copper, and molybdenum. The comparison of mineral content uptaked by *Gerbera* and *Cymbidium* plant showed the higher uptake of Ca, P, and Fe could be responsible for the better growth (Bouman and Tiekstra, 2001). The responses of microshoot growth and micronutrient acquisition in bitter almond (*Amygdalus communis*) and sour orange (*Citrus aurantium*) showed that Fe

and Mn acquisition of microshoots increment with increasing Fe concentration in all treatments when Cu and Zn acquisition were decreased (Shibli *et al*., 2002). The increasing of Zn level in the medium could increased fresh weight and dry weight of apple microshoot (Shibli *et al*., 2007). The optimum of micronutrients in MS basal medium for the improvement of *in vitro* plant regeneration of *Stevia rebaudiana* (Bert.). Bertotoni found the shoot bud induction response improved on medium with increased level of $MnSO_4$, KI and $CoCl_2$. Plant regenerated on higher levels of micronutrients showed significant increase in biomass and chlorophyll content of the plantlets (Jain *et al*., 2012). In micropropagation of banana shoots, $CuSO_4$ stimulated root induction, elongation, and shoot growth but the higher level of $CuSO_4$ (100 µM) had toxic effect on banana leaves and completely inhibited root formation (Nassar, 2004).

The vitamins required for plant growth and development as catalysts in various metabolic processes. They may act as limiting factors for cell growth and differentiation when plant cells and tissues are grown *in vitro*. The vitamins mostly used in cell and tissue culture media include thiamine HCl (vitamin B1), nicotinic acid and pyridoxine (vitamin B6). Thiamine is necessarily required for cell growth. Thiamine in the form of thiamine pyrophosphate is an essential co-factor in carbohydrate metabolism and is directly involved in the biosynthesis of some amino acid (Thorpe *et al*., 2008). Nicotinic acid and pyridoxine are often added to culture media but are not essential for cell growth in many species. Myo-inositol is vitamin which is usually added in a small quantity to stimulate cell growth of most plant species. Myo-inositol is believed to play a role in cell division because of its breakdown to ascorbic acid and pectin and incorporation into phosphoinositides and phosphatidyl-inositol (Saad and Elshahed, 2012). For *G. speciosum*, micronutrient and some vitamins are effective in PLB induction without any plant growth regulators.

4.2.2 Effects of types and concentrations of sugar

The source of carbon is a very important component in *in vitro* culture media. Carbon sources are added to the culture medium because of the light

energy deficiency and low CO_2 concentration present in *in vitro* condition (Faria *et al*., 2004). Carbon sources supply energy to the plants especially when they are not ready to photosynthesize their own food during the early stage of tissue culture. Sucrose has been widely used as the major carbon source to supply energy to cells in plant tissue culture because of its efficiency in being transported across the plasma membrane (Nambiar *et al*., 2012). Sucrose concentrations of 20 and 30 g/l are the most commonly used in orchid tissue culture studies. In the study of rooting and *in vitro* growth of *Dendrobium nobile* by using different sucrose concentration, the highest sucrose concentration in culture medium (60 g/l) presented the best result with the greatest mean plant height value (4.21±0.9 cm) even without the addition of plant hormones. Sucrose concentration in the culture medium did not influence in *in vitro* plant rooting (Faria *et al*., 2004). In *Dendrobium* Second Love, the number of roots formed per explant increase as sucrose concentration was raised up to 60 g/l and the addition of sucrose to the culture medium up to 20 g/l and 40 g/l was advantageous to the number of shoots produced per explant and the root longitudinal growth, respectively (Ferreira *et al*., 2011). The influence of sucrose was estimated on growth and development of shoot tip derived suspension cells of *Phalaenopsis* orchid. PLB formation was the most efficiently induced by sucrose at 14.6 mM (5 g/l) for *Phalaenopsis* Wedding Promenade. In *Doriataenopsis*, sucrose at 58.4 mM (20 g/l) Induced callus proliferation without PLB formation (Tokuhara and Mii, 2003). But only few PLBs produced plantlets and the most of PLBs regenerated yellowish or greenish callus in *Phalaenopsis* (*P.* Wedding Promenade and *P.* Hanaboushi X *P. equestris* 'Ilocos'). Almost 80 % of unrooted and 58 % of rooted plantlets developed yellowish green callus at the base of plantlet (Islam *et al*., 1998). Sucrose at 30 g/l gave the highest amount of embryos for *Oncidium* cv. Gower Ramsey and suitable ranges of sucrose concentration for direct embryo induction were found at 30-60 g/l and the suitable ranges for *Oncidium* cv. Sweet Sugar were at 20-60 g/l (Hong *et al*., 2008a).

Other sugars were used as carbon sources in orchid culture, such as glucose, fructose. In micropropagation of *Paphiopedilum* var *"densissimum"*, MS medium supplemented with 20 g/l glucose gave the highest seed germination at 19 % and leaf length and width (Long *et al*., 2010). In cell suspension culture of

Phalaenopsis Wedding Promenade, glucose at 58.4 mM (20 g/l) gave the highest efficiencies for PLB formation (Tokuhara and Mii, 2003). The promotion of direct somatic embryogenesis of *Oncidium* Sweet Sugar by adjusting carbon source, the medium supplemented with 20 g/l of glucose gave the highest embryogenesis (85 %) (Hong *et al*., 2008a). In *Dendrobium* Alya Pink, the medium supplemented with glucose gave the lowest average fresh weight of PLBs (0.94±0.55 g) and the highest average fresh weight of PLBs were found from the medium supplemented with fructose (9.1±0.82 g) (Nambiar *et al*., 2012).

Most of the PLBs of *Phalaenopsis* (*P*. Wedding Promenade and *P*. Hanaboushi X *P. equestris* 'Ilocos') developed to plantlets and a few additional PLBs on the medium supplemented with 10 g/l sorbitol. Sorbitol supported plantlet development the best in *in vitro* culture and proved to be the most suitable carbon source for plantlet initiation and development from PLBs (Islam *et al*., 1998). In *Doritaenopsis* New Totohashi, the medium supplemented with 58.4 mM (20 g/l) sorbitol resulted in little callus proliferation and a small amount of yellow PLBs (Tokuhara and Mii, 2003). The same as the study of proliferation of PLBs in *Dendrobium* Alya Pink, sorbitol and mannitol were not suitable to promote the growth of PLBs (Nambiar *et al*., 2012).

4.3 The encapsulation production and short-term storage conditions

4.3.1 Encapsulation production

The encapsulation has 3 factors that affected the bead formation and the germination rate of the explants. These compositions are the concentrations of sodium alginate, $CaCl_2$ solution, and polymerization exposure time. The germination decreased with either higher or lower concentrations of either sodium alginate or $CaCl_2$ solution. The sodium alginate at 5, 6, and 7 % was too viscous and the beads formation were harder and hindered the emergence of shoots and roots. Possible reasons cited for this inhibition of conservation are an unsuitable elasticity of the gel bead (Saiprasad and Polisetty, 2003). In the previous studies, the most commonly

used 2-4 % sodium alginate complexing with 50-100 mM $CaCl_2$ gave the best result in encapsulation. In the studies of the effect of concentration of gelling agent on *Dendrobium* 'Sonia'. The best encapsulation response was observed with 3 % sodium alginate upon complexation with 75 mM $CaCl_2$ solution (Saiprasad and Polisetty, 2003).

The combinations of sodium alginate, $CaCl_2$ solution, and exposure time on encapsulation affected the quality of beads. In the studies of *Flickingeria nodoza* (a medicinally important epiphytic orchid), various concentrations of sodium alginate and $CaCl_2$ solution were tested in order to optimize the shape, texture, and time required for complexing with alginate dipped in 100 mM $CaCl_2$ solution and incubated for 30 min in orbital shaker was found to be the best matrix and complexing agent respectively to produced firm, transparent, and uniform synthetic seeds. At 4 % sodium alginate polymerized in 75-100 mM $CaCl_2$ solution for 15-60 min gave slightly hard beads and when the concentration of sodium alginate was up to 6 %, all beads were hard to very hard after dropping in 25-100 mM $CaCl_2$ solution for 15-60 min (Nagananda *et al*., 2011). The encapsulation of somatic embryo of *Carica papaya*, sodium alginate was tested at 1.5, 2.0, and 2.5 %. Single coated somatic embryos were dropped into 0.05 mM $CaCl_2$ solution for 20 min. Beads of uniform size and shape were obtained when 2.5 % sodium alginate was tested but at low concentration (1.5-2.0 %), uniform, sufficiently firm beads were not found (Castillo *et al*., 1998). In the encapsulation of *in vitro* proliferated bulblets of garlic (*Allium sativum*), the increasing of sodium alginate from 1 up to 3 % polymerized in 2.5 % $CaCl_2$ solution for 20 min increased percentage of survival and conservation of encapsulated bulblets since smooth texture is appropriate for the storage, as well as for survival and conservation was observed with 3 % sodium alginate. At low concentration (1 %) of sodium alginate, beads were soft. However, at high level (4 %) of sodium alginate, beads were very hard and prevented bulblets proliferation. The effect of duration of exposure to $CaCl_2$ solution was studied and the results showed that the bulblets encapsulated with 3 % sodium alginate complexing with 2.5 % $CaCl_2$ solution for 30 min gave the highest percentage of survival and conversion to plantlets and more than 30 min of exposure time to $CaCl_2$ solution, a

very hard bead was formed and lower percentage of regeneration into plantlets was achieved (Bekheet, 2006).

4.3.2 Short-term storage conditions

Synthetic seeds of orchids are mostly produced by encapsulation of PLBs in alginate matrix. This system serves as a low-cost, high-volume propagation system. Advantages of synthetic seeds over somatic embryo for propagation include: (1) ease of handling during storage and transportation; (2) potential long-term storage without losing viability; (3) maintenance of the clonal nature of resulting plants (Saiprasad and Polisetty, 2003). The coating or synthetic seeds protect the PLBs from mechanical damage during handling and allow germination and conversion to occur without inducing undesirable variation (Nagananda *et al*., 2011).

If the encapsulated explants can be stored for a long duration and at different temperature, it will greatly enhance the efficiency of micropropagation by this system. In previous studies, the encapsulated beads were successful in short-time storage at 4°C and 25°C. The encapsulated PLBs of medicinally important vulnerable orchid *Flickingeria nodosa* stored at 4°C remained viable and germinated up to 90 days; however, poor germination of 8 % was observed after completion of 90 days of storage (Nagananda *et al*., 2011). The storage of encapsulated PLBs of *Coelogyne breviscapa* at 4°C and 25°C showed the encapsulated PLBs when stored at 4°C for 60 days showed no reduction in viability. And the germination percentage of encapsulated PLBs stored at 25°C was always much lower in comparison to those stored at 4°C. The encapsulated PLBs retain their viability even after storage for 90 days. However, the germination percentage of the encapsulated PLBs decreased gradually with increase in storage time (Mohanraj *et al*., 2009). The influence of storage temperature and duration of storage on viability of encapsulated PLBs of three orchid genera, *Dendrobium*, *Oncidium*, and *Cattleya* have been studied and they can be stored at 4°C for 75, 60, and 30 days, respectively with more than 88 % germination. At 4°C, the germination percentage decreased as the duration of storage was increase for all three orchid genera. At 25°C, complete germination (100 %) was

observed for up to 30 days of storage and when storage duration exceeded 30 days, germination declined and it was below 50 %. Desiccation of encapsulated PLBs was observed when stored for more than 75 days (Saiprasad and Polisetty, 2003).

In this study, the encapsulated protocorm of *G. speciosum* can be stored at 25°C up to 112 days (16 weeks). The successful of storage the encapsulated explants at 25°C were found in tree, such as the short-time storage of encapsulated shoot tips and nodal segments of the eucalyptus (*Corymbia torelliana* X *C. Citriodora*). Conversion of synthetic seeds into plantlets markedly decreased with the duration of storage at 4°C. High conversion frequency of synthetic seeds was obtained after 7 days, but these frequencies decreased to 0-24 % after 28 days (4 weeks). In contrast, synthetic seeds could be stored easily for 28 days (4 weeks) at 25°C and 68-84 % of encapsulated shoot tips and 50-70 % of encapsulated nodal segments still could be converted to plantlets after stored for 56 days (8 weeks) (Hung and Trueman, 2011a). *In vitro* conservation of *Cedrela fissilis*, a native tree of the Brazilian Atlantic forest. The encapsulated shoot tips stored for 180 days on 0.4 % (w/v) agar at 25°C showed the highest survival rates (44 %) (Nunes *et al*., 2003). Encapsulation technology was developed for short-term preservation of African mahogany (*Khaya senegalensis*) by using encapsulated shoot tips. Encapsulated shoot tips survived longer at 25°C than 4°C, with viability of 73-88 % after 56 days. At 4°C, regrowth capacity decrease after 28 days and was lost completely after 56 days (Hung and Trueman, 2011b). It is thought that the decline in the germination percentage observed among encapsulating propagules stored of a period of 90 days may be due to inhibited respiration of plant tissue by alginate leading to loss of viability (Nagananda *et al*., 2011).

4.4 Potential of the 2-step vitrification technique to cryopreserve the protocorms of *G. speciosum*

Vitrification requires a highly concentrated solution, which sufficiently dehydrates tissues without causing injury, enabling them to form a stable glass along with the surrounding PVS2 when plunged into LN. The grape (*Vitis vinifera*) was successfully cryopreserved by vitrification and a 2-step dehydration procedure

considerably improved the recovery. In the shoot tips of grape, preculture with sucrose alone did not produce a high level of recovery growth. Induction of osmotolerance to PVS2 was achieved by preculturing shoot tips with 0.3 M sucrose for 3 days followed by loading solution (a mixture of 2 M glycerol plus 0.4 M sucrose) for 20 min at 25°C. They were then dehydrated with PVS2 for 80 min at 0°C (1-step dehydration) before being plunged into LN. The recovery of the shoot tips amounted to approximately 60 %. A 2-step dehydration procedure was examined by dehydrated the osmoprotected shoot tips with ½ PVS2 for 30 min followed by PVS2 for 50 min at 0°C. This 2-step dehydration procedure improved shoot recovery from 60 to 80 %. The 2-step vitrification applied to ten cultivars of grape and successfully cryopreserved with and overall average of 63.8 % shoot recovery (Matsumoto and Sakai, 2003). In the vitrification protocol, direct exposure of less tolerant cells and meristems to highly concentrated PVS2 at 25°C may be harmful due to osmotic stress or chemical toxicity (Matsumoto and Sakai, 2003). This can be eliminated or reduced by a multi-step vitrification procedure with increasing concentration of PVS2. In *G. speciosum*, the preculture in sucrose was not necessary for cryopreserved by 2-step vitrification. Non-precultured protocorms dehydrated with ½ PVS2 for 60 min followed by PVS2 for 40 min gave the highest percenviability.

4.5 Potential of the vitrification technique to cryopreserve the protocorms of *G. speciosum*

In this study, the highest viability percentage of cryopreserved protocorms was 60 % observed in non-precultured protocorms osmoprotected with loading solution for 20 min before dehydrated with PVS2 for 60-80 min. In cryopreservation of grape shoot tips, shoot tips that precultured with sucrose alone did not produced a high level of recovery growth and the induction of osmotolerance to PVS2 was achieved by incubated precultured shoot tips in loading solution (2 M glycerol plus 0.4 M sucrose) for 20 min. During the treatment of loading solution, the meristematic cells are osmotically dehydrated and plasmolysed. The cells are then successively dehydrated with PVS2, producing concentrated spherical protoplasts. It is currently unknown what the actual protective mechanism is; however, the creation of these

plasmolysed cells may mitigate mechanical stress caused by severe dehydration process (Matsumoto and Sakai, 2003).

A mixture of 2 M glycerol and 0.4 M sucrose was reported to be very effective in inducing dehydration and freezing tolerance in various cells and meristems. Preculture with high sucrose concentration resulted in the accumulation of sugar and may increase the stability of membranes under severe dehydration condition (Ishikawa *et al*., 1997). A loading treatment increases the osmolarity of the cell and minimizes osmotic damage caused by the vitrification solution (Sakai *et al*., 2008).

One of the factors to successful cryopreservation is the careful control of dehydration and prevention of injury by chemical toxicity or excess osmotic stresses during treatment with PVS2. Optimizing the time of exposure or the temperature during exposure to PVS2 is important for producing a high level of explant formation after vitrification. PVS2 is the mixture of 30 % (v/v) glycerol, 15 % (v/v) ethylene glycol and 15 % (v/v) DMSO. This cryoprotectant is used to protect the plant material from damaging effects due to freezing. However, the use of cryoprotectant can be responsible for loss of viability on the cryopreserved cells. DMSO is toxic to plant cells and this toxicity may cause cell injury in association with distinct ultrastructural changes, especially in the plasma membrane. Therefore, it is vital to optimize the PVS2 exposure duration in order to reduce toxicity to the plant (Hooi *et al*., 2010). Incubation and temperature of vitrification solution are important factors affecting survival of cryopreserved plant tissues. Over-exposure of plant tissues to vitrification solution may lead to chemical toxicity and excessive osmotic stress (Yin and Hong, 2009). The optimum period of exposure to vitrification usually differs depending on the species, type of explants, and size of explants.

The exposure time for vitrification was optimized for seed cryopreservation of 3 species of *Cymbidium* and the optimum periods were found to be 60 min for *C. goeringii* and *C. macrorhizon* seeds and 30 min for *C. finlaysoniamum* seeds. The period of exposure to the vitrification solution varied with life-form. The period was optimized to 60 and 30 min for the terrestrial and epiphytic species, respectively (Hirano *et al*., 2011). Dehydration with PVS2 for 20 min at 0°C

was considered to be the best PVS2 treatment for PLBs cryopreservation of *Dendrobium* Sonia -28 by vitrification (Hooi *et al*., 2010).

The effect of the exposure time to PVS2 is associated with the size of excised meristem and appears to be species specific. In the cryopreservation of zygotic embryo of *Bletilla striata* study, embryo produced the highest survival when dehydrated in PVS2 solution for 3 h at 0°C (Ishikawa *et al*., 1997). Cryopreservation of *in vitro*-grown shoot tips of papaya (*Carica papaya*) with PVS2 at 4°C for 60 min gave high recovery percentage after cryopreserved (Wang *et al*., 2005). The shoot tips excised from dormant axillary buds of persimmon (*Diospyros kaki*) were successful cryopreserved when dehydrated in PVS2 for 20 min at 25°C (Matsumoto *et al*., 2001). Highly concentrated vitrification solutions have toxicity to plant cells but it is efficient to improve the recovery percentage after cryopreservation. To avoid the toxicity of cryoprotectant, the period of cryoprotectant exposure to cell need to be studied.

4.6 Potential of the encapsulation/vitrification technique to cryopreserve the protocorms of *G. speciosum*

Encapsulation/vitrification is a combination of the encapsulation/ dehydration and vitrification procedure, where samples are encapsulated in alginate beads, and then subjected to freezing following the vitrification approach. Encapsulation of explants allows the application of subsequent drastic dehydration processes prior to cryopreservation, which would otherwise be highly damaging or lethal to non-encapsulated samples (Arnao *et al*., 2008).

In cryopreservation by vitrification method with or without encapsulation, the explants are sufficiently dehydrated osmotically by exposure to highly concentrated vitrification solution. However the direct exposure of explants to vitrification solution causes harmful effect due to osmotic stress. Thus, the key to successful cryopreservation by vitrification is to induce the osmotolerence to vitrification solution by preconditioning. In this study, the encapsulated protocorms were precultured in 0.5 M sucrose for 2-3 days and incubated in loading solution for 20 min at 25°C before plunged into LN was effective to cryopreservation by

encapsulation/vitrification. PLBs of *Dendrobium candidum* were successfully cryopreserved using an encapsulation/vitrification method. The PLBs were precultured in 0.75 M sucrose for 5 days and osmoprotected with a mixture of 2 M glycerol and 2 M sucrose for 80 min before encapsulated and dehydrated with PVS2 (Yin and Hong, 2009). In *Dioscorea bulbifera*, embryogenic callus were precultured in MS liquid medium enriched with 0.75 M sucrose for 7 days. Then, osmoprotected with a mixture of 2 M glycerol and 1 M sucrose foe 80 min before encapsulated and dehydrated with PVS2. The survival rate of encapsulated vitrified embryogenic callus reaches over 70 % (Hua and Rong, 2010). Excised shoot tips of 'Troyer' citrange (*Poncirus trifoliate* X *Citrus sinensis*) were precultured with increasing sucrose concentration of 0.3-1 M sucrose for 4 days, followed by osmoprotected with a loading solution of 2 M glycerol and 1 M sucrose for 60 min during encapsulation. Dehydration by exposure to modified PVS2 for 90 min at 24°C and 180-210 min at 0°C were found to be optimum for survival of cryopreserved shoot tips (Wang *et al*., 2002).

The responses of different plant species to cryopreservation by encapsulation/vitrification are different and this could be attributed to genotypic differences, as well as differenced in incubation period and temperature of the vitrification solution. Osmotolerance of explants is rarely achieved by preculture with sucrose alone. Therefore, a loading treatment with loading solution containing various amounts of sucrose and glycerol is commonly used (Yin and Hong, 2009). During the treatment with loading solution, the meristem cells were dehydrated osmotically and plamolysed. These cells were further dehydrated with PVS2 solution and were capable of vitrifying upon rapid cooling into LN. The presence of loading solution in the periprotoplasmic space of plasmolysed cells may mitigate mechanical stress caused by severe dehydration and provide some protective action towards minimizing the injurious membrane changes (Hirai and Sakai, 1999).

CHAPTER 5
CONCLUSION

6-7 month-old seeds of *G. speciosum* were germinated on modified MS medium containing 30 g/l sucrose, 15 % (v/v) coconut water and solidified with 8.2 g/l agar after 5-6 months of culture.

The suitable medium for PLB induction of *G. speciosum* was MS solid medium containing 15 % (v/v) coconut water and 2 % (w/v) sucrose. The highest PLB formation rate was 84 % and produced PLBs about 3.1 PLBs per explant.

Protocorms of *G. speciosum* cultured in MS liquid medium supplemented with mannitol and sorbitol at 2-8 % (w/v) gave 100 % regeneration after transfer to induction medium (MS medium containing 15 % (v/v) coconut water and 2 % (w/v) sucrose) and incubated for 4 weeks.

Protocorms of *G. speciosum* encapsulated with 3 % (w/v) sodium alginate complexing with 75 mM $CaCl_2$ solution for 30 min gave firm round beads. The encapsulated protocorms did not lost viability after stored at 25°C in dark condition for 16 weeks.

The protocorms of *G. speciosum* gave 85 % viability rate after cryopreserved by 2-step vitrification technique, when dehydrated with ½ PVS2 for 460 followed by PVS2 for 60 min and plunged into LN for 1 h.

The protocorms of *G. speciosum* gave the highest viability at 60 % after vitrification by osmoprotected the protocorms with LS for 20 min followed by dehydrated with PVS2 for 60-80 min and plunged into LN for 1 h.

The encapsulated protocorms were precultured in modified MS liquid medium supplemented with 0.5 M sucrose for 2 days followed by osmoprotected the protocorms with LS for 20 min before dehydrated with PVS2 for 100 min and plunged into LN. The highest viability percentage at 66.67 %.

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APPENDICES

Composition of Murashige and Skoog (MS) medium (1962)

	1 liter
Macroelements	
Ammonium nitrate, $NH_4 NO_3$	1,650 mg
Potassium nitrate, KNO_3	1,900 mg
Calcium chloride, $CaCl_2.2H_2O$	440 mg
Potassium dihydrogen phosphate, KH_2PO_4	170 mg
Magnesium sulfate, $MgSO_4.7H_2O$	370 mg
Chelated iron	
Disodium ethylene diaminetetraacetate, Na_2-EDTA	37.3 mg
Ferrous sulfate, $FeSO_4.7H_2O$	27.8 mg
Microelements	
Boric acid, H_3BO_3	6.2 mg
Manganese sulfate, $MnSO_4.4H_2O$	16.9 mg
Zinc sulfate, $ZnSO_4.7H_2O$	6.14 mg
Potassium iodide, KI	0.83 mg
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.25 mg
Copper sulfate, $CuSO_4.5H_2O$	0.025 mg
Cobalt chloride, $CoCl_2.6H_2O$	0.025 mg
Growth factor	
Myo-inositol	100 mg
Organic addenda	
Glycine	2 mg
Vitamins	

Thiamine HCl	0.1 mg
Nicotinic acid	0.5 mg
Pyridoxin HCl	0.5 mg

Sugar

Sucrose	30,000 mg

VITAE

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