Cryopreservation of Protocorm-Like Bodies and Callus of Lady's Slipper Orchid (*Paphiopedilum niveum* (Rchb. f.) Stein) by Vitrification and Encapsulation-Vitrification

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ชื่อวิทยานิพนธ์ การเก็บรักษาโพรโทคอร์มไลค์บอดี และแคลลัส ของกล้วยไม้รองเท้านารีขาวสตูล (*Paphiopedilum niveum* (Rchb. f.) Stein) ในสภาพแช่แข็งโดยวิธี Vitrification และ Encapsulation-Vitrification

ผู้เขียน นายสุพรชัย ชัยฤกษ์

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บทคัดย่อ

กล้วยไม้รองเท้านารีขาวสตูล (*Paphiopedilum niveum* (Rchb. f.) Stein) ได้รับการจดทะเบียนเป็นกล้วยไม้ใกล้สูญพันธุ์ ในบัญชี CITES ว่าเป็นกล้วยไม้ทางการค้าและการอนุรักษ์ แต่การขยายพันธุ์ทำได้ยากและการหาเทคนิคการเก็บรักษาสายพันธุ์ที่เหมาะสมมีข้อจำกัด การศึกษาในครั้งนี้จึงมุ่งศึกษาแนวทางการเพิ่มจำนวนของกล้วยไม้รองเท้านารีขาวสตูลในสภาพปลอดเชื้อ และการศึกษาสภาวะที่เหมาะสมในการเก็บรักษาสายพันธุ์ในสภาพแช่แข็ง การศึกษาแบ่งเป็น 2 ขั้นตอน คือ การขยายพันธุ์กล้วยไม้รองเท้านารีขาวสตูลในสภาพปลอดเชื้อ และ การเก็บรักษาสายพันธุ์กล้วยไม้รองเท้านารีขาวสตูลในสภาพแช่แข็ง ในรูปแบบโพรโทคอมไลค์บอดี และแคลลัส รวมถึงการศึกษาความแปรผันของปริมาณดีเอ็นเอ ด้วยวิธี Flow cytometry

ศึกษาการเพิ่มจำนวนของโพรโทคอมไลค์บอดี ในสูตรอาหารดัดแปลง Vacin และ Went (VW) ที่ประกอบด้วย น้ำมะพร้าว (0 และ 10 %) และ ในอาหาร 3 รูปแบบ คือ อาหารเหลว (0 % Phytagel), อาหารกึ่งแข็ง (0.1 % Phytagel) และอาหารแข็ง (0.2 % Phytagel) พบว่า โพรโทคอมไลค์บอดี ที่เลี้ยงในสูตรอาหารดัดแปลง VW ที่ประกอบด้วยน้ำมะพร้าว 10 % และ 0.2 % Phytagel ให้น้ำหนักของ โพรโทคอมไลค์บอดีสูงสุด (159.40 ± 5.40 มิลลิกรัม) ทั้งนี้หลังจากการเพาะเลี้ยงเป็นเวลา 8 สัปดาห์ พบว่าโพรโทคอมไลค์บอดีมีการเจริญเติบโตต่อไปได้ ขั้นตอนต่อมา ศึกษาการชักนำแคลลัส โดยนำโพรโทคอมไลค์บอดีเลี้ยงบนอาหารดัดแปลง VW ที่ประกอบด้วย 2, 4-Dichlorophenoxyacetic acid (2, 4-D) ที่ระดับความเข้มข้น 0, 0.5 และ 1.0 มิลลิกรัมต่อลิตร และ Thidiazuron (TDZ) ที่ระดับความเข้มข้น 0, 0.1 และ 0.5 มิลลิกรัมต่อลิตร หลังการเพาะเลี้ยงเป็นเวลา 2 เดือน พบว่า น้ำหนักของแคลลัสที่ได้สูงที่สุด 312.70 ± 59.61 มิลลิกรัมต่อน้ำหนักเริ่มต้น 100 มิลลิกรัมของโพรโทคอมไลค์บอดี เมื่อย้ายเลี้ยงในอาหารสูตรดัดแปลง VW ที่ประกอบด้วย 2, 4-D 0.5 มิลลิกรัมต่อลิตร และ TDZ 0.1 มิลลิกรัมต่อลิตร เมื่อศึกษาการเพิ่มจำนวนของแคลลัสในอาหารสูตรดัดแปลง VW ที่ประกอบด้วย น้ำมะพร้าว (0, 10 และ 15 %) และ

น้ำตาลซูโครส (0, 10 และ 15 กรัมต่อลิตร) พบว่า สามารถเพิ่มจำนวนแคลลัสได้ดีที่สุดเท่ากับ 0.560±0.56 มิลลิกรัมต่อน้ำหนักเริ่มต้น 100 มิลลิกรัมของแคลลัส บนอาหารสูตรดัดแปลง VW ที่เติม น้ำมะพร้าว 10 % และ น้ำตาลซูโครส 15 กรัม/ ลิตร และหลังจากย้ายเลี้ยงในที่สว่าง พบว่า แคลลัสพัฒนาไปเป็นแคลลัสที่มีสีเขียวและเจริญเติบโตอย่างสมบูรณ์

การเก็บรักษาโพรโทคอร์มไลด์บอดีในสภาพแช่แข็งโดยใช้วิธี vitrification นำโพรโทคอมไลค์บอดีอายุ 8 สัปดาห์ที่ประกอบด้วย เนื้อเยื่อเจริญส่วนยอดและราก เลี้ยงในอาหารเหลวสูตรดัดแปลง VW ที่มีน้ำตาลซูโครสความเข้มข้นแตกต่างกัน (0, 0.25, 0.50, 0.75 และ 1.00 โมลาร์) เป็นเวลา 24 ชั่วโมง และ 5 วันที่มีการเพิ่มระดับความเข้มข้นของน้ำตาลในแต่ละวัน (วันละ 0.25 โมลาร์) ตามด้วยแช่ในสารละลาย Loading solution เป็นเวลา 30 นาที จากนั้นแช่ในสารละลาย plant vitrification solution 2 (PVS2) เป็นเวลา 0, 30, 60, 90 และ 120 นาที จากการทดสอบความมีชีวิต (TTC test) โดยใช้ สเปกโตรโฟโตมิเตอร์ ที่ความยาวคลื่น 530 นาโนเมตรพบว่า โพรโทคอมไลค์บอดีในสารละลายน้ำตาลซูโครส 0.75 โมลาร์ ที่เพิ่มระดับความเข้มข้นของน้ำตาลในแต่ละวัน เป็นเวลา 5 วันตามด้วยแช่ในสารละลาย PVS2 เวลา 90 นาที สูงที่สุด (0.57 ± 0.06) โพรโทคอมไลค์บอดีที่ถูกเก็บในสภาพแช่แข็งมีความเสียหายในระดับเนื้อเยื่อและมีการยุบตัวของผิวภายนอกในบางบริเวณ ไม่พบความแปรผันของปริมาณดีเอ็นเอ ที่เกิดขึ้นจากการเก็บรักษาสายพันธุ์ในสภาพแช่แข็ง และพบว่าความรอดของโพรโทคอมไลค์บอดี เท่ากับ 22.22 ± 15.71 % ส่วนการเก็บรักษาแคลลัสในสภาพแช่แข็งโดยวิธี encapsulation-vitrification และ vitrification โดยนำ แคลลัสเลี้ยงในอาหารเหลวสูตรที่มีน้ำตาลซูโครสความเข้มข้นแตกต่างกัน (0, 0.25, 0.50, 0.75 และ 1.00 โมลาร์) เป็นเวลา 5 วัน โดยทำการเพิ่มระดับความเข้มข้นของน้ำตาลในแต่ละวัน (วันละ 0.25โมลาร์) จากนั้น แช่ในสารละลาย PVS2 เป็นเวลา 0, 20, 40, 60, 80 และ 100 นาที พบว่า แคลลัสที่เก็บรักษาด้วยวิธี encapsulation-vitrification โดยผ่านการแช่ในน้ำตาลซูโครสความเข้มข้น 0.5 โมลาร์ และในสารละลาย PVS2 เป็นเวลา 100 นาที สูงที่สุด (0.237 ± 0.011) และมีปริมาณความชื้น 27.34 ± 0.96 % จากการศึกษาด้านเนื้อเยื่อวิทยา พบเซลล์มีลักษณะปกติ มีนิวเคลียสขนาดใหญ่อยู่ภายในเซลล์ ไม่มีการสลายตัวของผนังเซลล์ พบเม็ดแป้ง และโปรตีนภายในเซลล์เหมือนกับชุดควบคุม ในขณะที่การเก็บรักษาแคลลัสในสภาพแช่แข็งด้วยวิธี vitrification พบว่า แคลลัสที่แช่ในสารละลายน้ำตาลซูโครส 0.5 โมลาร์ เป็นเวลา 5 วัน ที่มีการเพิ่มระดับความเข้มข้นของน้ำตาลซูโครสในแต่ละวัน ตามด้วยการแช่ในสารละลาย PVS2 เป็นเวลา 60 นาที จะให้ค่าการดูดกลืนแสงเท่ากับ 0.216 ± 0.009 และมีปริมาณความชื้น 23.93 ± 2.05 % จากการศึกษาด้านเนื้อเยื่อวิทยาพบ ความเสียหายของผนังเซลล์ ปริมาณเม็ดแป้ง และโปรตีนมีการถูกทำลายในบางบริเวณไม่พบความแปรผันของปริมาณดีเอ็นเอ ในการเก็บรักษาสายพันธุ์แบบ encapsulation-vitrification และ vitrification และให้อัตรารอดของแคลลัสหลังการเก็บรักษาในสภาพแช่แข็ง 29.63 ± 10.31% และ 22.22 ± 7.85% ตามลำดับ

Thesis Title Cryopreservation of Protocorm-Like Bodies and Callus of Lady's Slipper Orchid (*Paphiopedilum niveum* (Rchb. f.) Stein) by Vitrification and Encapsulation-Vitrification

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ABSTRACT

Paphiopedilum niveum (Rchb. f.) Stein (*P. niveum*) is an endangered orchid species and has been listed in the Convention on International Trade in Endangered Species (CITES) Appendix I. Although this orchid species is marketed as a potted plant and preserved with high value, the propagation has been limited due to the fact that the explants are difficult to maintain in culture. The present study was to evaluate the increasing number of in vitro propagation and to investigate suitable conditions for cryostorage of *P. niveum* explants. This study was divided into two main parts. The first was in vitro propagation of *P. niveum* and the second was cryopreservation of protocorm-like bodies (PLBs) by vitrification technique and callus by encapsulation-vitrification and vitrification techniques. The DNA content was also evaluated by flow cytometry.

In order to proliferate the number of PLBs, PLBs were cultured on modified Vacin and Went (VW) medium supplemented with 10 % coconut water (CW) or without CW and Phytagel at 0%, 0.1 % and 0.2 % which were defined as liquid, semi-solid and solid culture medium, respectively. The highest increase in fresh weight was obtained from the PLBs cultured on solidified VW medium containing 10 % CW at 159.40 ± 5.40 61 mg/ 100 mg initial fresh weigh of PLB. After 8 weeks of culture, PLBs exhibited shoots and well grown PLBs. For callus induction, the proliferated PLBs were transferred to modified VW solid medium supplemented with 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (0, 0.5 and 1.0 mg L^{-1}) in combination with Thidiazuron (TDZ) (0, 0.1 and 0.5 mg L^{-1}). The highest increase in fresh weigh was obtained from medium supplemented with 0.5 mg L^{-1} 2, 4-D and 0.1 mg L^{-1} TDZ (312.70 ± 59.61 mg/ 100 mg initial fresh weigh of PLB) and the callus grew well after being transferred to light condition for 2 months. After that, PLBs-derived callus were inoculated on solidified VW solid medium supplemented with

coconut water (CW) (0, 10 and 15 %) combined with sucrose (0, 10 and 15 g L^{-1}) for callus proliferation. The highest callus growth rate was achieved from the medium containing 10 % CW and 15 g L^{-1} sucrose at 0.560 ± 0.56. The callus turned green and grew well after being transferred to light conditions for 2 months.

For cryopreservation of *P. niveum* PLBs by vitrification technique, 8-week-old PLBs which were composed of shoot and root apical meristems were precultured in modified VW liquid medium supplemented with various concentrations of sucrose (0, 0.25, 0.50, 0.75 and 1.00 M) for 24 h and 5 d with daily increasing sucrose concentration, then they were loaded in loading solution for 30 min followed by dehydrated in plant vitrification solution 2 (PVS2) with different exposure duration times (0, 30, 60, 90 and 120 min). Precultured PLBs in 0.75 M sucrose for 5 d with stepwise increased concentration of sucrose followed by 90 min dehydration in PVS2 gave the highest absorbance value (0.57 ± 0.06). Cryopreserved PLBs exhibited the tissue damage and starch grain accumulation in some areas, dimple exterior layers, stability of DNA content and the percentage of survival rate of 22.22 ± 15.71 %. Furthermore, the cryopreserved callus through encapsulation-vitrification and vitrification techniques was also investigated. The calli were precultured in modified VW liquid medium with various sucrose concentrations (0, 0.25, 0.50, 0.75 and 1.00 M) for 5 d with daily increasing sucrose concentration and dehydrated in PVS2 at different periods (0, 20, 40, 60, 80 and 100 min). The best result was obtained from the encapsulation-vitrification technique which the calli were precultured in 0.5 M sucrose followed by a 100-minute exposure to PVS2. They presented the highest viability absorbance value (0.237 ± 0.011) and moisture content at 27.34 ± 0.96 %. The histological and histochemical observations exhibited the normal cell with a large nucleus, a dense cytoplasm, non-degraded cell wall as well as non-disrupted starch grains (PAS reaction) and protein (Ninhydrin test). The cryopreserved calli via vitrification (0.5 M sucrose and in PVS2 for 60 minutes) presented high viability (0.216 ± 0.009) and moisture content at 23.93 ± 2.05 %. These vitrification-based calli displayed the cellular damages indicated by the degradation of cell wall and the disruption of both starch grain and protein. The percentage of survival rate of cryopreserved calli from the encapsulation-vitrification and vitrification techniques were 29.63 ± 10.31 % and 22.22 ± 7.85 %, respectively. In addition, no change in ploidy level of cryopreserved calli was observed.

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Supornchai Chaireok

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LIST OF ABBREVIATIONS AND SYMBOLS

$\mu molm^{-2}s^{-1}$	=	Micromole per second and square meter
2, 4-D	=	2, 4-Dichlorophenoxyacetic acid
AC	=	Activated charcoal
ANOVA	=	Analysis of variance
BA	=	N^6-Benzyladenine
BAP	=	6-Benzylaminopurine
BH	=	Banana homogenate
CITES	=	The Convention on International Trade in Endangered Species
CRD	=	Completely randomized design
CW	=	Coconut water
d	=	day
DMSO	=	Dimethy1-sulfoxide
ds	=	Dorsal sepal
ED	=	Encapsulation dehydration
FAA II	=	Formalin-acetic acid-alcohol II
HA	=	Hyaluronic acid
H&S	=	Hematoxylin and safranin stain
IAA	=	Indole-acetic acid
LN	=	Liquid nitrogen
M	=	Molar
MC	=	Moisture content
MS	=	Murashige and Skoog medium
Na_2HPO_4	=	Disodium hydrogen phosphate
NAA	=	1-Naphthaleneacetic acid
NDM	=	New Dogashima medium
p	=	probability level
PAS reaction	=	Periodic Acid Schiff reaction
PEG	=	Polyethylene Glycol
PGR	=	Plant growth regulator

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

pH	=	Hydrogen potential
PI	=	Propidium Iodide
PLBs	=	Protocorm like bodies
PVS2	=	Plant Vitrification Solution II
RAPD	=	Random amplified polymorphic DNA
rpm	=	Revolution per minute
SE	=	Standard error
SEM	=	Scanning electron microscope
TDZ	=	Thidiazuron
TTC	=	2, 3, 5-Triphenyl tetrazolium chloride
UV	=	Ultraviolet
VW	=	Vacin and Went medium

CHAPTER 1
INTRODUCTION

1.1 Introduction

The *Paphiopedilum* genus is commonly known as "Lady's Slipper" orchid because the pouch-shaped lip of this orchid is similar to that of a lady's slipper. This orchid is a terrestrial that is native to South and Southeast Asia and its distribution extends from the Himalayas and Burma to Indochina and the Asian region up to Papua New Guinea (Teob, 1989). Besides, this orchid appears popular in the international floricultural industry due to the variety of shapes, sizes, and colors of the flowers (Lee et al., 2011).

However, the Lady's Slipper orchid has been listed as one of the few 'truly' threatened orchid species by the Convention on International Trade in Endangered Species (CITES) Appendix I as a "world endangered orchid species" (Mcgough et al., 2006; Lee et al., 2011). Therefore, the conservation of this orchid is urgently needed and there are many reasons to prevent this kind of orchid species from extinction. The genus *Paphiopedilum* comprises of many commercially important species that have been extensively cultivated and have produced a wide range of attractive varieties, cultivars and hybrids as a result of intensive breeding (Cribb, 1998). In addition, the natural rarity, rapid decline and current low population numbers indicate that several species have already been damaged by collecting and by habitat destruction (Antonelli et al., 2011). Moreover, Lady's Slipper orchid conservation is complicated by the fact that there is no reliable method for vegetative propagation (Arditti, 2008).

The issue of conservation is necessary to maintain the orchid species and a propagation method is a good way to keep them from extinction (Lee et al., 2011; Ng et al., 2010; Vendrame et al., 2014). Tissue culture techniques for orchid propagation have been practiced for more than a century and uniform clones can be obtained. However, these have been used in some groups of *Paphiopedilum.* Ng et al. (2010) reported that a large number of plantlets of *P. rothschildianum* could be induced from nodal stem segments and shoot explants as well as *P. niveum* plantlets were obtained from

callus-derived PLBs (Kaewubon et al., 2010). Besides, the orchid propagation via in vitro culture faces many risks, such as contamination during frequent subculture or somaclonal variations during in vitro propagation.

In recent years, cryopreservation in liquid nitrogen (LN) at -196 °C has been the most promising choice for long-term storage of the plant genetic resources and the biological materials. In the cryopreserved materials, the biochemical process and all biological activities are arrested. So, these cryopreserved materials can be stored for unlimited periods without alteration. Chaudhury and Malik. (2004) revealed that this method ensures the safe and efficient long-term preservation of different types of seeds and vegetative tissues. Although the cryopreservation has many advantages, the freezing and thawing injuries resulting in dysfunction of cell membrane structure may cause a low survival percentage of cryopreserved material. Up to the present time, many cryopreservation methods, such as two-step freezing, encapsulation-dehydration, complete vitrification and encapsulation- vitrification have been used and developed for the success of preservation of different plant parts. (Sakai, 1995; Engelmann, 2000; Kaviani et al., 2010).

The aim of this study was to investigate (1) the optimal conditions for in vitro propagation and (2) the suitable procedure for the long-term storage of cryopreserved PLBs and callus of *P. niveum*. The effects of sucrose concentrations and dehydration periods (using PVS2 solution), the moisture content during cryopreservation procedure were examined. The histological and histochemical studies, scanning electron microscope (SEM) observation and DNA contents of non-cryopreserved and cryopreserved plant materials were also evaluated and compared.

1.2 Review of Literature

1.2.1 Background of *Paphiopedilum niveum* orchid

Paphiopedilum is known as Lady's Slipper orchid or Venus orchid (Figure 1.1). Their name is derived from the Greek "Paphian", an epithet for Aphrodite (the goddess known as Venus to the Romans) and "Pedilon" meaning slipper.

This *Paphiopedilum* is a special favorite of orchid lovers and has been included in the CITES appendix I.

Figure 1.1 A potted plant of the *Paphiopedilum niveum* (Rchb. f.) Stein.

The genus *Paphiopedilum* is native to Southeast Asia including Thailand, Malaysia, Indonesia, the Indian Subcontinent and the Southern part of China. The taxonomy of this orchid is illustrated in the Figure 1.2.

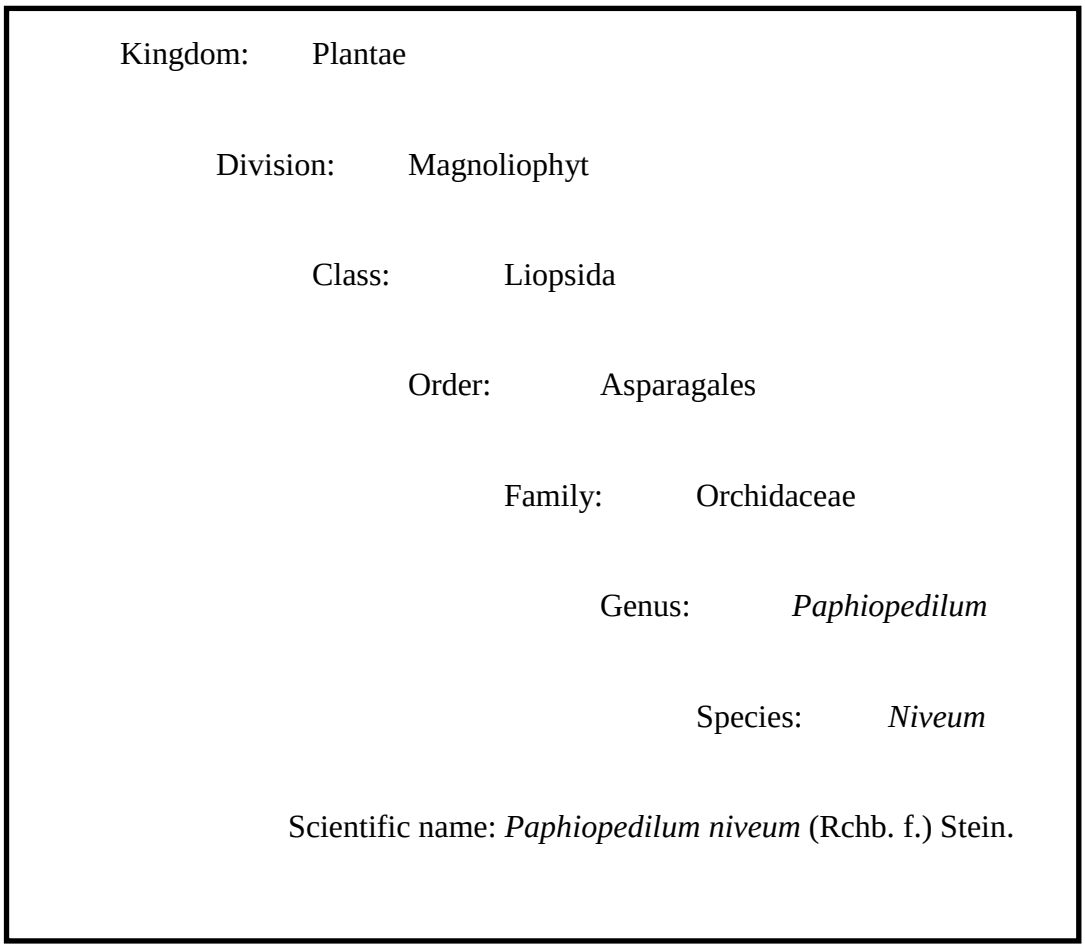

Figure 1.2 Taxonomy of the *Paphiopedilum niveum* (Rchb. f.) Stein.

The genus *Paphiopedilum* is of tropical Asiatic origin and is distributed among the countries in the Asiatic zone, especially in Southeast Asia. This genus can be found in China, Thailand, Malaysia, Indonesia and extend to the Philippines, including New Guinea and the Solomon Islands (Figure 1.3). No matter where the *Paphiopedilum* genus is found, they are still called endemic Lady's Slipper orchid (Cribb, 1998).

Paphiopedilum niveum is found in Southern Thailand and Northern Malaysia and the habitat is found in up to 200 m attitude from the sea level (Figure 1.3). The flower blooms from early May to early October. However, *P. niveum* which is found in both Thailand and Malaysia is an endangered species (Cribb, 1998).

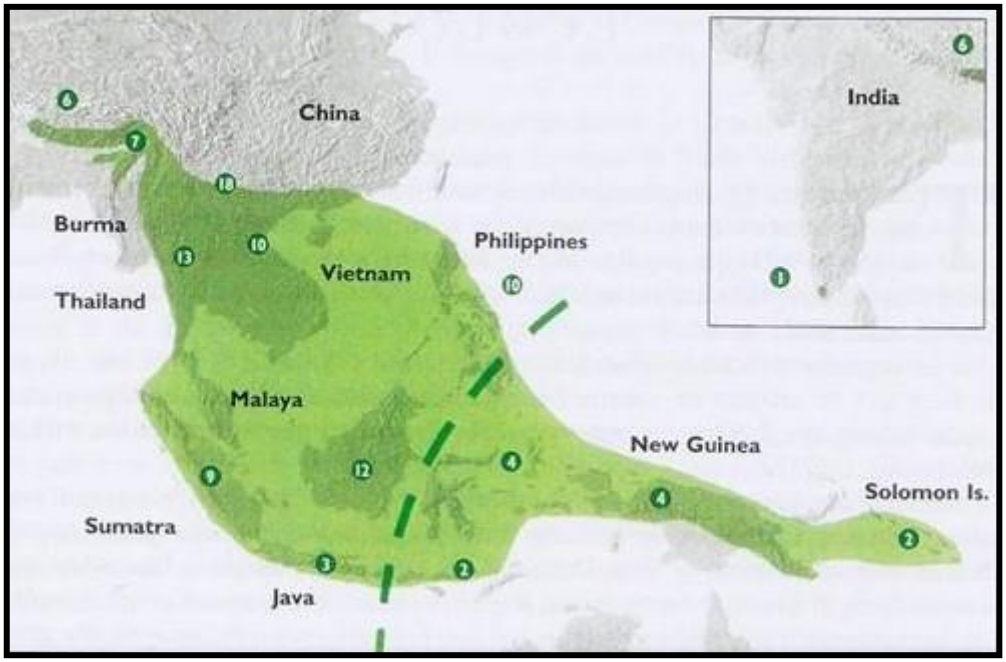

Figure 1.3 Distribution of the genus *Paphiopedilum* in India and Southeast Asia. The numeric numbers indicate the number of species in various parts of the range (Cribb, 1998).

The morphology of the *Paphiopedilum* orchid is an epiphytic, terrestrial or lithophyte plant that grows in a group or colony in the humus or the limestone area. All species of *Paphiopedilum* exhibit rhizome which are normally short. However, several species produce elongated rhizome, such as *P. robinsonii*, *P. armeniacum* and *P. micranthum.* The leaves are frequently notable and taxonomically valuable at the sub generic and the species level (Cribb, 1998). The inflorescences differ among the species and the flower number is an extremely useful diagnostic character. Normally, the *Paphiopedilum* genus produces a single flower and some well grown plants can produce two flowers. However, it is not uncommon if they produce two-flower inflorescence. The *P. niveum* exhibits taller inflorescences, with a 15-20 cm long scape, smaller shining white flowers usually lightly spotted with purple, a smaller ellipsoidal lip and transversely elliptic staminode (Figure 1.4-1.6). In some well-grown plants of *P. niveum* the inflorescence may bear two flowers, but one is usual in the wild. It also varies in the size of its flower and the degree of purple-spotting. *P.*

niveum has a pure white flower with a central yellow spot on the staminode (Cribb, 1998) (Figure 1.6).

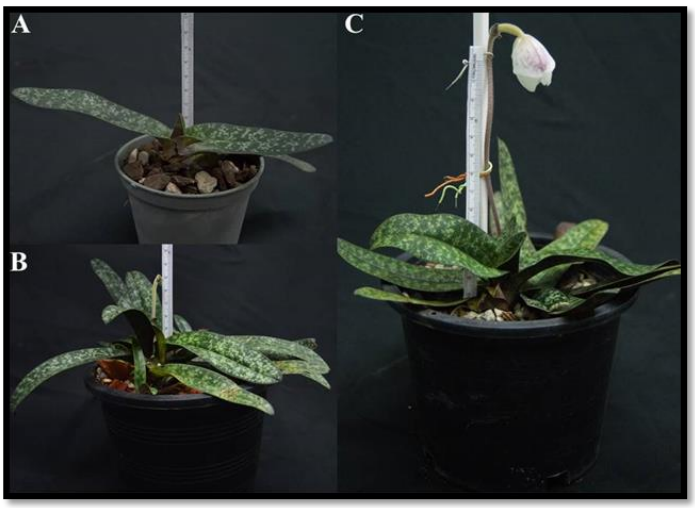

Figure 1.4 *Paphiopedilum niveum* grown in the greenhouse.

(A) A six-month-old plant exhibiting long leaves, (B) 8-10-month-old plant showing the flower stalk and (C) plant with bloomed flower.

Figure 1.5 Morphology of *P. niveum*.

(A) the underside surface of leaves exhibiting the purple color and some yellow spots (arrow-head), (B) the upper surface of leaves showing green color with some yellow spots and (C) the root surrounded by some fungi (arrow).

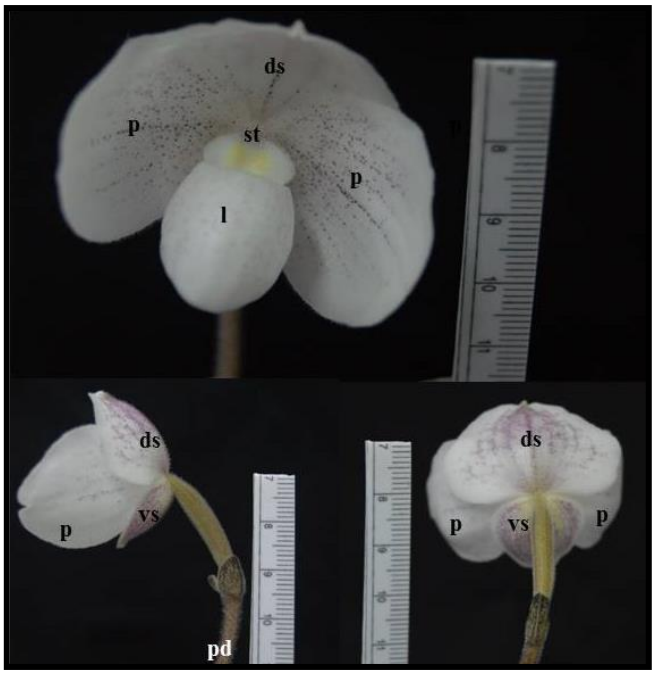

Figure 1.6 Flowers of *P. niveum* presenting dorsal sepal (ds), lip or pouch (l), petal (p), staminode (st), ventral sepal or synsepalum (vs) and peduncle (pd).

In addition, the cytology of *Paphiopedilum* exhibited that the chromosomes of *Paphiopedilum* are the largest and the most easily studied of all the *Orchidaceae*. Large chromosomes are found only in the other *Cypripedioideae* and in some genera of the *Neottioidae* (Duncan 1959). However, Shindo and Kamemoto. (1963). reported the karyotype investigation of *P. niveum* which the chromosome number at 2n = 26 (Figure 1.7A). Similarly, Lan and Albert. (2011) presented the study of chromosome number by using fluorescence in situ hybridization (FISH) technique that *P. niveum* had chromosome number at 2n = 26 (Figure 1.7B).

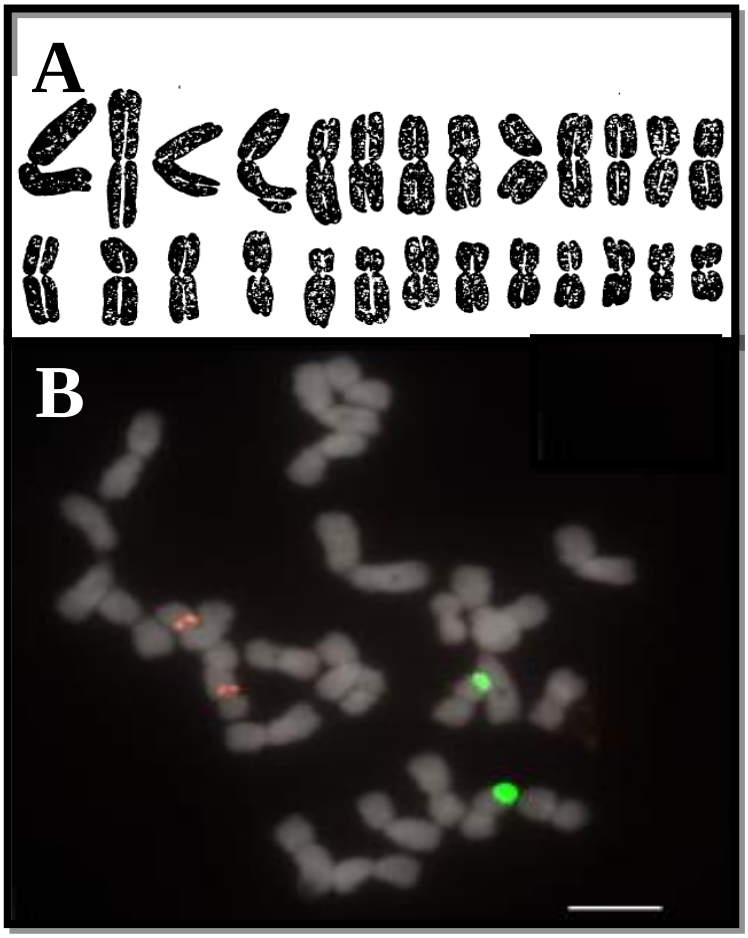

Figure 1.7 Chromosomes of *P. niveum* (Rchb.f.) Stein.

(A) Karyotype (Shindo et al., 1963), and (B) chromosomes using FISH technique (Lan et al., 2011).

1.2.2 In vitro propagation of orchid

Plant tissue culture technique plays an important role in the rapid clonal propagation of plant. This technique has been applied to many plant species including flowering plants. Cymbidium orchid was reported as the first orchid species which used this method for commercial propagation. There are many orchid species which were presented in vitro propagation by using plant tissue culture technique. However, the in vitro propagated mediums depend on plant species.

Many *Dendrobium* orchids were propagated by using plant tissue culture technique, such as *Dendrobium candidum* Wall. ex Lindl. It was cultured on MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg L^{-1} NAA (Shiau et al., 2005). Sunitibala and kishor. (2009) reported the culture of the *Dendrobium transparens* L. were maintained on MS medium supplemented with 1 mg L^{-1} NAA and 2 mg L^{-1} BAP. In addition, *Dendrobium* hybrids were also reported to culture on MS medium supplemented with the combination of 1 mg L^{-1} BAP and 1 mg L^{-1} IBA (Khatun et al., 2010). *Dendrobium fimbriatum* which were cultured on MS medium supplemented with 2 mg L^{-1} BAP and 0.01 mg L^{-1} IBA could produce multiple shoots (Kabir et al., 2013).

While, Lin et al. (1986) reported in vitro culture of *Phalaenopsis* and *Doritaenopsis* on VW medium with 1 mg L^{-1} or 5 mg L^{-1} BA. *Phalaenopsis* Richard Shaffer 'Santa Cruz were cultured on VW medium supplemented with 0.1 2, 4-D mg L^{-1} and 0.01 mg L^{-1} BA (Ishii et al., 1998). In contrast, *Phalaenopsis gigantea* were cultured on ND medium supplemented with the combination of 1 mg L^{-1} NAA and 0.1 mg L^{-1} TDZ (Niknejad et al., 2011).

For *Vanda* orchid, *Vanda testacea* (Lindl.) Reichb.f. The best results in proliferation of regenerated plantlets were cultured on Mitra medium with 1 mg L^{-1} BAP and 1 mg L^{-1} NAA (Kaur and Bhutani., 2010). Tee et al. (2010) reported that *Vanda* Somsri Pink gave a good results in protocorm-like bodies (PLBs) induction on MS medium supplemented with 1 mg L^{-1} NAA and 1 mg L^{-1} picloram.

In addition, *Cypripedium* orchids which are closed to *Paphiopedelum* orchid were also reported to use plant tissue culture technique for rapid clonal propagation (Zhang et al., 2010). *Crypripedium calceolus* L. were cultured on MS medium with 0.1 mg L^{-1} NAA and 1.0 mg L^{-1} BA (Pindel and Pindel., 2004). While, *Paphiopedilum rothschildianum* was cultured on MS medium supplemented with 1 g L^{-1} peptone and 2 g L^{-1} trytone-peptone (Ng et al., 2010). Furthermore, *Paphiopedilum callosum* wes cultured on MS medium supplemented with 0.5 µM TDZ and 50 µM 2, 4-D (Wattanawikkit et al., 2011). Udomdee et al. (2012) also reported the culture of

Paphiopedilum Hsinying Rubyweb on MS medium supplemented with 10 g L^{-1} sucrose, 1 g L^{-1} peptone and 50 g L^{-1} banana homogenate.

1.2.2.1 Callus induction and proliferation

Callus has the ability to differentiate from many plant parts. So, many orchid species have been reported for callus induction and proliferation. PLB-derived-callus of *Phalaenopsis* were induced on VW medium supplemented with 40 g L^{-1} sucrose and 20 % CW (Ishii et al., 1998). Teixera da Silva et al. (2006) reported callus induction from PLBs of *Cymbidium* Twilight Moon 'Day Light' on modified VW medium supplemented with 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} kinetin, 2 g L^{-1} tryptone and 20 g L^{-1} sucrose. Besides, PLBs of *Phalaenopsis* was cultured on 1/ 2 MS medium supplemented with 30 g L^{-1} sucrose was effective for callus induction (Ling et al., 2007). PLBs-derived-callus of *Dendrobium* Sonia 28 were cultured on half strength MS semi-solid medium supplemented with 1.0 mg L^{-1} NAA combined with 0.1 mg L^{-1} 2, 4-D (Mei et al., 2012).

In addition, the mediums for callus proliferation were reported in many orchid species, such as callus of *Rhynchostylis gigantea* provided the best callus formation on VW and NDM medium containing 2 % sucrose and 15 % CW (Rittirat et al., 2011). *Dendrobium* Serdang Beauty orchid callus were proliferated on MS medium containing 1.5 mg L^{-1} IBA (Khosravi et al., 2008). Moreover, Callus of *Cymbidium* orchid very well proliferated on modified VW medium containing the combination of 0.1 mg L^{-1} NAA and 0.01 mg L^{-1} TDZ (Teixera da Silva et al., 2006).

1.2.2.2 Protocorm-like body (PLB) proliferation

PLBs is the small swollen organell which is consisted of undifferentiated cells that have the potential to produce in vitro roots or shoots. PLBs have been widely used for plant material in orchid propagation. The success of in vitro propagation via PLBs explant was reported in many orchid species. Because the advantages of PLB are clonal propagation, conserved germplasm and

commercial value. Besides, PLBs can be induced form various plant part such as the flower stalk internodes (*Epidendrum radicans*) (Chen et al., 2002), leaf apices (*Oncidium flexuosum*) (Mayer et al., 2010), seedlings (dwarf *Dendrobium*) (Sujjaritthurakarn and Kanchanapoom., 2011) and shoot tip (*Rhynchostylis gigantea*) (Prasongsom et al., 2012). However, the *Paphiopedilum* has been rarely reported over the years. Hong et al. (2008) presented the induction of callus from seed of *Paphiopedilum* Alma Gavaert and maintained on 1/2 MS medium supplemented with 2, 4 D and TDZ. While, Kaewubon et al. (2010) reported that the PLB induction from seed-derived callus of *Paphiopedilum niveum* which were maintained on a modified VW solid medium supplemented with 10 g L^{-1} sucrose, 2 g L^{-1} Phytagel and 2 g L^{-1}AC. Furthermore, the in vitro formations of multiple shoots of *Paphiopedilum rothschildianum* were induced from stem which were maintained on the 1/2 MS medium (Ng et al., 2010).

In addition, the PLB proliferations are widely reported in many orchid species. For instance, Khoddamzadeh et al. (2011) reported that proliferation of PLBs of *Phalaenopsis bellina* (Rchb. f.) Christenson on modified MS medium supplemented with 3 mg L^{-1} TDZ. Nambiar et al. (2012) exhibited the proliferation of PLBs from new *Dendrobium* hybrids and maintained on MS medium supplemented with glucose, fructose and sucrose, and they also mentioned that CW was the best organic additive for the proliferation of *Dendrobium* hybrid PLBs. Furthermore, Samarfard et al. (2013) reported that the highest PLB multiplication of *Phalaenopsis gigantea* on VW and NDM supplemented with 10 mg L^{-1}chitosan. *Dendrobium* Sonia-28 was maintained on MS medium supplemented with 10 g L^{-1} sucrose and devoid of exogenous 6-benzylaminopurine (BAP) or naphthalene acetic acid (NAA) (Julkiflee et al., 2014).

Although the proliferation of *Paphiopedilum niveum* PLBs is rarely reported, Ng et al. (2010) presented the proliferation of secondary PLBs of *Paphiopedilum rothschildianum* by using modified MS medium supplemented with 4.0 μM kinetin.

1.2.3 Plant conservation

There are two basic approaches for plant conservation, namely in situ and ex situ conservations. In situ conservation means the conservation of the plant genetic resources in their environments, such as in natural habitat. While ex situ conservation the plant genetic resource is conserve outside their natural habitats, such as in a greenhouse or in vitro culture conditions. (Maxted et al., 1997; Benito et al., 2004; Rao, 2004; Edagbo, 2013)

1.2.3.1 In situ conservation

In situ conservation focuses on the conservation of plant germplasm in their natural habitats. The use of this approach is to maintain plant population in surrounding areas, where they have developed their distinctive properties. This approach needs management. Effectively, the problem of in situ conservation is the cost of maintenance, error in the labeling and the deterioration of material due to abiotic and biotic stresses.

1.2.3.2 Ex situ conservation

This conservation is the process to protect an endangered species by removing them from the natural habitats and placing them or parts of them in the unnatural habitat. So, the diversity acquisition, the maintenance, the distribution and the evaluation are needed. The maintenance of individual grown in these conditions is subjected to risk of stock loss due to the culture contaminations, the technique failure, and the genetic in stability.

Botanic gardens can be used to maintain germplasm as a living collection. Most of the botanic gardens conserve the wild relatives of cultivated crops, the medicinal and the forestry species, as well as diversity of ornamental plants. Many botanic gardens also maintain the collections of threatened and/or rare plants as well as minor/underutilized crops can be maintained in botanical gardens. Such species are often conspicuously absent in other ex situ collections.

Seeds are generally stored for a long time without losing their fertility if they are in standard conditions and can withstand conventional storage condition (5 % water content at -18 °C). However, seed conservation is an ineffective for recalcitrant seeds which do not remain fertile for a long time. The advantages of the seed storage are economy of space, relative low labor demands and consequently, the capacity to maintain large samples at economically viable cost (Whighama et al., 2006). Pollen is a product of genetic recombination and can provide a reliable source of nuclear genetic diversity at the haploid stage.

In vitro conservation is varied according to the storage duration requested. Based on plant tissue culture technique, plant can be conserved in the aseptic area. However, the problem of culture contamination and the variation of plant germplasm can be observed. In the latter case, the appearances depend on the frequency subculture of plant material. So, plant tissue culture technique can be maintained for medium- term conservation. In the same way, cryopreservation were applied for long-term of conservation at ultra-low temperature, usually that of liquid nitrogen (-196 °C). This method is currently available because all metabolic processes and cell division are stopped at this low temperature.

1.2.4 Plant cryopreservation

Cryopreservation was developed and examined for preserving plant cells and tissues. The repetitive storage of plant germplasms other than the seeds in the liquid nitrogen (LN) is a qualified new practice (Engelmann, 2004). Many procedures were applied for cryopreservation techniques such as encapsulation, vitrification, encapsulation-vitrification and droplet-vitrification. The processes of cryopreservation technique are not difficult but the initial implementation of cryopreservation procedures are limited (Reed et al., 1998). The success of cryopreservation also depends on storage location, and number of replication, viability testing and type of material to be kept. Moreover, cryopreservation is valuable as a secondary backup for primary collection of

clonally-propagated plants or as a secure system for maintaining the embryonic and metabolic potentials of many important plant species.

1.2.4.1 Principles of cryopreservation

Cryopreservation is the long-term storage of the plant material including cell suspension, pollen, embryonic culture, somatic and zygotic embryo, shoot apices or meristematic cell at ultra-low temperature (-196 °C) in liquid nitrogen (LN). At this temperature, cell division, the metabolic activities and the biochemical process are arrested and the plant materials can be stored for an infinite period of time. In this way, subcultures are not required and the treat of somaclonal variation is reduced. (Niino et al., 1992; Bajaj, 1995; Niino et al., 1997; Towill, 1996; Engelmann, 2000; Sakai et al., 2000; Helliot et al., 2002; Benito et al., 2004; Rao et al., 2004; Burritt, 2008).

Many factors, such as source-plant status, culture conditions, personnel, starting materials, pretreatment conditions, cryopreservation methods, cryogenic facilities, regimes and post-thawing are involved for successful cryopreservation (Reinhoud et al., 2000; Reed et al., 2005). One of the vital factors for cryopreservation is the property of water which occurs as ice crystals in plant cell. The physiological factor is also the most influential determinants of the survival of plant material. Cryopreservation procedures require the stability of plant germplasm and the minimal space for storage in LN where plant materials can be reduced according to size. The advantage of minimal space applies for many kinds of explant such as PLBs, callus and shoot tip with meristematic cells. In addition, the investigation of cryopreservation reported in the past has not influenced genetic variation (Engelmann et al., 1997; Sakai, 2000). The two main types of the plant cryopreservation method are classical and new method. The classical method contains a cryoprotective substances followed by slow freezing (Kartha, 1985). The basic cryoprotective substances are sucrose, polyethylene glycol (PEG), dimethylsulfoxide (DMSO), sorbitol and mannitol. All of constituents as previously mentioned have the osmotic engagements. However, DMSO can pass into the cell and shield cellular reliability during cryopreservation (Rajasekharan, 2006). The classical methods are

mainly used for freezing the undifferentiated cultures calli and cell suspension (Kartha and Engelmann., 1994) and differentiated tissues and organs (embryonic axes, shoot tips, seed and somatic and zygotic embryos). New methods have been developed based on the classical method including pre-growth, pregrowth-desiccation, desiccation, encapsulation-dehydration (ED), vitrification, encapsulation-vitrification, and droplet freezing. (Englemann et al., 1997).

1.2.4.2 Techniques for plant cryopreservation

1.2.4.2.1 Encapsulation-dehydration (ED)

The ED procedure which is based on artificial seed technology was developed by Fabre and Dereuddre (1990). This procedure was proposed to encapsulate single somatic embryo inside an artificial seed coat (Dereuddre, 1992; Engelmann et al., 1997; Sakai, 2004). Various other explants, for instance shoot tips, nodal segments, PLBs and callus samples can be used to produce synthetic seeds (Murashige, 1977). This procedure involves the incubation of plant materials in Na-alginate solution and then immersed into a complexity agent as a $CaCl_2$ solution where the completing agent is formed (Figure 1.8). After that, Na-alginate solution is mixed with $CaCl_2$ solution through the formation of ionic linkages between carboxylic groups on the guluronic acid molecules of alginate (Redenbaugh et al., 1993) (Figure 1.8). These alginate beads are precultured in a high concentration of sucrose depending on explant and plant species. In order to decrease the moisture content (20-30 %), these beads then dehydrated using either air-drying in a laminar flow or exposure to silica gel. After that, the beads are imersed in LN (-196 °C) for at least 1 h followed by thawing on the water bath (35-40 °C). Finally, beads are placed on the regrowth medium. The procedures of the encapsulation-dehydration are showed in Figure 1.9. Encapsulation-dehydration technique was used for storage the germplasm of many plant species such as hairy root cultures of Vinca minor L. (Hirata et al., 2002), and Spanish plant (*Antirrhinum microphyllum*) (González-Benito et al., 2011). This technique was used in many orchid

species for example the protocorms of *Vanda coerulea* (Jitsopakul et al., 2008), PLBs of *Brassidium* Shooting Star (Yin et al., 2011), PLBs of *Dendrobium* Sonia 28 (Pouzi et al., 2013).

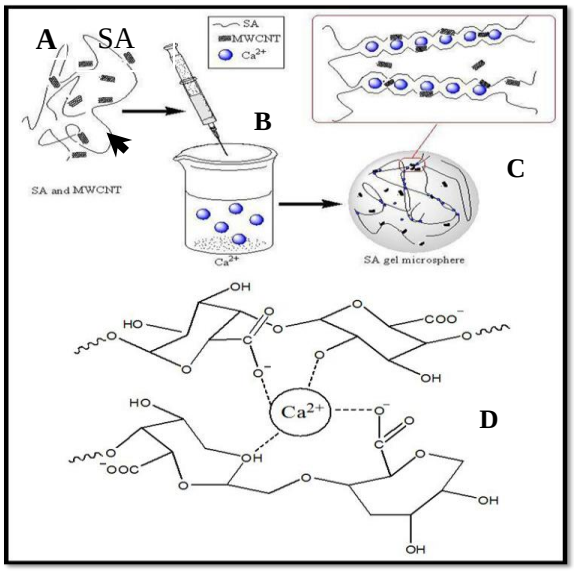

Figure 1.8 The reactive process of sodium alginate.

(A) Na-alginate (SA) with the material (arrow-head), (B) Dropping sodium alginate solution into Ca solution for forming sodium alginate bead, (C) microsphere of sodium alginate bead with molecule of Ca reacted with Na-alginate and (D) molecular structure of sodium alginate bead (Tsai et al., 2013).

1.2.4.2.2 Vitrification

Vitrification is based on the tissues being dehydrated by high osmoticum concentration followed by ultra-rapid freezing to avoid the risk of ice crystal formation which are detrimental to cellular structure integrity during cryopreservation and thawing. In this technique the explants were put into a high concentration of cryoprotective solutions for short periods. To induce the desiccation tolerance, the explants are cultured on medium with high concentration of sucrose or sorbitol. However, the suitable concentration depends on type of plant materials.

Subsequently, they are transferred to a glycerol-sucrose solution, called loading solution (Sakai, 2000). In addition, Sakai et al. (1990) was the first to report that osmoprotection with 2 M glycerol and 0.4 M sucrose is the effective in enhancing the capacity of cell to tolerate severe dehydration. However, Kim et al. (2009) developed the alternative loading solutions and reported that a loading solution comprising 1.9 M glycerol and 0.5 M sucrose was the most effective for plant vitrification protocols. Besides, plant vitrification solution 2 (PVS2) was developed for the cells are osmotically dehydrated which consists of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol and 15 % (w/v) DMSO (Sakai et al., 1990). The vitrification procedure was shown in Figure 1.9.

Cryopreserved orchid species by using vitrification technique, such as *Brassia rex* orchid hybrid was precultured in 0.5 M sucrose and dehydrated in PVS2 for 10 min (Shuhaida et al., 2009). Thammasiri (2000) reported that the successfully cryopreserved seeds of a Thai orchid (*Doritis pulcherrima* Lindl.) which were dehydrated in PVS2 for 50 min. In addition, *Vanda* Kaseem's Delight orchid PLBs were precultured in 0.1 M sucrose for 24 h followed by a loading solution treatment and they were the dehydrated in PVS2 for 20 min (Poobathy et al., 2012). Cryopreserved seeds of *Vanda coerulea* Griff. ex Lindl which they were treated with PVS2 solution for 70 min exhibited normal growth of seedlings in vitro (Thammasiri et al., 2007)

1.2.4.2.3 Encapsulation-vitrification

The encapsulation-vitrification procedure is a combination of the advantages of encapsulation-dehydration and vitrification whereby samples are encapsulated in alginate bead, then submitted to vitrification. The encapsulation-vitrification method is easy to handle and saves time which is needed for dehydration. In addition, the recovery growth is much earlier than encapsulation-dehydration technique (Lipavska and Vreugdenhil., 1996; Ashmore and Engelmann., 1997; Hirai et al., 1998; Sakai and Engelmann., 2007). For the encapsulation-vitrification procedure, plant materials are precultured in the hypertonic solution medium and then maintained in the solution of sodium alginate with loading solution.

After that, the plant materials are sucked into a pipette or small tube and put in $CaCl_2$ solution where the beads are formed. Then, these beads are immersed in PVS2 at different periods of time (depending on explant and plant species). The procedure of encapsulation-vitrification technique is shown in Figure 1.9.

Orchid species have been reported to use encapsulation- vitrification technique method such as the PLBs of *Dendrobium candidum* which were precultured in 0.75 M sucrose and followed by dehydration in PVS2 solution for 150 min at 0 °C (Yin and Hong., 2009). Ching et al. (2012) reported the optmal parameter of cryopreserved *Dendrobium* Sonia 28 PLBs precultured in 0.5 M sucrose for six days and followed by dehydration in PVS2 at 0ºC for 150 minutes. Furthermore, seed of *Dendrobium cariniferum* exhibited successful cryopreservation by using this encapsulation-vitrification technique (Thammasiri, 2008).

1.2.5 Technique for evaluation the cryopreservation efficiency

The conservation of plant germplasm is essential to protect biodiversity and to prevent the extinction in plants. Cryopreservation is one of the technique have great potential for plant conservation. However, the cryopreservation procedure is often ineffective because of slow or poor regrowth of explant. Consequently, no less than one technique, that permits a quick and precise prediction of viability after cryopreservation, is necessary.

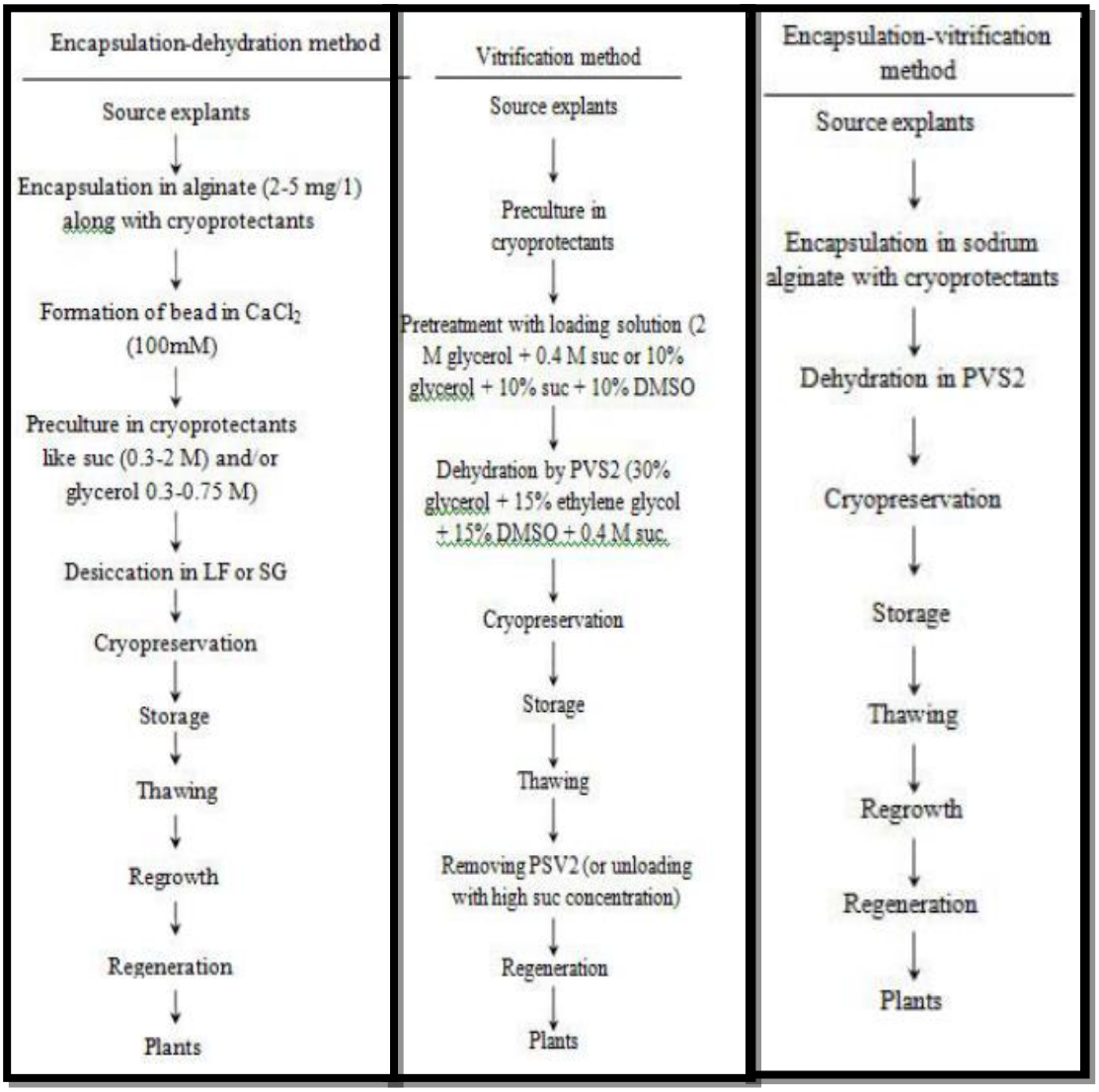

Figure 1.9 Different procedure of plant cryopreservation; Encapsulation- dehydration, vitrification and encapsulation-vitrification (Kaviani, 2011).

Many techniques were used for evaluation of cryopreservation efficiency such as 2,3-triphenyl-tetrazolium chloride (TTC) staining (both visual and spectrophotometric analysis), a mathematical model that relates 'water content' to the weight of encapsulated material as well as histological and histochemical analyses. In

addition, the genetic stability of the cryopreserved material is also examined by many techniques such as flow cytometry and also random amplified polymorphic DNA (RAPD).

1.2.5.1 Determination of viability by TTC testing

The TTC based on the enzymatic activity of living plant cells with the level of cell respiration (Whiters, 1985). The dehydrogenase activity in the mitochondria reduce colorless TTC to red triphynylformazan and the living cell is stained-red (Figure 1.10). This technique can be used to assess seed viability and freezing-caused damage in plant (Steponkus and Lanphere., 1967). The TTC assay has been widely used for many years as a fast and intensive test. However, some data showed that the TTC test give variable and erroneous results from the TTC able to penetrate into the tissue by limited factor that the most reaction intents the cell surface or a cut edges of tissue (Verleysen et al., 2004). Therefore, TTC assay was applied by using spectrophotometer for measuring the red-color. Verleysen et al. (2004) also reported the wavelength of TTC reaction-red color at 530 nm. Cryopreserved orchid species were presented by using TTC-spectrophotometer test such as cryopreserved *Brassia rex* (Johari et al., 2009), *Dendrobium* Bobby Messina (Antony et al., 2011), *Brassidium* Shooting Star (Yin et al., 2011), *Dendrobium* Bobby Messina hybrid (Zainuddin et al., 2011) and *Vanda* Kaseem's Delight (Poobathy et al., 2012).

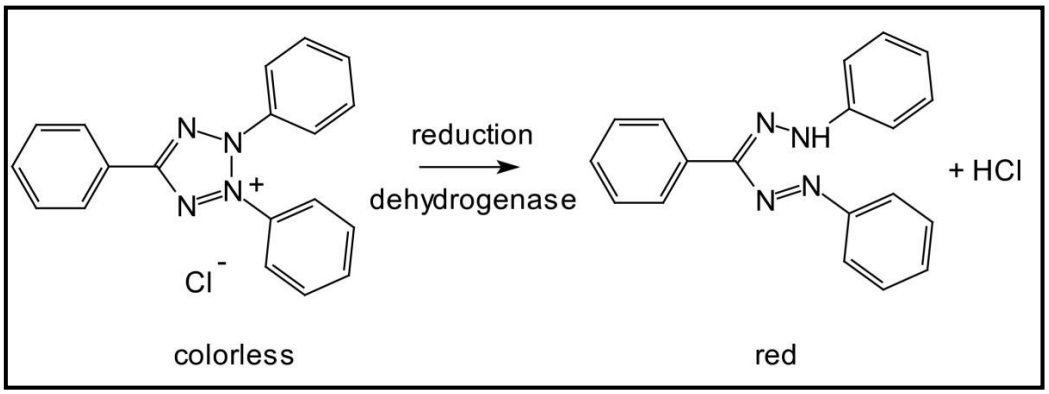

Figure 1.10 The 2,3,5-Triphenyl tetrazolium chloride (TTC) reaction structure (Opwis et al., 2012).

1.2.5.2 Estimation of DNA content by flow cytometry

The in vitro regenerated plants from tissue culture concerns somaclonals variations as a result from genetic or epigenetic modifications. Many studies have been performed on the genetic stability of plant material regenerated after using cryopreservation. Moreover, the protocols for cryopreservation have usually been claimed as a guarantee for genetic stability, in comparison with other in vitro long term storage procedures. However, the genetic variation in cryopreserved materials have been occasionally reported (Harding 2004). The cause of this variation is generally not attributed to the cryopreservation treatments themselves but to the in vitro proliferation or regeneration step, which are necessary before or/and during the cryopreservation process (Harding, 1997). The exposure to extreme physical condition (i.e. very low temperature, high osmotic pressure) and to certain chemicals employed as cryoprotectants (i.e. DMSO) may result in physiological stress and, consequently in genetic instability.

Fukai et al. (1994) reported that adult plant of *Apricot* Marble obtained from cryopreserved shoot tips under cryoprotechtant (DMSO) provided the change of flower color. Besides, the change in phenotype may be due to some kind of damage suffered by the shoot tips during freezing and thawing (Fukai et al., 1994).

Flow cytometry is increasingly employed as the method of choice for determination of nuclear DNA content and ploidy level in plants (Galbraith et al., 1998). DNA content or ploidy is one of the most frequent genetic variation in vitro systems. Estimation of nuclear DNA content is one of the important applications of flow cytometry. The principle of flow cytometry involves the movement of suspended cells flowing through a thin orifice, sequentially probed by a light source to excite the attached fluorescent label. Flow cytometry is typically used in configurations for either quantitative analysis or physical cell sorting. The most common type of quantitative analysis using cytometry data involves creating a histogram of fluorescence events to count the number of

cells with the attached probe. This effectively creates a set of data which gives a ratio of the cells in a population with a particular surface protein, enzyme, or other analyst.

The estimation of nuclear DNA content is performed with flow cytometer, using the fluorescent stain such as propidium iodide (PI). The samples of growing leaf tissue of plant materials are prepared together. Leaf material is chopped with a sharp razor blade for 30-60 s, in a petri dish containing 0.4 mL of extraction buffer. The resulting extract is passed through a 30-μm filter into a 3.5 mL plastic tube, to which is then added 1.6 mL of staining buffer, to give final stain and RNase concentrations. Samples are kept in the dark for 30 min and then analysed by flow cytometry. All stages of the extraction and staining are performed at 4 °C (Dolezel and Göhde., 1995). Flow cytometry technique has been used for evaluation of the genetic variation in many cryopreserved plant species such as *Oryza sativa* L (Moukadiri et al., 1999), *Quercus suber* somatic embryos (Fernandes et al., 2008), *Gentiana tibetica* (Mikula et al., 2008), and *Aerides multiflora* Roxb (Bunnag et al., 2014).

1.3 Objectives

1.3.1 To investigate the suitable medium for PLB proliferation of *P. niveum.*

1.3.2 To study the suitable medium for callus induction and proliferation from PLBs of *P. niveum.*

1.3.3 To obtain the best cryopreservation protocol for cryopreserving PLBs and callus of *P. niveum.*

CHAPTER 2
RESEARCH METHODOLOGY

The successful cryopreservation of tropical Lady's Slipper orchid, *P. niveum* was investigated. The research was divided into 2 parts, as follows:

I) In vitro propagation of plant materials required for cryopreservation experiment.

This part was carried out to increase the number of PLBs and callus which were used as plant materials in the cryopreservation processes. Two approaches in this part were performed, the PLB proliferation and the callus induction and proliferation.

II) Cryopreservation of PLBs and callus.

This part was determined to investigate the suitable conditions for cryopreserved PLBs by vitrification technique and cryopreserved callus by both vitrification and encapsulation-vitrification techniques. However, the cryopreserved PLBs was based on the preliminary process. The viability, the moisture content, the histological, histochemical and SEM studies were determined. The ploidy level of cryopreserved materials was also evaluated using flow cytometry.

2.1 In vitro propagation of *P. niveum*

2.1.1 PLB proliferation and plant regeneration

2.1.1.1 Plant materials

PLBs of *P. niveum* which were obtained from seed-derived callus were maintained at the Plant Tissue Culture Research Unit, Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. Eight-week old PLBs (approximately 3-4 mm in size) consisting of both shoot apical meristem and root apical meristem were used as plant materials. They were cultured on modified Vacin and Went (VW) solidified medium supplemented with 10 g L^{-1} sucrose, 2 g L^{-1} Phytagel and 2 g L^{-1} Activated Charcoal (AC). They were maintained

in a culture room under light (intensity at 23 µmol $m^{-2}s^{-1}$) provided by Phillips white fluorescent light with a 16 h light/ 8 h dark photoperiod.

2.1.1.2 PLB proliferation

PLBs of *P. niveum* (3-4 mm in length) were cultured on modified VW medium supplemented with coconut water (CW) (0, 10 %) at 3 culture states of Phytagel: 0 %, 0.1 %, and 0.2 %, which were defined as liquid state, semi-solid state and solid state, respectively. The experiment was performed in a completely randomized design (CRD) with 12 replications and repeated thrice. The pH of the medium was adjusted to 5.3 with 1 N NaOH or HCl prior to autoclaving at 121 °C for 20 min. The PLBs were incubated at 25 ± 1°C, and maintained under light intensity of 23 µmol $m^{-2}s^{-1}$ provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod. After 8 weeks of culture, the increased fresh weight and the morphotype were measured and recorded. Photographs were taken using a digital camera (Panasonic DMC-FZ 18).

2.1.1.3 Plant regeneration

The proliferated PLBs with the highest increase in fresh weight were transferred to modified MS medium for plantlet regeneration (Kaewubon et al., 2010). The medium consisted of modified MS medium supplemented with 20 g L^{-1} sucrose, 50 g L^{-1} banana homogenate (BH), 6.8 g L^{-1} agar and 2 g L^{-1}AC. They were maintained at 25 ± 2 °C under light intensity at 23 µmol $m^{-2}s^{-1}$ provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod. The increased fresh weight and the PLBs regeneration were observed after 4 months of culture.

2.1.1.4 Statistical analysis

Each experiment was performed in a completely randomized design (CRD) with 12 replications and repeated thrice. The mean of increased fresh weight of PLBs was subjected to an analysis of variance (ANOVA) and compared using Duncan's Multiple Range Tests (DMRT) at $P \leq 0.05$.

2.1.2 Callus induction and proliferation

2.1.2.1 Plant materials

Five-month-old PLBs which were cultured on modified VW solidified medium supplemented with 10 g L^{-1} sucrose 2 g L^{-1} Phytagel and 2 g L^{-1} AC for callus induction.

2.1.2.2 Callus induction from PLBs

PLBs of *P. niveum* were transferred to modified VW solid medium containing 15 g L^{-1} sucrose, 1 g L^{-1} Phytagel, 5.5 g L^{-1} agar and 2 g L^{-1} AC. The medium as supplemented with various concentrations of 2, 4-D (0, 0.5 and 1 mg L^{-1}) and in combination with TDZ (0, 0.1 and 0.5 mg L^{-1}). The pH of the medium was adjusted to 5.3 with 1 N NaOH or HCl prior to autoclaving at 121 °C for 20 min. The initial fresh weight was recorded and the cultures were incubated at 25 ± 1°C under dark conditions for 2 months. After that, they were transferred to maintain in a light conditions at intensity of 23 µmol $m^{-2}s^{-1}$ provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod. The increased fresh weight of PLBs-derived callus, the percentage of PLBs formed callus, and percentage of dead PLBs were recorded after 4 months of culture.

$$\text{Increased fresh weight} = (\text{Final fresh weight of callus - initial fresh weight of PLBs})$$

$$\text{Percentage PLBs formed callus} = \frac{\text{Number of PLBs forming callus}}{\text{Total number of cultured PLBs}} \times 100$$

$$\text{Percentage of dead PLBs} = \frac{\text{Number of dead PLBs}}{\text{Total number of cultured PLBs}} \times 100$$

2.1.2.3 Callus proliferation

Callus of *P. niveum* (approximately 20 mg, 1-2 mm in size) was cultured on modified solidified VW medium containing 1 g L^{-1} Phytagel 5.5 g L^{-1} agar, 2 g L^{-1} AC. The medium as supplemented with different concentration of coconut water (CW) (0, 10 and 15 %) combined with different sucrose concentrations (0, 10 and 15 g L^{-1}). All cultures were maintained in the dark for 4 weeks, then transferred to light conditions (intensity at 23 µmol $m^{-2}s^{-1}$) provided by Philips white

fluorescent light with a 16 h light/ 8 h dark photoperiod. The increased fresh weight of callus, the callus growth rate and the color of callus were observed and recorded every month for 2 months.

Increased fresh weight after culture for a month = (one-month old fresh weight - initial fresh weigh)

Increased fresh weight after culture for 2 months = (two-month old fresh weight - initial fresh weigh)

$$\text{Callus growth rate} = \frac{\text{Final fresh weight of callus - Initial fresh weight of callus}}{\text{Initial fresh weight of callus}}$$

2.1.2.4 Statistical analysis

Each experiment was performed in a completely randomized design (CRD) with 12 replications and repeated thrice. The mean values were subjected to an analysis of variance (ANOVA) and compared using DMRT at $P \leq 0.05$

2.2 Investigation of cryopreserved PLBs via vitrification and encapsulation techniques

2.2.1 Plant material

PLBs of *P. niveum* (Rchb.f.) Stein were obtained from seed-derived callus. Eight-week-old PLBs (approximately 3-4 mm in size) consisting of both shoot apical meristem and root apical meristem were used as explants (Figure 2.1). These PLBs were cultured on modified solidified VW medium containing 10 g L^{-1}sucrose, 2 g L^{-1} peptone, 5 g L^{-1} chitosan, 2 g L^{-1} Phytagel and 2 g L^{-1} AC (Kaewubon et al., 2010). They were maintained in a culture room under light intensity of 23 µmol $m^{-2}s^{-1}$) provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod.

2.2.2 Cryopreserved PLBs using vitrification technique

2.2.2.1 Preculture treatment

The PLBs clumps (approximately 30 mg/ clump) were transferred to modified VW medium supplemented with various concentrations of sucrose (0, 0.25, 0.50, 0.75 and 1.0 M) for 24 h and 5 d. In the latter case, the step of sucrose concentration was changed from an initial concentration of 0.25 M and the concentration was raised daily until the designated final sucrose concentration. The cultures were placed on a rotary shaker with agitation at 75 rpm. After a culture for 24 h and 5 d with the daily increasing sucrose concentration, the moisture contents (MC) and the viability of PLBs were examined.

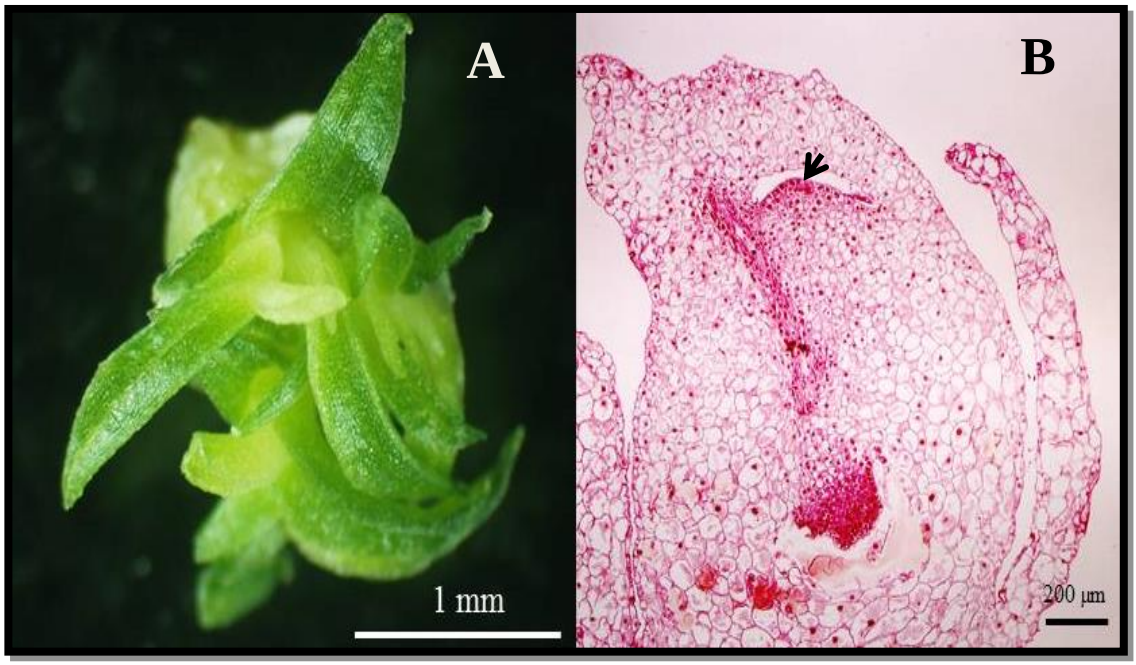

Figure 2.1 Explants for the cryopreservation experiment.
(A) Four-month-old PLB of *P. niveum* exhibiting well grown shoot. (B) Micrograph of 4-month-old PLB showing shoots apical meristem (arrow).

2.2.2.2 Loading solution and dehydration treatment

Three clumps of precultured-PLBs were placed in a 2 mL cryotube containing 1.0 mL of loading solution (LS) (modified VW liquid medium, 2 M glycerol and 0.5 M sucrose) for 30 min at room temperature. They were then removed and placed in plant vitrification solution 2 (PVS2) at 25 ºC for different periods of time (0, 30, 60, 90, 120 min). PVS2 contains 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO supplemented with 0.5 M sucrose in modified liquidified VW medium (pH 5.3). After dehydration in PVS2, the PLBs were directly plunged into liquid nitrogen (-196 ºC) for 1 h.

2.2.2.3 Rewarming and regrowth of cryopreserved PLBs using vitrification technique

After storage in LN, the cryopreserved PLBs were rewarmed rapidly in a water bath at 40 ºC for 2-3 min. Subsequently, PVS2 was drained from the cryotube and replaced with modified liquidified VW medium supplemented with 0.5 M sucrose and kept in darkness for 7 d. After a week of culture, the viability was determined by 2, 3, 5-Triphenyl tetrazolium chloride (TTC). The absorbance value was used as the condition for survival rate investigation. Then, these cryopreserved PLBs were transferred to modified Murashige and Skoog (MS) solid medium supplemented with 20 g L^{-1} sucrose, 50 g L^{-1} BH, 6.8 g L^{-1} agar and 2 g L^{-1} AC. They were maintained in culture room at a light intensity of 23 µmol $m^{-2}s^{-1}$provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod. The survival rates of both cryopreserved and non-cryopreserved PLBs (control) were also evaluated based on visual observation after 4 weeks of culture on modified solidified MS medium. The diagram of cryostorage PLBs using vitrification is shown in Figure 2.2.

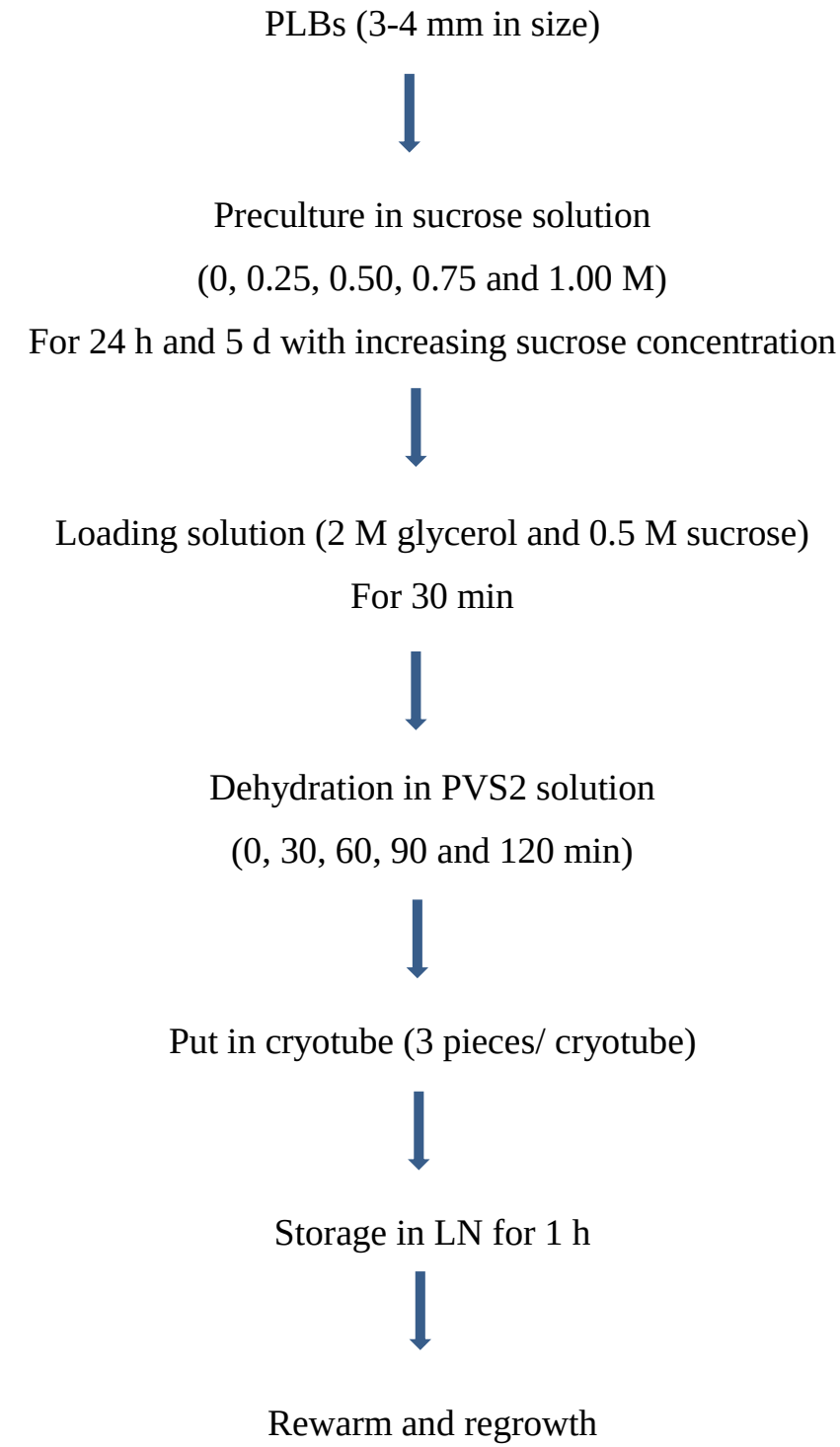

Figure 2.2 Cryopreservation procedures of PLBs base on vitrification technique.

2.2.3. Cryopreserved PLBs using encapsulation-vitrification technique

2.2.3.1. Preculture treatment

The PLBs clumps (approximately 30 mg/ clump) were transferred to modified VW medium supplemented with various concentrations of sucrose (0, 0.25, 0.50, 0.75 and 1.0 M) for 24 h and 5 d with daily increasing sucrose concentration. The cultures were placed on a rotary shaker with agitation at 75 rpm. After that the precultured PLBs were encapsulated in modified VW liquid medium {without $Ca_3(PO_4)_2$} containing 2.5 % (w/v) sodium alginate, 2 M glycerol and 0.5 M sucrose (loading solution) for making an alginate bead. The PLBs clumps were sucked up into wide-bore pipette, and then dropped into a $CaCl_2$ solution (0.1 M $CaCl_2$, 2 M glycerol, 0.5 M sucrose) where it remained to complete polymerization for 20 min. The obtained beads (about 4-5 mm in diameter) were washed with modified liquidified VW medium (pH 5.8). After that, they were dehydrated in PVS2 for different period of time (0, 20, 40, 60, 80, 100 min) at 25 ºC. PVS2 was composed of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO in modified liquidified VW medium with 0.5 M sucrose, pH 5.8. After PVS2 treatment, the beads were put into 2 mL cryotubes (3 beads / cryotube) and directly immersed into LN for 1 h. The diagram for PLBs through encapsulation-vitrification was shown in Figure 2.3.

2.2.3.2 Rewarming and regrowth of cryopreserved PLBs using encapsulation-vitrification

After storage in LN, the cryopreserved PLBs were rewarmed rapidly in a water bath at 40 ºC for 2-3 min. Subsequently, PVS2 was drained from the cryotube and replaced with modified liquidified VW medium supplemented with 0.5 M sucrose and kept in darkness for 7 d. After a week of culture, the viability was determined by 2, 3, 5-Triphenyl tetrazolium chloride (TTC) test.

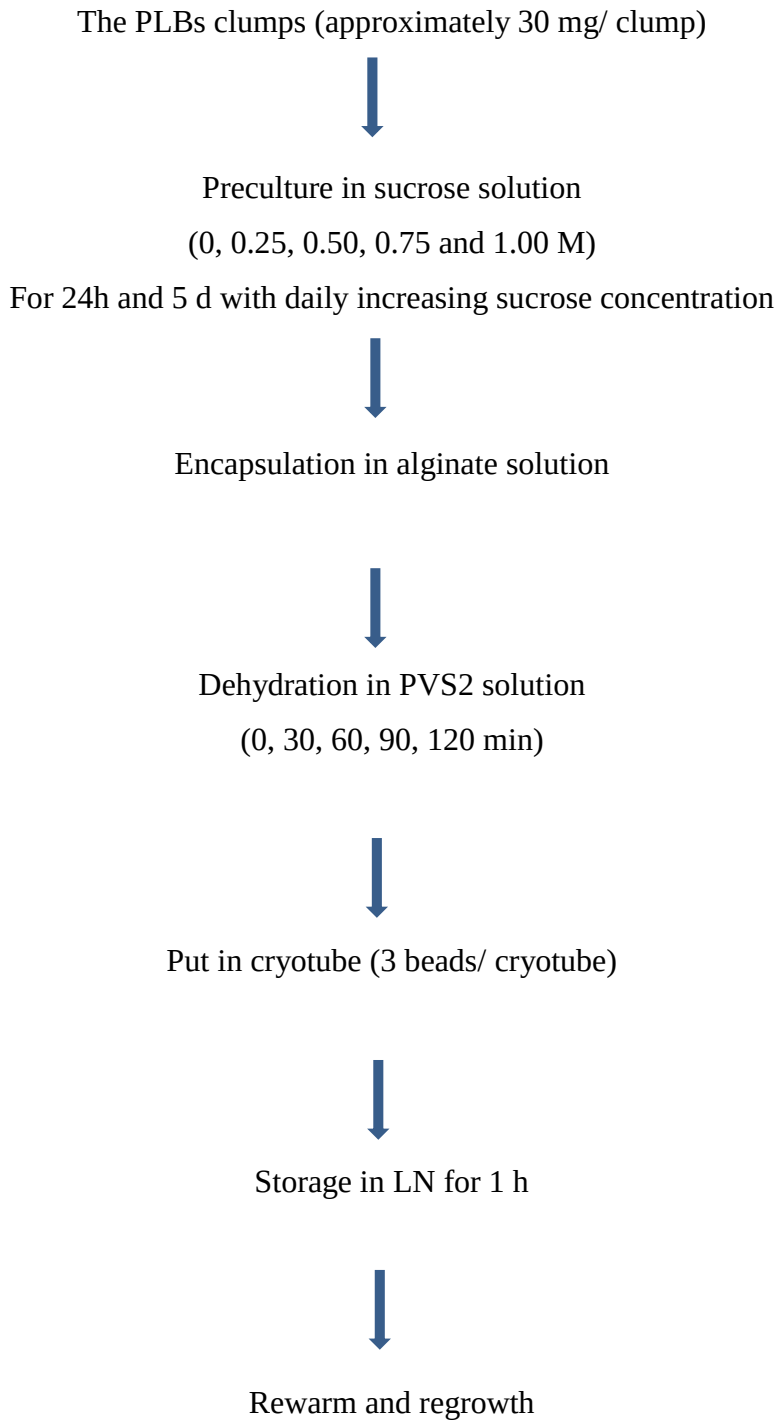

Figure 2.3 Cryopreservation procedures of PLBs base on encapsulation- vitrification technique.

2.3 Investigation of cryopreserved callus via encapsulation-vitrification and vitrification techniques

2.3.1 Plant material

PLBs-derived callus were cultured on modified VW supplemented with 15 g L^{-1} sucrose, 1 g L^{-1} Phytagel, 5.5 g L^{-1} agar and 2 g L^{-1} AC. They were maintained in the culture room under light intensity at 23 µmol $m^{-2}s^{-1}$ provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod. Six-month-old PLBs-derived calli were used as plant material.

2.3.2 Preculture treatment

The callus clump each with approximately 20 mg and 1-2 mm in size were suspended in modified liquidified VW medium added with various sucrose concentrations (0, 0.25, 0.50, 0.75 and 1.00 M) for 5 d with daily increasing sucrose concentrations. They were cultured on an agitation rotary shaker at 75 rpm and maintained under conditions as previous conditions.

2.3.3 Cryostorage of callus by encapsulation-vitrification method

The precultured calli were encapsulated in modified VW liquid medium [without $Ca_3(PO_4)$] containing 2.5 % (w/v) sodium alginate, 2 M glycerol and 0.5 M sucrose (loading solution) for making an alginate bead. Callus pieces were sucked up into wide-bore pipette, and then dropped into a $CaCl_2$ solution (0.1 M $CaCl_2$, 2 M glycerol, 0.5 M sucrose) where it remained to complete polymerization for 20 min. The obtained beads (about 3-4 mm in diameter) were washed with modified liquidified VW medium (pH 5.8). After that, they were dehydrated in PVS2 for different period of time (0, 20, 40, 60, 80, 100 min) at 25 ºC. PVS2 was composed of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO in modified liquidified VW medium with 0.5 M sucrose, pH 5.8. After PVS2 treatment, the beads were put into 2 mL cryotubes (3 beads / cryotube) and

directly immersed into LN for 1 h. The diagram for callus via encapsulation-vitrification was shown in Figure 2.4.

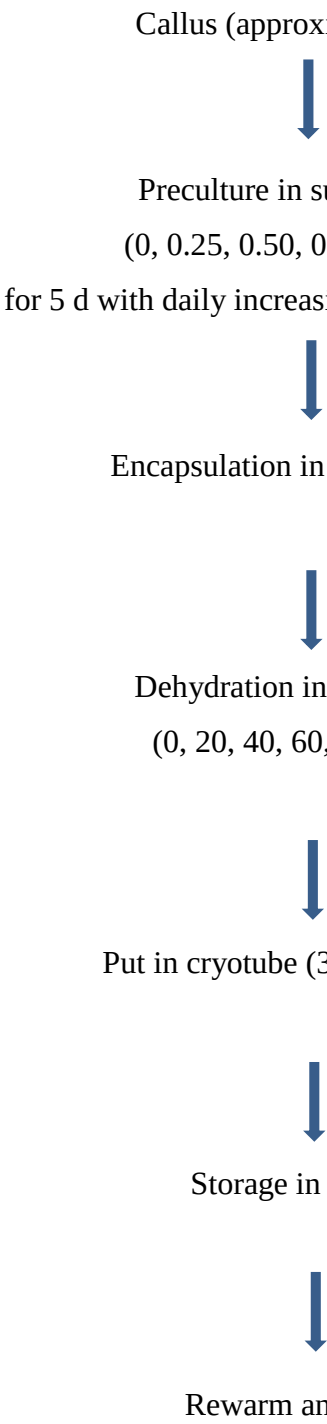

Figure 2.4 Cryopreservation procedures of callus base on encapsulation- vitrification technique.

2.3.4. Cryostorage of callus by vitrification method

The precultured calli were placed in cryotube and added with 1.0 mL LS for 30 min at room temperature. After LS was removed, these calli were dehydrated in PVS2 (30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO for different periods of time (0, 20, 40, 60, 80, 100 min) at 25 ºC. They were then placed in a cryotube (3 pieces / cryotube) and plunged into LN for 1 h. The diagram of callus cryostorage using vitrification technique was shown in Figure 2.5.

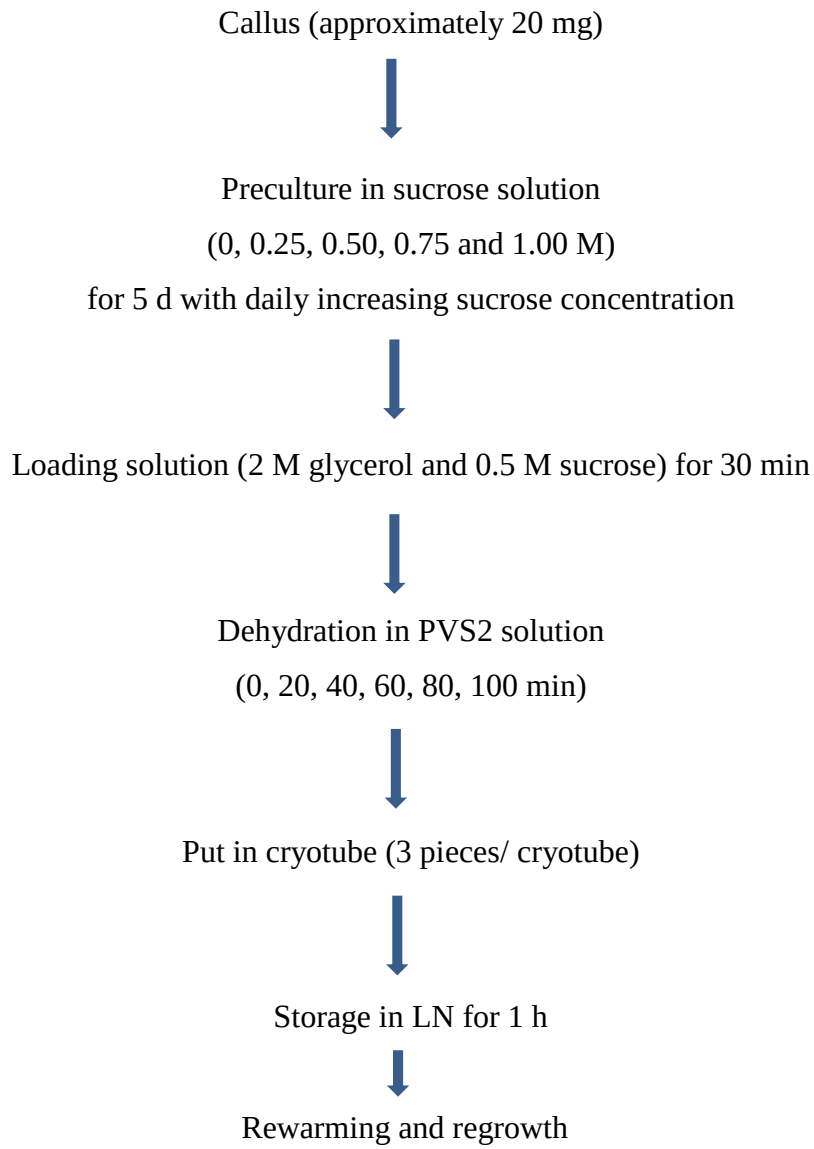

Figure 2.5 Cryoprestorage procedures of callus based on the vitrification technique.

2.3.5 Rewarming and regrowth of cryopreserved callus

After storage in LN for 1 h, the samples from both techniques (as previously described) were rewarmed rapidly in a water bath at 40 ºC for 2-3 min. Subsequently, PVS2 was drained from the cryotube and replaced with modified VW liquid medium. Then, these cryopreserved calli were transferred to dark conditions for a week followed by light conditions (intensity at 23 µmol $m^{-2}s^{-1}$) provided by Philips white fluorescent light with a 16 h light/ 8 h dark photoperiod. After a week of culture, the viability was determined by TTC. The survival rate was investigated based on the viability absorbance values. The survival rates of cryopreserved and non-cryopreserved callus (control) were also evaluated based on visual observation after 4 weeks of culture on modified MS solid medium supplemented with 20 g L^{-1} sucrose, 50 g L^{-1} BH, 6.8 g L^{-1} agar and 2 g L^{-1} AC.

2.4 Evaluation for the success of cryopreservation

2.4.1 Viability assessment

The viability of cryopreserved PLBs and callus of *P. niveum* was determined based on a TTC- spectrophotometric assay. The TTC assay was conducted according to the protocol described by Verleysen et al. (2004). The samples were immersed in 1.5 mL TTC solution (1 % TTC in buffer solution: 0.05 M KH_2PO_4 and 0.05 M Na_2HPO_4) and kept in the dark for 18 h at room temperature. After the TTC solution was drained off, these samples were washed with distilled water for 3 times, placed in cryotubes filled with 2 mL of 95 % ethanol and boiled in a water bath at 80°C for 10 minutes. After that, the obtained extract was cooled and the intensity of the redness of the extract was measured with a UV-spectrophotometer (Shimadzu UV-1601; Shimadzu corporation, Japan) at 530 nm (Yin et al., 2011), using 95 % ethanol as the blank.

2.4.2 Determination of moisture content (MC)

The MC of the cryopreserved materials was determined using the high constant temperature oven method (Khoddamzadeh et al., 2011). Plant

materials were pre-weighed and recorded for initial fresh weigh and then placed in the oven at 130 ºC for 24 h. Upon removal from the oven, they were placed in a desiccator for 24 h and reweighed. The percentage of MC was calculated using the formula below (Khoddamzadeh et al., 2011).

$$\text{Percentage of MC} = \frac{\text{Initial fresh weight of PLBs - Dry weight of PLBs}}{\text{Initial fresh weight of PLBs}} \times 100$$

2.4.3 Histological, histochemical and scanning electron microscope (SEM) observations

The cryopreserved and non-cryopreserved plant materials, which were cultured for 7 d in dark conditions, were randomly collected and fixed in FAA II (formaldehyde (Ajax Finechem, Taren Point, Australia) : glacial acetic acid (J.T. Baker, Phillipsburg, NJ, USA) : 70 % ethyl alcohol (Merck, Billerica, MA, USA) ; 5: 5: 90 v/v/v) for 48 h (Ruzin, 1999). They were dehydrated in a tertiary-butyl-alcohol series and embedded in Histoplast PE (ThermoShandom Limited, USA), cut (6 µm) and stained using hematoxylin and safranin for the observation of general features and Periodic acid-Schiff (PAS) for the detection of carbohydrate accumulation (Ruzin, 1999; Feder and Brien., 1968). Moreover, the cryopreserved calli were stained with Ninhydrin to investigate the protein accumulation.

For SEM observation, samples (cryopreserved and non-cryopreserved PLBs) were fixed in SEM fixative (10 % formaldehyde, 5 % acetic acid, 45 % ethanol and 1 % Triton X-100) at 4 ºC for 2 h. They were washed in 0.1 M phosphate buffer (pH 7.2) and dehydrated through a graded ethanol series (including 30 %, 50 %, 70 %, 80%, 90 %, 95 % and 100 % ethanol) for 15 min each. They were critical dried under liquid CO_2 using a Polaron CPD 7501 critical point drying apparatus (VG. Microtech, East Sussex, UK) and coated with gold by a sputter coater 11425 (SPI Supplies Division of Structure Probe Inc., West Chester, PA, USA). Samples were examined and photographed with a scanning electron microscope (Quanta 400; FEI Company, USA) at 10 kV.

2.4.4 Determination of DNA content

The ploidy levels of 4- week-old cryopreserved plant materials (under 1-week in the dark, followed by a 16 h light/ 8 h dark photoperiod for 3 weeks) were examined by flow cytometric analysis. The non- cryopreserved plant materials were used as control. *Vicia faba* CV. (2c = 26.90 pg.) was used as an internal reference standard. Clumps of PLBs (10-20 mg) or callus (approximately 20 mg) were finely chopped with a sharp razor blade in a 1.0 mL stock solution of Otto's buffer [Otto I: 100 mM citric acid, 0.5 % Tween 20 (pH 2-3) and Otto II: 400 mM $Na_2PO_4.12H_2O$ (pH 8-9)] (Otto, 1992; Dolezel and Göhde., 1995). After extraction, 50 µl of RNase (Sigma-Aldrich Co. P.O. box, USA) and propidium iodide (PI) (Sigma Chemical Co. P.O. box, USA) were added immediately prior to filtering through 42 µm nylon mesh. The samples were measured on a BD FACSCanto II flow cytometer (BD Biosciences, Belgium).

2.4.5 Experimental design and data analysis

Each experiment was performed in a completely randomized design (CRD) with 12 replications and repeated thrice. The mean values were subjected to an analysis of variance (ANOVA) and compared using DMRT at $P \leq 0.05$**.**

Furthermore, T-test was used to compare means of the viability absorbance value of cryopreserved based on encapsulation-vitrification and vitrification techniques. The means of DNA contents between non-cryopreserved and cryopreserved materials were also determined using a T-test.

CHAPTER 3
RESULTS

3.1 PLB proliferation and plant regeneration of *P. niveum*

3.1.1 Effects of CW and type of culture conditions on the PLB proliferation

Four-month-old PLBs of *P. niveum* were cultured on modified VW medium supplemented with coconut water (CW) (0, 10 %) at 3 culture conditions of Phytagel: 0 %, 0.1 %, and 0.2 %, which were defined as liquid-state, semi-solid-state and solid-state, respectively. After 8 weeks of culture, the highest increased fresh weight was obtained from the treatment containing 10 % CW combined with a solid-state (159.40 ± 5.40) (Table 3.1). It was found that the treatment that was supplemented with 10 % CW combined with a semi-solid-state gave a high increase in fresh weight (109.37 ± 4.11). However, PLBs on solidified medium provided higher weight than those of semi-solid medium (Table 3.1). All the treatments without CW which were in solid and semi-solid-state exhibited the low increasing fresh weight and there were no significant difference. Meanwhile, all treatments were in liquid state gave low increase fresh weight (Table 3.1).

PLBs which were cultured in the solid-state supplemented with CW exhibited a green color and grew well (Figure 3.1A-3.1B). For instance, these PLBs also presented healthy green PLBs with shoots (Figure 3.1A). However, the browning of PLBs was observed in liquid-state for 8 weeks (Figure 3.1E-3.1F).

3.1.2 Plant regeneration of PLBs of *P. niveum*

Two-month-old PLBs were obtained from the best result of PLB proliferation that provided the highest increase fresh weight. These PLBs were then transferred to plant regeneration medium. Shoots occurred and differentiated to yellow-green color leaves after 12 weeks of culture, (Figure 3.2A). However, after 16

weeks of culture, they were transferred to fresh medium in which their leaves turned green and healthy (Figure 3.2B).

Table 3.1 Effect of CW and culture conditions on the PLB proliferation.

Treatment	Cultural states	CW (%)	Increased fresh weight of PLBs (mg) / 100 mg of initial PLBs (means ± SE)	Morphotype (color)
1	Liquid	0	13.28 ± 9.99^{d}	Brown
2	Liquid	10	5.72 ± 5.72^{d}	Brown
3	Semi-solid	0	52.48 ± 10.79^{c}	Yellow
4	Semi-solid	10	109.37 ± 4.11^{b}	Green-yellow
5	Solid	0	60.30 ± 7.80^{c}	Yellow
6	Solid	10	159.40 ± 5.40^{a}	Green-yellow

Data were scored after 8 weeks of culture and expressed as increased fresh weight of PLBs per 100 mg of initial PLBs. The different letters in a column are the significant differences at $P \leq 0.05$ with Duncan's Multiple Range Test.

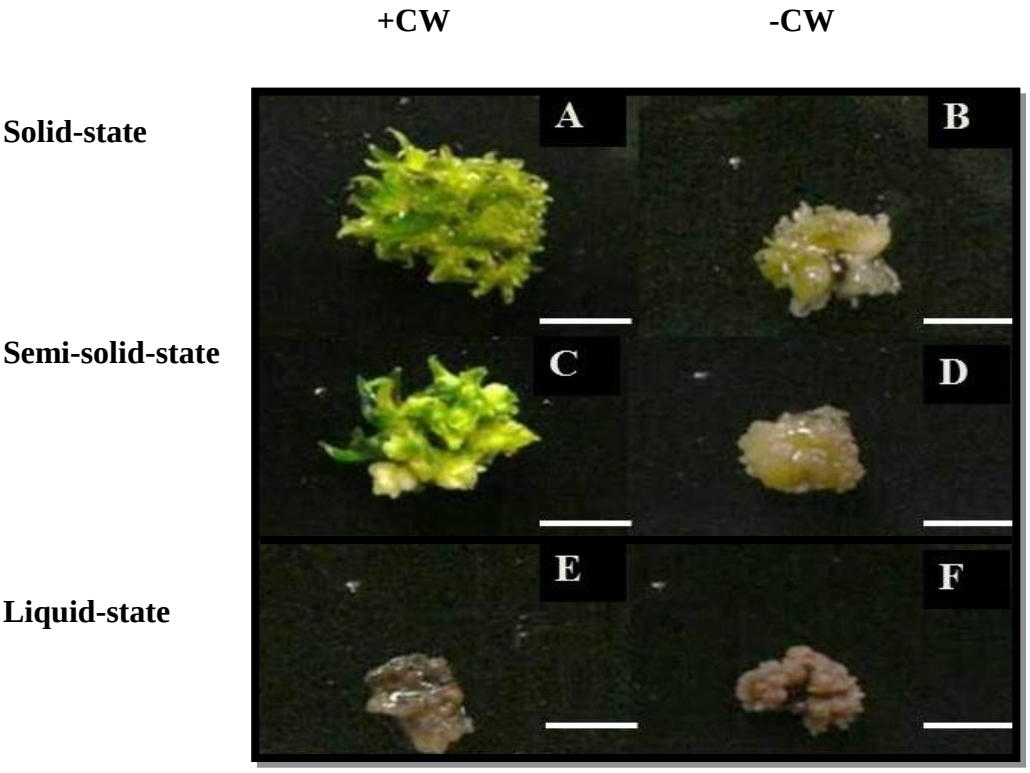

Figure 3.1 PLBs clumps of *P. niveum* cultured on modified VW medium supplemented with different concentration of coconut water (CW) in combination with different cultural states defined as liquid (0 % Phytagel), semi-solid-state (0.1 % Phytagel) and solid-state (0.2 % Phytagel).

(A) PLBs clump on solid-state supplemented with 10 % CW showing well grown shoot, (B) PLBs on solid-state with no CW exhibiting lower number of shoot than solid-state. (C) PLBs on semi-solid state in combination with 10 % is not healthy. (D) Yellow PLBs on semi-solid-state without CW. (E, F) Browning PLBs on liquid cultural state. Bar = 3 mm.

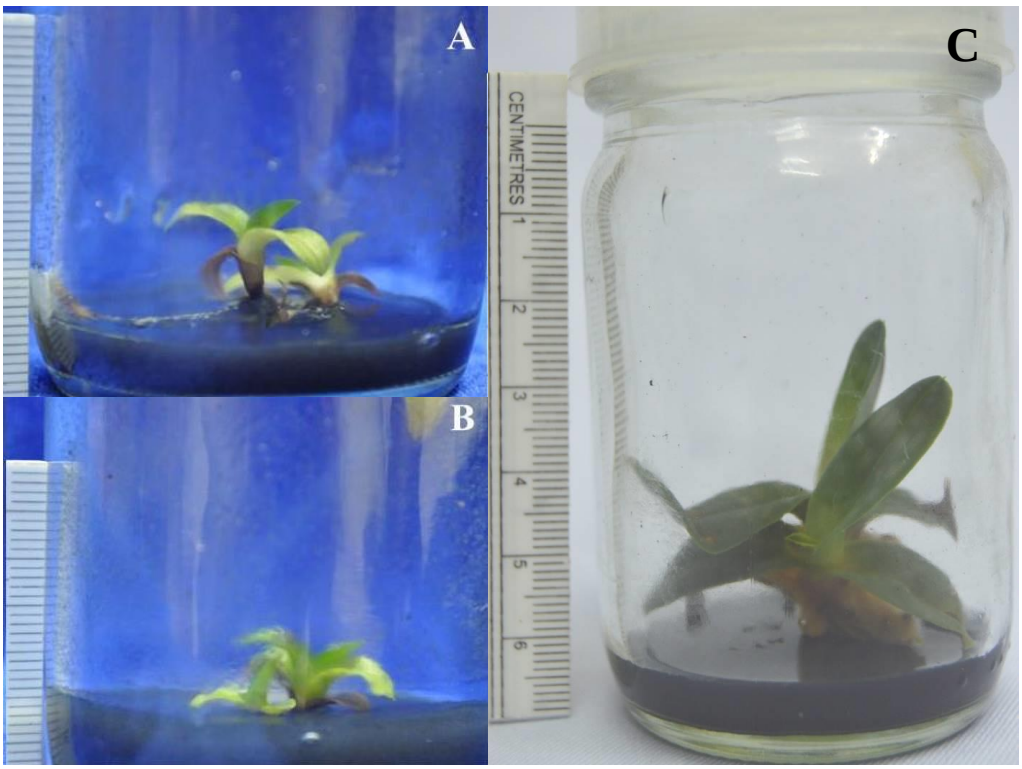

Figure 3.2 PLBs-derived plantlets of *P. niveum* on modified MS medium.
(A) Plantlets with yellow-green leaves after culture for 12 weeks.
(B) Healthy green plantlets on fresh regeneration medium, after 16 weeks of culture. (C) Illustrated the healthy green plantlets after maintained for 8 months

3.2 Induction and proliferation of PLB-derived callus of *P. niveum*

3.2.1 Effects of 2, 4-D and TDZ on callus induction

PLBs maintained on modified VW medium in the dark for 2 months died and the exterior layer of PLBs withered with the leaves dying first. The PLBs which were cultured on modified VW medium supplemented with 2, 4-D or TDZ alone exhibited the callus browning and eventually the callus dead. These results were similar to that of control. However, the PLBs cultured on medium supplemented with 2, 4-D in combination with TDZ exhibited the PLBs forming whitish yellow and compact callus at the base of PLBs (Figure 3.3). PLBs-derived callus after being transferred to light conditions for 8 weeks showed green and grew well (Figure 3.3). The highest increase in fresh weight was obtained from the treatment that was maintained on modified VW solidified medium supplemented with 0.5 mg L^{-1} 2, 4-D

combined with 0.1 mg L^{-1} TDZ (312.70 ± 59.61 mg per 100 mg of initial PLBs). This treatment gave the highest percentage of callus formation (38.58 ± 31.11 %) and exhibited the lowest percentage of the dead PLBs (Table 3.2). Followed by the medium supplemented with the combination of 0.5 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} TDZ provided high increase in fresh weight (162.99 ± 50.57 mg per 100 mg of initial PLBs) (Table 3.2). The application of high level of 2, 4-D (1.0 mg L^{-1}) and TDZ (0.5 mg L^{-1}) exhibited the browning PLBs and the callus dead.

3.2.2 Effects of CW and sucrose on the proliferation of callus of *P. niveum*

Four-month-old callus was transferred to callus proliferation medium. The callus was maintained in darkness for a month followed by a 16 h light/ 8 h dark photoperiod. The highest increase in fresh weight of one-month-old (95.67 ± 13.82 mg per 100 mg of initial callus) and 2-month-old (57.49 ± 7.56 mg per 100 mg of initial callus) calli were presented in Table 3.3. The proliferated calli were achieved from the medium supplemented with 10 % CW and 15 g L^{-1} sucrose. The growth rate of these calli also gave the highest growth rate (0.560±0.56) (Table 3.3).

The color of PLBs-derived callus exhibited a green color and grew very well in light conditions. However, the calli that were maintained on modified solidified VW medium supplemented either CW or sucrose exhibited lower callus growth rate.

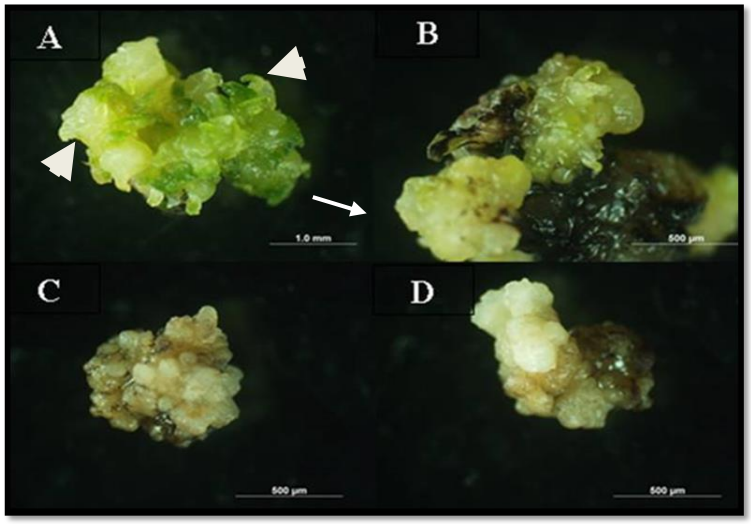

Figure 3.3 PLB-derived callus of *P. niveum* on modified Vacin and Went solid medium supplemented with 0.5 mg L^{-1} 2, 4-D in combination with 0.1 mg L^{-1} TDZ. (A) Five-month-old PLBs with small shoots (arrow-head) at the beginning of culture. (B) PLBs eventually died after maintained in dark conditions for 4 months and new callus formation (arrow). (C) New white callus originated from the base of PLBs. (D) Well grown PLBs-derived callus after being transfer to light conditions for 6 months.

Table 3.2 Effects of 2, 4-D and TDZ on callus induction and callus color of *P.niveum*.

Treatment No.	Plant growth regulator		Increased fresh weight* (mg) (mean ± SE)	Number of segments	Percentage of explants Forming callus	Percentage of dead PLBs	Color of callus
	2,4-D	TDZ					
1	0	0	0.00±0.00[c]	36	0.00±0.00[c]	100±0.00[a]	Browning
2	0	0.1	0.00±0.00[c]	36	0.00±0.00[c]	100±0.00[a]	Browning
3	0	0.5	0.00±0.00[c]	36	0.00±0.00[c]	100±0.00[a]	Browning
4	0.5	0	0.00±0.00[c]	36	0.00±0.00[c]	100±0.00[a]	Browning
5	0.5	0.1	312.70±59.61[a]	36	38.58±31.11[a]	60.75±31.27[c]	Green
6	0.5	0.5	162.99±50.57[b]	36	19.25±22.06[b]	80.25±22.53[b]	Yellow-green
7	1.0	0	0.00±0.00[c]	36	0.00±0.00[c]	100±0.00[a]	Browning
8	1.0	0.1	107.71±46.53[b]	36	13.75±16.99[b]	85.83±17.50[b]	Yellow
9	1.0	0.5	0.00±0.00[c]	36	0.00±0.00[c]	100±0.00[c]	Browning

Data were taken after PLBs were cultured on callus induction medium for 16 weeks.* Increased fresh weight of callus per 100 mg of initial PLBs. Each value represents mean ± SE. means followed by a different letter are significantly different at $P \leq 0.05$ according to one-way ANOVA with Duncan's Multiple Range Test.

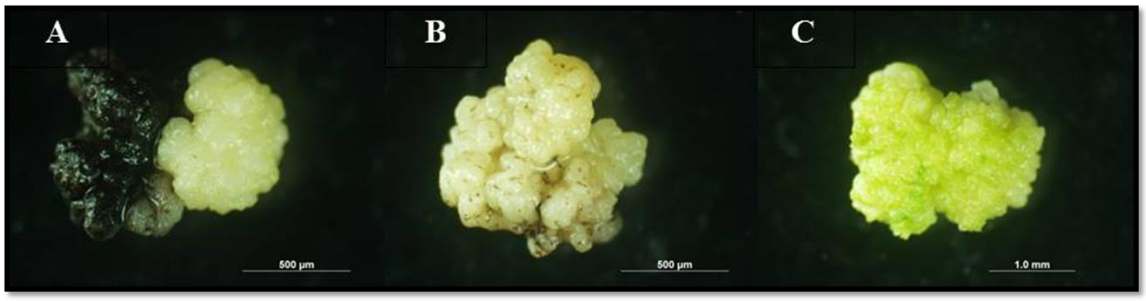

Figure 3.4 Proliferated callus of *P. niveum* on modified VW solid medium supplemented with 10 % CW and 15 g L^{-1} sucrose.
(A) Initial PLBs-derived callus on induction medium, (B) well grown yellow callus after culture for a month. (C) Green callus growing well after culture for 2 months.

Table 3.3 Effects of coconut water (CW) in combination with sucrose on callus proliferation of *P. niveum.*

Treatment	CW (%)	Sucrose (g L^{-1})	Increase fresh weight (mg) (means ± SE)		Callus growth rate	Callus color
			1st month*	2nd month*		
1	0	0	2.55±0.78^{c}	5.93±1.87^{c}	0.053±0.05^{c}	Yellow-brown
2	10	0	3.19±1.66^{c}	4.94±1.32^{c}	0.045±0.05^{c}	Yellow-brown
3	15	0	2.39±0.72^{c}	6.74±1.87^{c}	0.060±0.06^{c}	Yellow-brown
4	0	10	3.32±1.10^{c}	7.43±2.21^{c}	0.065±0.06^{c}	Yellow-brown
5	10	10	49.50±6.61^{b}	83.32±11.31^{b}	0.429±0.43^{b}	Yellow-brown
6	15	10	66.47±14.45^{b}	103.09±21.39^{b}	0.423±0.42^{b}	Yellow
7	0	15	57.49±7.56^{b}	80.19±8.65^{b}	0.424±0.42^{b}	Yellow
8	10	15	95.67±13.82^{a}	160.52±25.02^{a}	0.560±0.56^{a}	Yellow-green
9	15	15	5.27±1.41^{c}	11.41±3.10^{c}	0.095±0.10^{c}	Yellow

*Increased fresh weight of callus per 100 mg of initial callus

Each value represents mean ± SE, Means followed by a different letter are significantly different at $P \leq 0.05$ according to one-way ANOVA with DMRT analysis.

3.3 Cryopreservation of PLBs of *P. niveum*

3.3.1 Cryopreservation of PLBs of *P. niveum* by encapsulation-vitrification techniques

The bead of PLBs were precultured in modified VW liquid medium supplemented with various concentrations of sucrose (0, 0.25, 0.50, 0.75 and 1.00 M) for 24 h and 5 d with daily increasing sucrose concentration (Figure 3.5 A-3.5B).

It was found that there were not successful in the preliminary process after cryostorage in the LN. The viability of cryopreserved PLBs was evaluated by TTC test which were given the dead PLBs in both 24 h and 5 d with daily increasing sucrose concentration. Cryopreserved PLBs exhibited white colors which were not subjected to formazan reaction with the TTC test (Figure 3.5 C). In addition, no viability of cryopreserved PLBs was confirmed by Evan blue test exhibiting dark blue color (Figure 3.5 D). According to that result, the PLBs were not chosen as plant materials for cryopreservation by encapsulation-vitrification.

3.3.2 Cryopreservation of PLBs of *P. niveum* by vitrification techniques

3.3.2.1 Effects of sucrose concentrations and preculture time on viability and moisture content (MC) of PLBs

The PLBs were maintained in modified VW liquid medium supplemented with various concentrations of sucrose (0, 0.25, 0.50, 0.75 and 1.00 M) for 24 h and 5 d with daily increasing sucrose concentration. The PLBs which were precultured in the liquidified medium without sucrose (control) gave the lowest absorbance value in both 24 h (0.04 ± 0.03) and 5 d (0.05 ± 0.00) with daily increasing sucrose concentration (Table 3.4). These PLBs also provided the highest percentage of MC with non-significant difference at 24 h (82.77 ± 2.87 %) and 5 d with daily increasing of sucrose (70.00 ± 7.42 %). In contrast, the highest absorbance values were obtained when PLBs were precultured in 0.75 M sucrose at both 24 h (0.93 ± 0.53) and 5 d with daily increasing sucrose concentration (0.98 ± 0.14) (Table 3.4). It was noticed that the precultured PLBs in all sucrose concentrations

(hypertonic solution) exhibited the higher absorbance value than those of the control. However, the PLBs which were precultured in 0.75 M sucrose for 5 d with increasing sucrose concentration presented the lowest percentage of MC (25.23 ± 1.21 %).

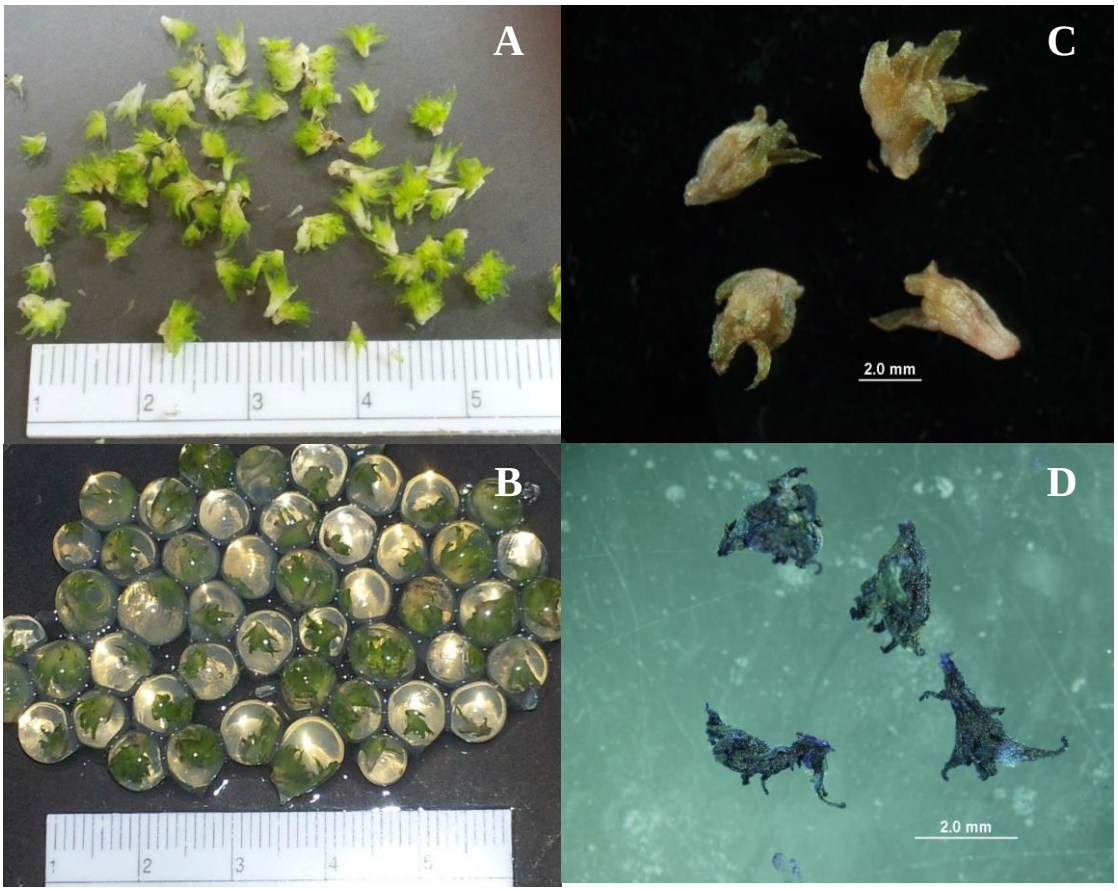

Figure 3.5 Cryopreserved PLBs of *P. niveum* using encapsulation-vitrification. (A) 4 months old PLBs obtained from seed-derived callus (B) encapsulated PLBs. (C) Cryopreserved PLBs evaluated by TTC test showing white color, (D) Cryopreserved PLBs determined by Evan blue test exhibiting dark blue color.

3.3.2.2 Effects of preculture protocols and exposure time to PVS2 on PLBs viability

The suitable sucrose concentration (0.75 M) from the previous experiment was chosen to investigate the best preculture protocol and exposure time for cryostorage. Thus, PLBs were precultured in 0.75 M sucrose for 24 h and 5 d with daily increasing sucrose concentration followed by different exposure time to PVS2 (0, 30, 60, 90 and 120 min). It was found that precultured PLBs from both protocols without PVS2 treatment provided the lowest absorbance values from 0.03 ± 0.01 to 0.04 ± 0.01 (Table 3.5). However, the PVS2-treated PLBs from both protocols exhibited the higher absorbance values than those of PLBs excluding PVS2 step. The stepwise increasing sucrose concentrations followed by the exposure time to PVS2 for 90 min gave the highest absorbance value (0.57 ± 0.06) while the 0.75 M sucrose precultured PLBs for 24 h followed by PVS2 for 90 min showed a significant difference of the absorbance value (0.36 ± 0.16).

Table 3.4 Effects of different sucrose concentrations and incubation periods on the viability and moisture content of *P. niveum* PLBs.

Incubation periods	Sucrose concentration (Molar)	Moisture contents (%)	Viability Absorbance (at 530 nm)
24 h	0	82.77 ± 2.87^{a}	0.04 ± 0.03^{e}
	0.25	83.60 ± 2.58^{a}	0.13 ± 0.06^{de}
	0.50	71.32 ± 1.11^{b}	0.53 ± 0.14^{bc}
	0.75	54.06 ± 2.32^{d}	0.93 ± 0.53^{a}
	1.00	58.01 ± 4.43^{cd}	0.34 ± 0.07^{cde}
5d. with a daily increase of sucrose concentrations	0	70.07 ± 7.42^{b}	0.05 ± 0.00^{e}
	0.25	61.32 ± 2.75^{c}	0.13 ± 0.03^{de}
	0.50	35.43 ± 2.26^{e}	0.81 ± 0.17^{ab}
	0.75	25.23 ± 1.21^{f}	0.98 ± 0.14^{a}
	1.00	32.38 ± 2.27^{e}	0.42 ± 0.15^{cd}

A viability assessment of precultured PLBs was illustrated in the absorbance value expressed by UV-spectrophotometer at 530 nm. The different letters in a column are significantly different at $P \leq 0.05$ with DMRT analysis.

Table 3.5 Effects of different preculture protocols and exposure time to PVS2 on the viability of *P. niveum* PLBs after cryostorage (at -196 °C).

Time	PVS 2 Time (Min)	Viability after LN Absorbance (530_{nm})
24 h	0	0.03 ± 0.01^{e}
	30	0.08 ± 0.01^{cde}
	60	0.12 ± 0.05^{cd}
	90	0.36 ± 0.06^{b}
	120	0.05 ± 0.03^{de}
5 d with a daily increase of sucrose concentrations	0	0.04 ± 0.01^{e}
	30	0.14 ± 0.02^{c}
	60	0.34 ± 0.12^{b}
	90	0.57 ± 0.06^{a}
	120	0.06 ± 0.02^{de}

A viability assessment of PVS2-treated PLBs was illustrated in the absorbance value expressed by UV-spectrophotometer at 530 nm. The different letters in a column are significantly different at $P \leq 0.05$ with DMRT analysis.

3.3.2.3 Survival percentage after thawing and regrowth

The PLBs precultured in 0.75 M sucrose with daily increasing sucrose concentration for 5 d followed by PVS2 treatment for 90 min were chosen for cryostorage. These PLBs were then cultured on the regrowth medium for 1 week in the dark followed by 3 weeks with a 16 h light/ 8 h dark conditions. It was found that non-cryopreserved PLBs exhibited healthy green PLBs (Figure 3.6A) and cryopreserved PLBs became green and healthy after 4 weeks of culture (Figure 3.6B). Although, they had a lower survival percentage (22.22 %) than those of non-cryopreserved PLBs (66.66 %) (Figure 3.6). Their green plantlets could be observed after a culture for 8 weeks on regrowth medium.

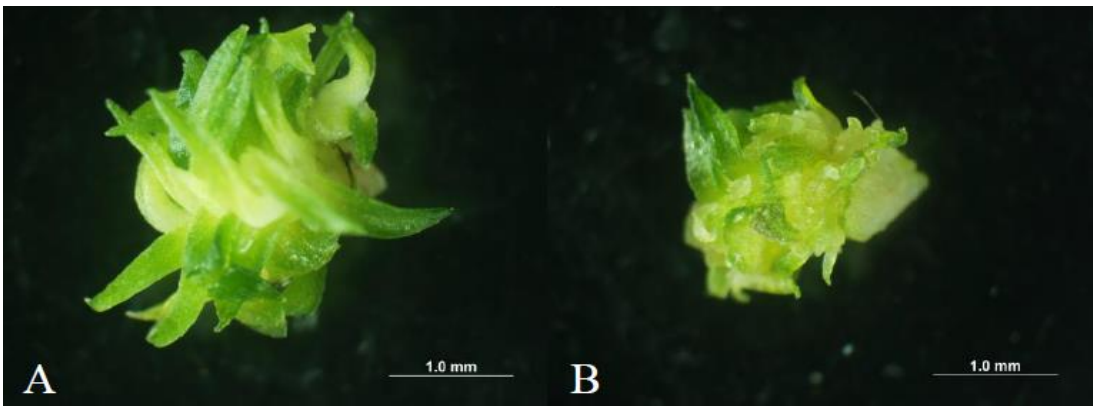

Figure 3.6 Clumps of in vitro PLB-derived plantlets of *P. niveum* on the regrowth medium.

(A) 4 months old non-cryopreserved PLBs-derived plantlets served as a control (B) 4 months old plantlets derived from cryopreserved PLBs preculturing in 0.75 M sucrose for 5 d. with a daily gradual increase of sucrose concentrations followed by PVS2 for 90 min and being plunged in LN at -196 °C for 1 h.

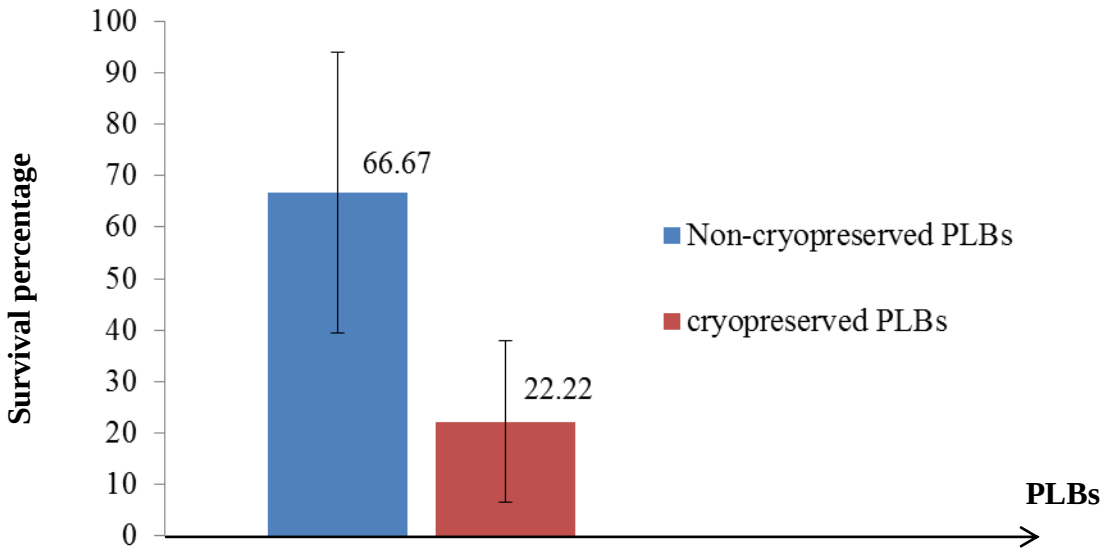

Figure 3.7 Survival percentage of non-cryopreserved and cryopreserved PLBs.

They were precultured in 0.75 M sucrose for 5 d with a daily increase of sucrose concentrations followed by PVS2 for 90 min were observed after 4 months of culture. Bar represents the standard error (SE).

3.3.2.4 Histological, histochemical and SEM analyses

The comparison of histological, histochemical and SEM studies were observed between non-cryopreserved PLBs (control) and cryopreserved PLBs (precultured PLBs in 0.75 M sucrose for 5 d with daily increasing of sucrose concentration followed by PVS2 for 90 min). It was found that both non-cryopreserved and cryopreserved PLBs exhibited darkly stained nuclei in the meristematic cells (Figure. 3.8A, 3.8B) and the reserved starch grains. However, cryopreserved PLBs presented lower amount of nuclei and starch grains than those of non-cryopreserved PLBs (control) (Figure. 3.8C, 3.8D). SEM study illustrated that the non-cryopreserved PLBs displayed smooth epidermal layer (Figure. 3.9A) while, the cryopreserved PLBs showed shrinking of epidermal cells (Figure. 3.9B).

3.3.2.5 Determination of the genetic stability of non-cryopreserved and cryopreserved PLBs via flow cytometry

After culture for 4 weeks on regrowth medium, the cryopreserved PLBs (in 0.75 M sucrose for 5 d with daily increasing sucrose concentration followed by PVS2 for 90 min) were determined for the genetic stability. The DNA contents these cryopreserved PLBs exhibited at 39.69 ± 0.82. In addition, there was no significant difference between these cryopreserved PLBs and non-cryopreserved PLBs served as the control (40.60 ± 0.24) (Table 3.6).

Table 3.6 Determination of DNA contents of *P. niveum* PLBs. Before and after storage into LN for 1 h.

PLBs	2C DNA content (pg.)	Mbp
Non-cryopreserved	40.60±0.24 [a]	19589.50
Cryopreserved	39.69±0.82 [a]	19150.43

Mean followed by the same letters in a column are not significantly different at $P \leq 0.05$ with DMRT analysis.

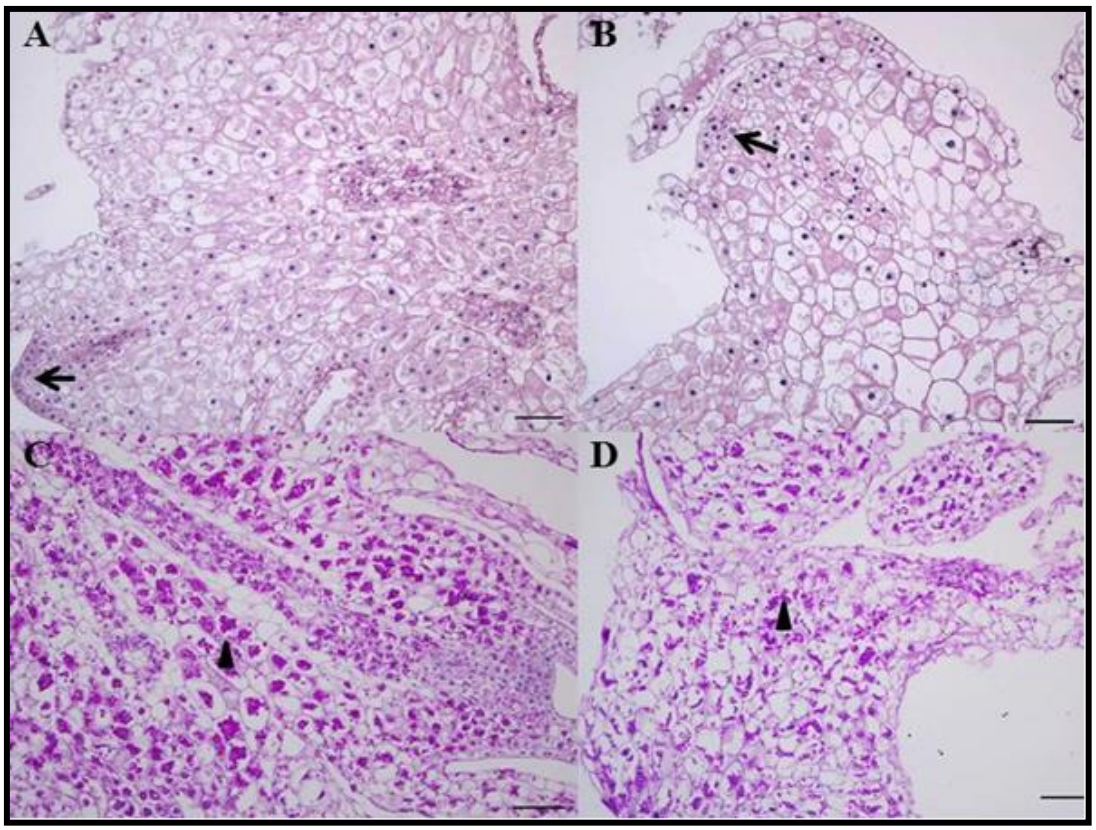

Figure 3.8 Histological and histochemical observations of (A, C) non-cryopreserved PLBs and (B, D) cryopreserved PLBs by preculturing in 0.75 M sucrose for 5 d with a daily increase of sucrose concentrations followed by PVS2 treatment for 90 min.

(A) Non-cryopreserved PLBs and (B) cryopreserved PLBs showing cells with common characteristics of parenchymatous cells and area of meristematic tissues which are actively dense nuclei (arrow) (Hematoxylin and safranin staining). (C) Non-cryopreserved PLBs present more numerous starch grains (arrow head) than in (D) cryopreserved PLBs resulting from cryoinjury during being plunged in LN (PAS reaction). Explants were observed after 7 d of culture in the dark.

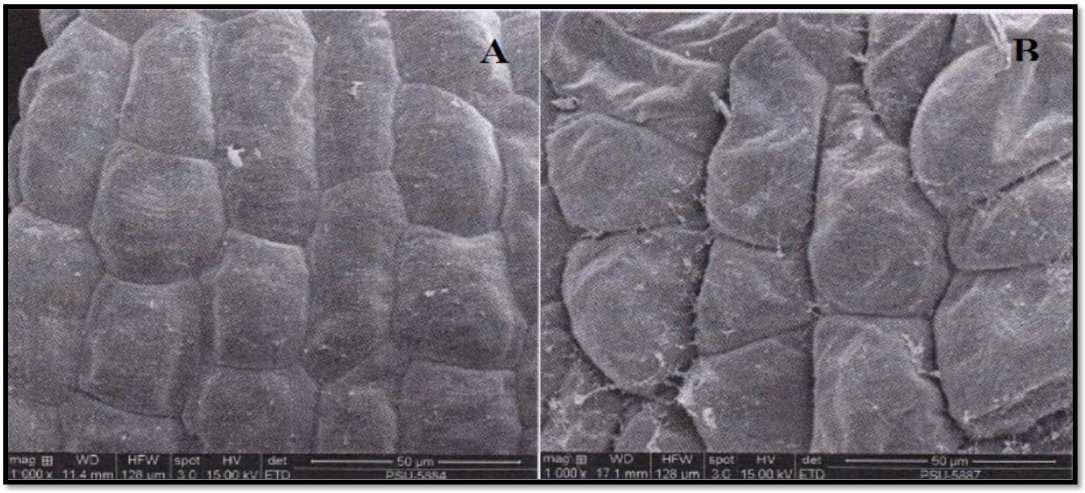

Figure 3.9 SEM micrographs display the surface views of PLBs.
(A) Non- cryopreserved PLBs exhibit normal surface. (B) Cryopreserved PLBs preculturing in 0.75 M sucrose for 5 d. with a daily increase of sucrose concentrations followed by PVS2 treatment for 90 min show some shrunken area on the exterior surface.

3.4 Cryopreservation of callus of *P. niveum*

3.4.1 Cryopreservation of callus of *P. niveum* by vitrification and encapsulation-vitrification techniques

3.4.1.1 Effects of sucrose concentrations in combination with dehydration duration time

The calli were maintained on modified VW solid medium supplemented with 15 g L^{-1} sucrose, 1 g L^{-1} Phytagel 5.5 g L^{-1} agars and 2 g L^{-1} AC. They were transferred to preculture in the modified liquidified VW medium supplemented with various concentrations of sucrose (0, 0.25, 0.50 and 0.75 M) for 5 d with daily increasing sucrose concentration. The precultured calli were dehydrated in PVS2 for different time (0, 20, 40, 60, 80 and 100 min). It was found that calli

were successfully cryopreserved by both encapsulation-vitrification and vitrification techniques.

During the encapsulation-vitrification technique, calli which were precultured in modified liquidified VW medium without sucrose and no dehydration (control) gave low viability absorbance value after storage in LN (0.083 ± 0.009) (Table 3.7). In addition, there were no significant differences among all treatments that precultured in the medium without sucrose. These calli also provided high percentage of MC (75.16 ± 1.52 %). In contrast, the highest absorbance values of cryopreserved calli (after storage in LN) weas obtained from the treatment which was precultured in 0.50 M sucrose with the stepwise increasing sucrose concentrations followed by dehydration in PVS2 at 100 min (0.237 ± 0.011). This treatment also gave a low percentage of MC (27.34 ± 0.96). In case of the vitrification technique, the calli precultured in the medium without sucrose concentration and no exposure to PVS2 gave low viability absorbance value (0.076 ± 0.005) and also gave a high percentage of MC (65.86 ± 1.71). Meanwhile, the highest absorbance value was obtained from the calli that were precultured in 0.50 M sucrose followed by dehydration in PVS2 at 60 min (0.216 ± 0.009) (Table 3.7). In this treatment, they gave a low percentage of MC (23.93 ± 2.05 %) (Table 3.7).

3.4.1.2 Effects of cryopreservation techniques on the cryopreserved calli of *P. niveum*

The cryopreserved material providing the highest viability absorbance value was chosen to compare with non-cryopreserved and cryopreserved calli. The encapsulation-vitrification technique showed no significant difference in viability absorbance value between non-cryopreserved and cryopreserved calli (Table 3.8). In contrast, the vitrification technique exhibited significant difference in viability absorbance value between non-cryopreserved and cryopreserved calli (Table 3.8). The comparison of cryopreservation techniques illustrated that both encapsulation-vitrification and vitrification techniques were successful in cryopreserving callus of *P.niveum*.

Table 3.7 Effect of sucrose concentrations in combination with dehydration duration time on the viability and moisture contents of cryopreserved and non-cryopreserved calli using encapsulation-vitrification and vitrification. The absorbance value was measured by UV-spectrophotometer at 530 nm.

Treatment	Sucrose concentration (M)	Dehydration duration time (min)	Encapsulation- vitrification			Vitrification		
			Moisture contents (%)	Viability assessment (Absorbance 530 nm)		Moisture contents (%)	Viability assessment (Absorbance 530 nm)	
				Non-LN	LN		Non-LN	LN
1	0	0	75.16 ± 1.52^{a}	0.120 ± 0.009^{i}	0.083 ± 0.009^{ijk}	65.86 ± 1.71^{ab}	0.132 ± 0.007^{ijk}	0.076 ± 0.005^{ghi}
2	0	20	74.73 ± 1.09^{a}	0.129 ± 0.020^{hi}	0.084 ± 0.009^{ijk}	60.68 ± 0.81^{abc}	0.097 ± 0.004^{k}	0.070 ± 0.003^{ghi}
3	0	40	75.19 ± 2.07^{a}	0.120 ± 0.004^{i}	0.078 ± 0.008^{k}	59.23 ± 0.77^{bc}	0.091 ± 0.002^{k}	0.063 ± 0.007^{hi}
4	0	60	74.80 ± 2.81^{a}	0.115 ± 0.006^{i}	0.079 ± 0.009^{k}	58.34 ± 0.63^{c}	0.089 ± 0.006^{k}	0.073 ± 0.005^{ghi}
5	0	80	73.71 ± 0.81^{a}	0.123 ± 0.006^{hi}	0.076 ± 0.008^{k}	55.41 ± 1.39^{cd}	0.092 ± 0.002^{k}	0.069 ± 0.001^{ghi}
6	0	100	62.02 ± 5.07^{b}	0.113 ± 0.004^{i}	0.104 ± 0.004^{fghijk}	50.68 ± 1.10^{de}	0.092 ± 0.006^{k}	0.060 ± 0.001^{i}
7	0.25	0	75.55 ± 1.03^{a}	0.115 ± 0.005^{i}	0.080 ± 0.007^{k}	67.18 ± 3.96^{a}	0.090 ± 0.002^{k}	0.061 ± 0.002^{i}
8	0.25	20	74.13 ± 2.19^{a}	0.163 ± 0.025^{ghi}	0.086 ± 0.005^{ijk}	57.45 ± 3.97^{cd}	0.119 ± 0.024^{jk}	0.065 ± 0.006^{ghi}
9	0.25	40	79.17 ± 4.47^{a}	0.527 ± 0.075^{a}	0.092 ± 0.008^{fghijk}	57.07 ± 0.60^{cd}	0.187 ± 0.053^{fghij}	0.071 ± 0.006^{ghi}
10	0.25	60	32.09 ± 1.37^{efg}	0.297 ± 0.027^{cdefg}	0.153 ± 0.021^{cd}	46.48 ± 1.81^{ef}	0.205 ± 0.054^{defghi}	0.085 ± 0.004^{efghi}
11	0.25	80	36.18 ± 0.91^{de}	0.553 ± 0.149^{a}	0.168 ± 0.012^{cd}	43.51 ± 1.47^{fg}	0.128 ± 0.012^{jk}	0.093 ± 0.010^{efg}
12	0.25	100	30.79 ± 1.62^{efg}	0.404 ± 0.055^{abcd}	0.121 ± 0.009^{efg}	35.39 ± 6.61^{h}	0.137 ± 0.024^{ijk}	0.113 ± 0.002^{de}
13	0.50	0	65.34 ± 1.30^{b}	0.174 ± 0.003^{fghi}	0.111 ± 0.005^{efghij}	60.12 ± 3.32^{bc}	0.163 ± 0.001^{ghijk}	0.123 ± 0.005^{cd}
14	0.50	20	38.60 ± 1.94^{de}	0.192 ± 0.016^{fghi}	0.122 ± 0.015^{ef}	40.56 ± 1.70^{fgh}	0.181 ± 0.012^{fghij}	0.106 ± 0.005^{def}
15	0.50	40	34.26 ± 4.18^{ef}	0.346 ± 0.011^{bcdef}	0.115 ± 0.002^{efghi}	38.67 ± 0.76^{gh}	0.355 ± 0.017^{a}	0.113 ± 0.011^{de}
16	0.50	60	24.59 ± 1.16^{g}	0.471 ± 0.076^{abc}	0.176 ± 0.010^{c}	23.93 ± 2.05^{i}	0.312 ± 0.005^{ab}	0.216 ± 0.009^{a}
17	0.50	80	25.94 ± 1.72^{g}	0.492 ± 0.171^{ab}	0.207 ± 0.012^{b}	24.13 ± 0.91^{hi}	0.265 ± 0.017^{bcde}	0.206 ± 0.017^{a}
18	0.50	100	27.34 ± 0.96^{fg}	0.460 ± 0.054^{abc}	0.237 ± 0.011^{a}	24.26 ± 0.77^{i}	0.244 ± 0.008^{bcdef}	0.204 ± 0.026^{a}
19	0.75	0	65.05 ± 1.90^{b}	0.197 ± 0.015^{fghi}	0.088 ± 0.002^{hijk}	54.80 ± 1.45^{cd}	0.120 ± 0.006^{jk}	0.095 ± 0.001^{defg}
20	0.75	20	58.28 ± 3.70^{bc}	0.329 ± 0.051^{bcdefg}	0.087 ± 0.001^{ijk}	33.24 ± 5.25^{h}	0.253 ± 0.022^{bcdef}	0.095 ± 0.002^{defg}
21	0.75	40	43.66 ± 1.81^{c}	0.385 ± 0.018^{bcde}	0.140 ± 0.007^{de}	22.92 ± 1.06^{i}	0.269 ± 0.013^{bcd}	0.146 ± 0.019^{g}
22	0.75	60	35.78 ± 0.73^{de}	0.349 ± 0.124^{bcdef}	0.165 ± 0.019^{cd}	24.46 ± 0.27^{i}	0.193 ± 0.018^{efghi}	0.147 ± 0.007^{g}
23	0.75	80	36.01 ± 2.46^{de}	0.395 ± 0.031^{abcde}	0.119 ± 0.005^{efgh}	23.72 ± 1.31^{i}	0.288 ± 0.017^{abc}	0.178 ± 0.014^{b}
24	0.75	100	26.95 ± 1.98^{fg}	0.339 ± 0.012^{bcdef}	0.090 ± 0.004^{hijk}	24.38 ± 2.21^{i}	0.304 ± 0.048^{ab}	0.092 ± 0.003^{efgh}
25	1.00	0	58.55 ± 2.00^{b}	0.189 ± 0.013^{fghi}	0.092 ± 0.007^{fghijk}	46.25 ± 1.25^{ef}	0.215 ± 0.048^{defgh}	0.079 ± 0.003^{ghi}
26	1.00	20	53.94 ± 3.92^{c}	0.282 ± 0.021^{defghi}	0.088 ± 0.001^{hijk}	37.93 ± 1.97^{gh}	0.120 ± 0.002^{jk}	0.083 ± 0.002^{fghi}
27	1.00	40	38.81 ± 4.85^{de}	0.279 ± 0.043^{defghi}	0.086 ± 0.001^{ijk}	34.51 ± 1.54^{h}	0.133 ± 0.001^{ijk}	0.084 ± 0.002^{efghi}
28	1.00	60	36.46 ± 2.95^{de}	0.229 ± 0.039^{efghi}	0.080 ± 0.001^{k}	24.76 ± 0.53^{i}	0.224 ± 0.017^{cdefg}	0.085 ± 0.003^{efghi}
29	1.00	80	25.40 ± 1.47^{g}	0.223 ± 0.010^{efghi}	0.090 ± 0.008^{ghijk}	25.67 ± 1.48^{i}	0.144 ± 0.005^{hijk}	0.088 ± 0.001^{efghi}
30	1.00	100	24.97 ± 0.96^{g}	0.307 ± 0.021^{cdefg}	0.094 ± 0.002^{fghijk}	23.66 ± 1.15^{i}	0.121 ± 0.012^{jk}	0.092 ± 0.001^{efgh}

A viability assessment of precultured PLBs was illustrated in the a4bsorbance value expressed by UV-spectrophotometer at 530 nm. The different letters in a column are significantly different at $P \leq 0.05$ with Duncan's Multiple Range test.

Table 3.8 The mean comparison of the highest absorbance values of non-cryopreserved and cryopreserved calli via encapsulation-vitrification and vitrification methods.

P. niveum		Absorbance 530_{nm} mean ± SE	df	t
Vitrification	Non- cryopreserved	0.312 ± 0.051	2	7.039^{ns}
	Cryopreserved	0.216 ± 0.095		
Encapsulation-vitrification	Non- cryopreserved	0.460 ± 0.054	2	3.388^{ns}
	Cryopreserved	0.237 ± 0.011		

Comparison of the mean value was analyses using the t-test at $P \leq 0.01$. ns = not significant difference.

3.4.1.3 Histological and histochemical observations

One-week old (under darkness) cryopreserved and non-cryopreserved calli from encapsulation-vitrification and vitrification techniques were used as plant material for histological and histochemical observations. Histological studies reveal that non-cryopreserved calli had exhibited darkly stained nuclei and complete tissue layers.While, cryopreserved calli showed the breakage of tissue layers. However, the nuclei within the cryopreserved cell was still darkly stained. Histochemical examination showed a reduction in the content of starch grains and protein on the cryopreserved calli (Figure. 3.10-3.11).

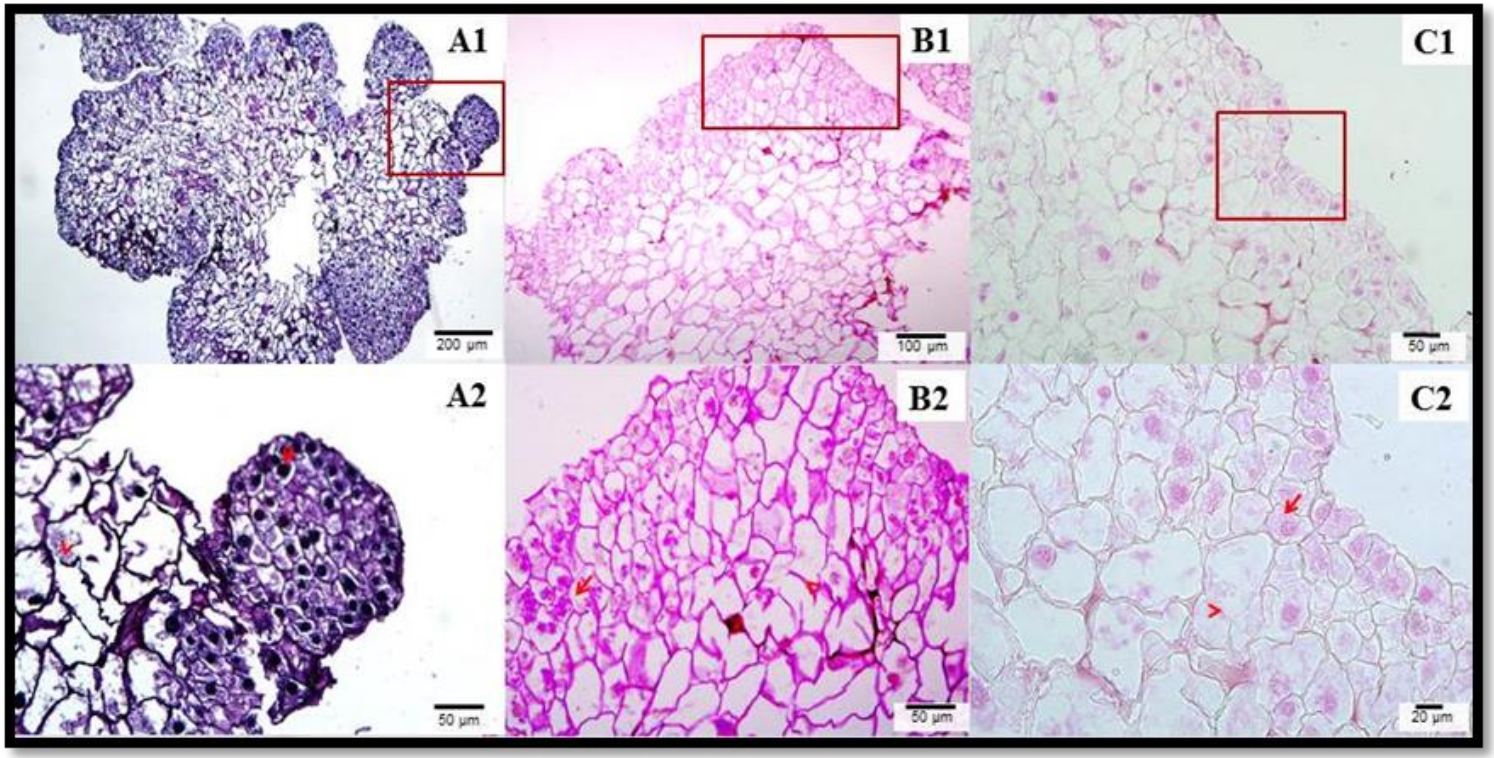

Figure 3.10 Histological characteristics of cryopreserved callus through vitrification technique.

(A) Meristematic cells exhibiting large nuclei (arrow) and dense cytoplasm (H&S staining). (B) Tissue containing less carbohydrate (arrow, PAS reaction) and (C) protein (arrow, Ninhydrin staining). A2, B2 and C2 are magnified images of A1, B1 and C1, respectively.

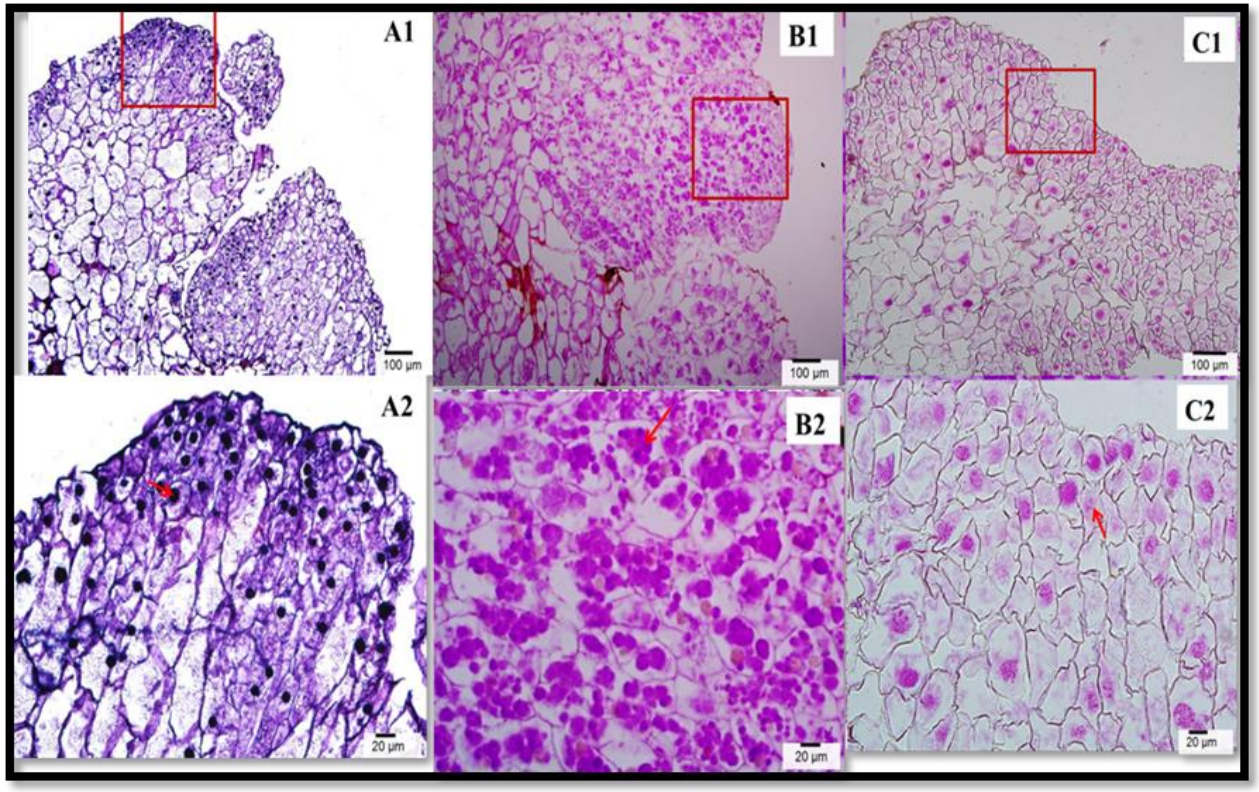

Figure 3.11 Histological characteristics of encapsulated callus after cryopreservation using encapsulation-vitrification technique. (A) Meristematic cells showing large nuclei and dense cytoplasm. The greater accumulations of (B) carbohydrate (PAS reaction) and (C) protein (Ninhydrin). A2, B2 and C2 are magnified image of A1, B1 and C1, respectively.

3.4.1.4 DNA contents of non-cryopreserved and cryopreserved callus.

Flow cytometric analysis was applied to study the DNA contents of non-cryopreserved and cryopreserved callus of *P. niveum* using *Vicia faba* CV. (2c = 26.90 pg.) as the internal standard plant. The 2C DNA content from non-cryopreserved and cryopreserved based on encapsulation-vitrification (precultured in 0.5 M sucrose and dehydrated in PVS2 for 100 min) and vitrification (precultured in 0.5 M sucrose and dehydrated in PVS2 for 60 min) were 39.39 ± 0.07, 38.56 ± 0.25 and 38.23 ± 0.56 (Table 3.9) respectively. There were no a significant difference in 2C DNA content value among these samples.

Table 3.9 The result of DNA content of *P. niveum* as revealed by flow cytometer.

P. niveum explant	2C DNA content (pg.) (mean ± SE)	Mbp
Non-cryopreserved callus	39.39±0.07 [a]	19005.675
Cryopreserved callus via vitrification method	38.56±0.25 [a]	18605.200
Cryopreserved callus via encapsulation-vitrification method	38.23±0.56 [a]	18445.975

The same letters in a column are not significantly different at $P \leq 0.05$ with Duncan's Multiple Range tests analysis.

3.4.1.5 Survival rate of cryopreserved and non-cryopreserved calli using encapsulation-vitrification and vitrification

In the case of encapsulation-vitrification, the survival rate (29.63 ± 10.31 %) was obtained from calli precultured in 0.5 M sucrose followed by dehydration in PVS 2 for 100 min (Figure 3.12). The morphotype of those cryopreserved calli exhibited yellow green (Figure 3.13). Whereas the vitrification-based cryopreserved calli presented calli presented the colorless (Figure 3.12) with lower survival presentege (22.22 ± 7.85 %) (Figure 3.13).

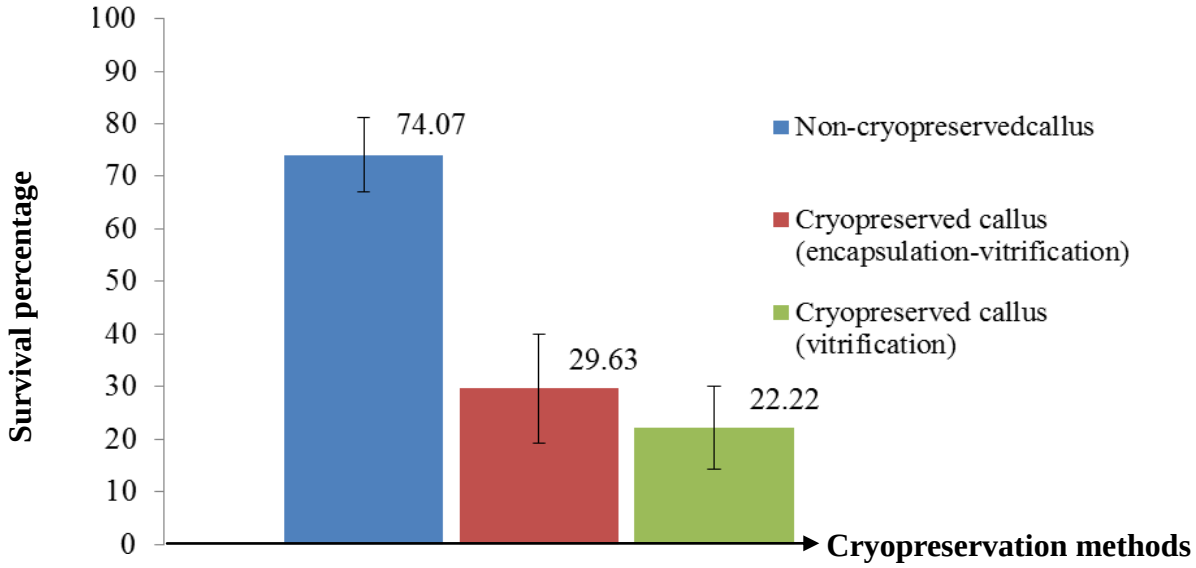

Figure 3.12 The survival rate of non-cryopreserved and cryopreserved calli of *P. niveum*. Bar represents the standard error (SE).

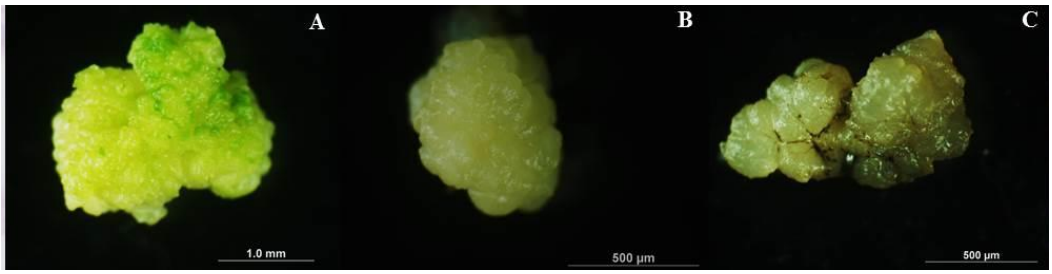

Figure 3.13 Morphological features of (A) initial non-cryopreserved callus. (B) Cryopreserved callus via encapsulation-vitrification and (C) vitrification. Photographs taken after 4 weeks of culture on recovery medium.

CHAPTER 4
DISCUSSION

4.1 In vitro propagation of *P. niveum*

4.1.1 PLB proliferation and plant regeneration of *P. niveum*

The proliferated PLBs of *P. niveum* which were cultured on modified VW supplemented with 10 % CW and 0.2 % Phytagel (defined as solidified culture state presented the highest increase in fresh weight. Similarly to PLBs of *Cymbidium pendulum* (Kaur et al., 2012) and *Dendrobium* Alya Pink PLBs (Nambiar et al., 2012), they reported that CW was the most suitable carbohydrate source for proliferation. In addition, CW influenced on PLB proliferation in many orchid species. Gnasekaran et al. (2012) reported that the PLB proliferation of *Vanda* Kasem's Delight exhibited the highest proliferation rate when they were cultured on medium supplemented with 15 % CW. CW contains many types of biochemical compounds, such as sodium, potassium, iron, calcium, copper, magnesium, phosphorous, ascorbic acid and sulphur. CW also consists of diphenyl urea which functions like a cytokinin hormone that can enrich the growth of PLBs by encouraging cell division (Teixera da Silva et al., 2006; Gnasekaran et al., 2010).

However, medium types also had an influence role on PLB proliferation. This study found that solid state was the best suitable type of medium for the proliferation of *P. niveum* PLBs, similar to the *Dendrobium* Orchid (Aktar et al., 2008), *Vanda* Kasem's Delight (Gnasekaran et al., 2012) and *Cymbidium pendulum* (Kaur et al., 2012). Park et al. (2002) presented that PLBs of *Phalaenopsis* which were cultured on the solidified medium gave higher proliferation rate than the liquidified medium. Julkiflee et al (2014) reported PLBs of *Dendrobium* Sonia-28 gave higher percentage of growth on the semi-solid medium rather than liquidified medium. Mbiyu et al. (2012) reported the advantages of the solid medium are support the plant material into an upright position, plant can absorb enough aeration and high biomass accumulation.

4.1.2 Induction and proliferation of PLB-derived callus of *P. niveum*

The highest increase fresh weight of callus induction was obtained from the callus cultured on modified solidified VW medium supplemented with 0.5 mg L^{-1} 2, 4-D and combined with 0.1 mg L^{-1} TDZ. The effects of 2, 4-D on the callus induction have been presented in many plant species, such as, pakistani wheat (*Triticum aestivum*) (Ali et al., 2009), mature chickpea (*Cicer arietinum* L.) (Zaman et al., 2010), sugarcane (*Saccharum officinarum*) (Tahir et al., 2011), spring wheat (*Triticum aestivum* L.) (Zheng et al., 2011) and potato (*Solanum tuberosum* L.) (Sherkar and Chavan, 2014). The present study also found that 0.5 mg L^{-1} 2, 4-D was the optimal concentration for callus induction, similar to Zaman et al. (2010) who reported that the best concentration of 2, 4-D on the callus induction of mature chickpea (*Cicer arietinum)* was the 0.5 mg L^{-1}. In contrast, using 0.5 mg L^{-1} 2, 4-D in callus induction of spring wheat (*Triticum aestivum* L.) exhibited low callus induction yield (Zheng et al., 2001). However, Sherkar et al. (2014) presented that callus induction was dependent on the type of explant, the composition of media and plant growth conditions. Raina. (1989) reported that 2, 4-D was the most suitable auxin for callus induction of rice in the plant tissue culture, however the optimal concentration of 2, 4-D varies depending on the explant sources and genotype. However, this present study revealed that the suitable medium for callus induction consisted 2, 4-D in combination with TDZ. Similar to Li et al. (2006) who reported that the combination of auxin and cytokinin has been widely used for callus induction. However, the callus induction medium which was supplemented with 2, 4-D alone gave the best results in the suitable concentration of plant growth regulator of Bambara groundnut (*Vigna subterranean* L) (Konate et al., 2013).

Moreover, TDZ is one of the PGR used in callus induction of many species for example, *Astragalus nezaketae* (Erisen et al., 2010), *Astragalus cariensis* (Erisen et al., 2011), *Falcaria vulgaris* (Hamideh et al., 2012) and *Musa* sp. (Srangsam and Kanchanapoom, 2003). Mok et al. (1982) also reported that TDZ has a high cytokinin activity and can promote growth of cytokinin-dependent callus cultures. However, Hong et al. (2008) reported that TDZ inhibited shoot proliferation and rooting of *Paphiopedilum* orchid. Many reports also revealed that TDZ had a positive effect on shoot regeneration in *Musa* sp. (Srangsam and Kanchanapoom.,

2003) and *Astragalus cariensis* (Erisen et al., 2011). The combination of 2, 4-D and TDZ in culture medium was found in callus induction of *Swertia chirayita* (Kumar and Chandra., 2013), *Cordeauxia edulis* (Seyoum and Mekbib., 2014) and *Solanum tuberosum* (Abdelaleem, 2015). However, the callus induction was also depended on the ratio of auxin and cytokinin (Su et al., 2011). In this study established that the amount of auxin and cytokinin on callus induction was 0.5 mg L^{-1} 2, 4-D and 0.1 mg L^{-1} TDZ. In contrast, callus induction of *Falcana Vulgaris* was 0.5 mg L^{-1} 2, 4-D and 1.0 mg L^{-1} TDZ (Hamideh et al., 2012).

Callus could be proliferated using the different concentrations of organic additive such as CW. The highest increase in fresh weight of callus was obtained from callus which was maintained on modified solidified VW medium supplemented with 10 % CW in combination with 15 g L^{-1} sucrose. Rittirat et al. (2011) reported that callus of Chang Daeng (*Rhynchostylis gigantea* var. Sagarik) was proliferated on medium containing CW and sucrose. Ishii et al. (1998) who reported callus induction of *Phalaenopsis* on medium containing CW and sucrose. Both sucrose and CW play important roles on callus proliferation, because CW contains complex organic compounds such as sugars, vitamins, minerals and amino acid (Young et al., 2009) while sucrose is one source of carbon for use in plant tissue culture and is widely used for additive organic material in many plant species (Konate et al., 2013).

4.2 Cryopreservation of PLBs of *P. niveum* by vitrification technique

The lowest absorbance value could be obtained when PLBs were precultured in modified VW medium without sucrose. Sucrose is a sugar compound which is widely used for preculturing in cryopreservation technique due to its ability to protect the cellular membrane and cytoplasm by transition into a vitrified state (Khoddamzadeh et al., 2011). Khoddamzadeh et al. (2011) also reported that non-precultured PLBs of *Phalaenopsis bellina* were not successfully cryopreserved by encapsulation-dehydration technique and provided low viability as examined by TTC test with spectrophotometer. Similar results were gained in the cryopreserved PLBs of *Dendrobium candidum* via encapsulation-vitrification (Yin and Hong., 2009). They exhibited the features of non-survive and later dead of PLBs when they were no

preculturing with sucrose (Yin and Hong., 2009). This present study confirms that preculturing with sucrose required for cryopreservation. Therefore, the optimal conditions in this step played an important role in successful cryostorage, especially preventing injury from freezing and avoiding the formation of ice crystals. This step has provided the opportunity for enhancing the stress tolerance from both dehydration and freezing during cryostorage (Hong et al., 2009; Khoddamzadeh et al., 2011; Melo et al., 2011).

The high absorbance values could be observed when PLBs were precultured in 0.75 M sucrose for 24 h and 5 d with daily increasing concentration of sucrose. Both preculture protocols presented the highest viability with no statistically significant difference which was based on comparison with preculturing without sucrose. However, the percentages of MC from both protocols were significantly different at $P \leq 0.05$. The present result was similar to those of *Dendrobium candidum* and *Phalaenopsis bellina* PLBs which were precultured in 0.75 M sucrose for 5 d with daily increasing concentration of sucrose (Yin and Hong., 2009) and 3 d (Khoddamzadeh et al., 2011), respectively. It was possible that, preculture in 0.75 M was the suitable concentration of sucrose for preculturing in the cryopreservation process. Khoddamzadeh et al. (2011) also reported sucrose might be the optimal conditions required for many explant types prior to cryostorage in LN.

This present result also showed the high absorbance values inversely correlated with the low MC. Thus, the preculture PLBs in 0.75 M sucrose with the stepwise increasing concentrations of sucrose gave the highest absorbance value (0.98 ± 0.14) and the lowest percentage of MC (25.23 ± 1.21 %). Many reports revealed that a progressive increase in concentration of sucrose was used to reduce the toxic effect of high sucrose concentration in *Arachis pintoi* (Rey et al., 2009) and to produce the better protection of *Nandina domestica* shoot tip (Ozudogru et al., 2013) during cryostorage. The low percentage of MC indicated that the precultured explants having low water contents could avoid the tissue injury from formation of ice crystals in the cell leading to cell death (Melo et al., 2011). Hajari et al. (2011) reported that the MC percentage was the most critical factor for a successful cryopreservation protocol of zygotic embryonic axes of *Ekebergia capensis*. The present study was similar to that of Jitsopakul et al. (2008) who reported that the highest regrowth rate of *Vanda*

coerulea was obtained when cryopreserved seeds presented the lowest percentage of 35 % water. Moreover, successfully cryopreserved of the somatic embryos of horse-chestnut (*Aesculus hippocastanum* L.) was obtained at a lower MC percentage (Jekkel et al., 1998).

The precultured PLBs in 0.75 M sucrose from both protocols (for 24 h and 5 d increasing sucrose concentration) without PVS2 treatment provided the lowest absorbance value due to the cryo-injuring during storage in LN. This present result was similar to Antony et al. (2011) who reported that the non PVS2-treated PLBs of *Dendrobium* Bobby exhibited the low viability after cryostorage in LN. Hong et al. (2009) also reported that PVS2 was an essential factor to prevent the formation of ice crystal during cryostorage. The cryopreservation techniques with dehydration in PVS2 exhibited higher survival rate of cryopreserved materials than those of non-PVS2 treated explants. Moreover, the exposure duration time for PVS2 is one of the factors that could be lethal to cells and varies considerably among species (Panis et al., 2011). Many reports revealed that the suitable time to PVS2 treatment in embryonic cells of *Vitis spp* (Wang et al., 2004), shoot tips of *Thymus cariensis* and *Thymus vulgaris* (Ozudogru and Kaya, 2012) and *Dendrobium* Sonia-28 (Poobathy et al., 2013).

However, PVS2 solution containing DMSO and glycerol, which play an important role in the cell osmolality before freezing process, may cause cell rupture after injury (Meryman and Williams, 2008; Sakai et al., 2008; Kim et al., 2009). The toxicity of PVS2 also caused some delay in growth recovery of embryonic cultures of *Persea americana* Mill (Guzman-Garcia et al., 2013) and oil palm (*Elaeis guineensis*) (Suranthran et al., 2012) after storage in LN.

Both non-cryopreserved PLBs and cryopreserved PLBs (0.75 M sucrose using stepwise increasing sucrose concentrations followed by PVS2 for 90 min) gave the same results including dark stained nuclei and accumulated starch grains. However, a higher number of these nuclei and starch grains could be observed in non-cryopreserved than another one. It was possible that cells of cryopreserved PLBs with a few dark stained nuclei had still some meristematic cells that might be able to divide and survive after cryostorage. Poobathy et al. (2012) revealed that cells with dark stained nuclei had still the hereditary materials and metabolism exhibiting the

efficiency to be necessary for regrowth. Similarly, darkly staining nuclei could be found in cells of cryopreserved PLBs of *Dendrobium* Sonia-28 (Julkifle et al., 2012; Poobathy et al., 2013) and *Ascocenda Wangsa* Gold (Rajasegar et al., 2015), as well as shoot of *Malus domestica* Borks (Kushnarenko et al., 2010). In addition, the reduction of starch grains in tissue of the cryopreserved PLBs presented here might be affected by dehydration process (Poobathy et al., 2013). According to Melo et al (2011), the accumulating starch grains could help the cell recovery from stress and reactivate metabolism. So, few reserved starch grains in cryopreserved PLBs presented here were still enough for the cellular reawakening. SEM observation revealed that there was no damage on the epidermal layer of both non-cryopreserved and cryopreserved PLBs except for the appearance of cell shrinking on cryopreserved PLBs. This result was consistent with no aberration on exterior layer of cryopreserved PLBs of *Dendrobium* Sonia-28 (Poobathy et al., 2013). However, the internal damage resulting from osmotic and freezing injuries during cryopreservation process might occur (Poobathy et al., 2013).

Flow cytometry analysis exhibited no change in ploidy levels between cryopreserved PLBs and control. This result was consistent with the stability in ploidy level when comparing cryopreserved materials and controls which were reported in *Quercus suber* somatic embryos (Fernandes et al., 2008) *Vanda coerulea* protocorms (Jitsopakul et al., 2008) and *Grammatophyllum speciosum* PLBs (Sopalun et al., 2010). In addition, cryopreserved PLBs were morphologically similar to healthy non-cryopreserved PLBs which all indicated the success of cryopreservation protocol for *P. niveum* PLBs.

4.3 Cryopreservation of callus of *P. niveum* by vitrification and encapsulation-vitrification techniques

The highest absorbance values of TTC reaction (530 nm) using vitrification technique were obtained from calli precultured in 0.50 M sucrose with daily increasing sucrose concentrations followed by dehydration in PVS2 for 60 min. Meanwhile, encapsulation-vitrification gave the highest viability absorbance value in calli precultured in 0.50 M sucrose with the stepwise increasing sucrose concentrations followed by dehydration in PVS2 at 100 min. It was found that 0.50 M sucrose was the

optimal concentration for preculture treatment for both encapsulation-vitrification and vitrification. Similar to Melo et al (2011) who reported the cryopreserved shoots of sugar cane (*Saccharum* spp.) which preculture in 0.50 M sucrose gave the highest survival rate. In addition, the maximum regrowth of shoot tips of sour orange (*Citrus aurantium* L.) was obtained from the treatment that was precultured in 0.50 M sucrose (Samia et al., 2002). The cryopreserved PLBs of *Dendrobium* Sonia-28 were also precultured in 0.50 M sucrose for the optimal preculture concentration (Ching et al 2012). The best results were also obtained when PLBs of *Vanda* Kaseem's Delight were precultured in 0.1 M sucrose (Poobathy et al., 2012) and PLBs of *Phalaenopsis bellina* were precultured in 0.75 M sucrose. (Khoddamzadeh et al., 2011).

Clearly, preculture treatment plays an important role on the viability of cryopreserved materials, not only for callus but also for various explants of many plant species. This treatment affected on the viability, regrowth or the regeneration of plantlets. Moreover, the preculture process is an essential step in the experiment of cryopreservation, because the preculture process prevents the occurrence of ice crystal during storage under low temperatures (Jisopakul., 2008). In addition, sucrose is one of the sugars that have been used in the preculture process and it acts to protect cells from dehydration injury from the loss of water and to tolerate the desiccation and the freezing (Sakai et al., 2000; Uragami et al., 1990). Many plant species have been reported to use sucrose solution in the preculture process, including *Vanda coerulea* (Jisopakul et al., 2008), *Dendrobium candidum* (Yin and Hong, 2009), *Dendrobium* Bobby Messins (Antony et al., 2011) and *Vanda* Kaseem's Delight (Poobathy et al., 2012).

Moreover, the preculture treatment with stepwise sucrose concentrations also plays an important role in this cryopreservation, similar to Samia et al. (2002) who reported that survival and regrowth rate of the shoot tips of sour orange (*Citrus aurantium* L.) increased with preculturing in increasing sucrose concentrations.

The cryopreserved PLBs of *Dendrobium candidum* and *Phalaenopsis bellina* exhibited the highest viability when they were precultured in stepwise sucrose concentration (Yin and Hong, 2009; Khoddamzadeh et al., 2011). A progressively increase in sucrose concentration to reduce the toxic effect of high sucrose

concentration in the preculture process was illustrated in *Arachis pintoi* (Rey et al., 2009).

The low percentage of MC was correlated with the high viability absorbance value for supporting the viable cryopreserved materials. The cryopreservation of *Vanda coerolea* gave the highest regrowth rate when the moisture content was low (35 %) (Jitsopakul et al., 2008). The suitable rage of moisture content required for cryopreserved explants was approximately 15-25 % (Uragami et al., 1990; Jekkel et al., 1998; Sherlock et al., 2005).

In this study it was found that the encapsulation-vitrification technique exhibited a higher viability absorbance value than that of the vitrification technique. This result coincided with the cryopreserved protocorm using encapsulation-vitrification of *Cymbidium eburneum* L which the provided a higher regeneration than vitrification (Gogoi et al., 2012). It was possible that the encapsulation-vitrification technique had achieved better results due to the presence of an alginate capsule which was able to protect the explant from direct contact with PVS2 and LN (Gogoi et al., 2012). Besides, the encapsulation-vitrification callus was encapsulated in a nutritive bead surrounding the explant that could promote the regrowth after thawing while the vitrification-based cryopreserved materials are osmotically dehydrated by PVS2 at a nonfreezing temperature, where the main problem is the toxicity of the concentrated vitrification solution. (Sakai et al., 1990; Kaviani, 2011).

Histological and histochemical studies presented the dark stained nuclei and the accumulation of starch grains and protein of cryopreserved calli. The vitrification-based cryopreserved callus exhibited some damage area in the tissue layer. Many researchers reported that the large stained nuclei can be found in the cells during cryopreservation. The cryopreserved PLBs of *Vanda* Kaseem's Delight had still dark stained nuclei and had the hereditary materials and the metabolism exhibiting the efficiency necessary for their regrowth. In addition, many plant species still illustrated darkly-stained nuclei in the cryopreservation, such as cryopreserved *Malus domestica* Borks (Kushnarenko et al., 2010), *Dendrobium* Sonia-28 (Julkifle et al., 2012) and *Ascocenda Wangsa* Gold (Rajasegar et al., 2015).

The cryopreserved callus presented here exhibited some damage area similarly to Poobathy et al. (2013) who reported that the injury of the cryopreserved *Dendrobium* Sonia-28 occurred in the cryostorage procedure of preculture, dehydration and freezing. Ding et al. (2008) reported that cryopreserved *Candidatus Liberobacter asiaticus* exhibited damage and dead cells in the meristematic tissues after recovering from thawing. The histochemical study showed more content of starch grains and protein in callus tissue of the cryopreserved callus in the encapsulation-vitrification than the vitrification technique. The low amount of this substance might be affected by the toxicity of PVS2 (Poobathy et al., 2013).

From both techniques of cryostorage, flow cytometry analysis showed no change in ploidy level between the non-cryopreserved and the cryopreserved callus. Similar to Fernandes et al. (2008) who reported that the cryopreserved somatic embryo of *Quercus suber*. They showed the consistent in the stability in ploidy levels between non-cryopreserved and cryopreserved. This study was similar to that of *Vanda coerulea* protocorms (Jitsopakul et al., 2008) and *Grammatophyllum speciosum* PLBs (Sopalun et al., 2010) who reported that there was no variation in ploidy level after cryostorage.

CHAPTER 5
CONCLUSIONS

PLBs on modified VW medium supplemented with 10 g L^{-1} sucrose, 0.2 % Phytagel, 2 g L^{-1} AC and 10 % CW while, PLBs-drived callus were obtained on modified VW medium supplemented with 0.5 mg L^{-1} 2, 4-D in combination with 0.1 mg L^{-1} TDZ. Callus could be proliferated on modified VW medium supplemented with 10% CW and 15 g L^{-1} sucrose.

The success of cryopreservation of *P. niveum* was presented. PLBs which were precultured in 0.75 M sucrose for 5 d with daily increasing sucrose concentration followed by dehydration in PVS2 for 90 min. The vitrification technique was employed. Callus could be used for cryopreserved via both encapsulation-vitrification and vitrification techniques. In case of encapsulation-vitrification, was precultured in 0.5 M sucrose for 5 d with stepwise concentration of sucrose followed by dehydration in PVS2 for 100 min. For vitrification, callus was precultured in 0.5 M sucrose for 5 d with gradually increased sucrose concentrations followed exposure to PVS2 for 60 min.

The histological and histochemical observations of both cryopreserved PLBs and callus illustrated darkly stained nuclei, starch grain and protein accumulations. However, those substances more exhibited in the non-cryopreserved than cryopreserved plant materials.

Both cryopreserved PLBs and callus could be maintained on regrowth medium. They exhibited healthy plants with no genetic variation in the ploidy level detected by flow cytometry.

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APPENDICES

APPENDIX 1

Media composition

Table 1 The compositions of VW medium (Vacin and Went, 1949).

Components	Chemical formula	Quantity mg L^{-1}
Macro elements		
Calcium phosphate	$Ca_3(PO_4)_2$	200
Potassium nitrate	KNO_3	525
Potassium dihydrogen phosphate	KH_2PO_4	525
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	250
Diammonium sulphate	$(NH_4)_2SO_4$	500
Micro elements		
Manganese sulphate tetrahydrate	$MnSO_4 \cdot 4H_2O$	250
Ferus elements		
Disodium salt Ethlenediaminetetra acetate	Na_2-EDTA	37
Other additions		
Sucrose	$C_{12}H_{22}O_{11}$	20,000

Table 2 The composition of Murashige and Skoog medium (Murashige and Skoog, 1962).

Component	Chemical formula	Quantity mg L^{-1}
Macro elements		
Ammonium nitrate	NH_4NO_3	1,650
Potassium nitrate	KNO_3	1,900
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	440
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	370
Potassium dihydrogen phosphate	KH_2PO_4	170
Micro elements		
Manganese sulphate tetrahydrate	$MnSO_4 \cdot 4H_2O$	6.9
Zinc sulphate heptahydrate	$ZnSO_4 \cdot 7H_2O$	6.14
Boric acid	H_3BO_3	6.2
Copper sulphate	$CuSO_4 \cdot 5H_2O$	0.025
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.025
Potassium iodide	KI	0.83
Organics additive		
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine HCl	$C_8H_{11}NO_3$	0.5
Thiamine HCl	$C_{12}H_{17}ClN_4O_5$	0.1
Glycine	$C_2H_5NO_2$	0.1
Sucrose	$C_{12}H_{22}O_{11}$	30,000
Ferus elements		
Disodium salt Ethlenediaminetetra acetate	Na_2-EDTA	37.25
Iron(II) Sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	27.85

APPENDIX 2

Staining solution

Hematoxylin staining solution		
Ammonium aluminium sulphate	16	g
Hematoxylin	8	g
Ethanol 95 %	250	mL
Grecerine	400	mL
Potassium permanganate	0.4	g
Distill water	800	mL
Safranin staining solutions		
Safranin O	2	g
Methyl cellusolve	100	mL
(Ethylene glycol monoethyl ether)		
Ethanol 95%	50	mL
Sodium acetate	2	g
Formalin	4	mL
Periodic and Schiff's reagent reaction (PAS)		
Periodic acid solution		
Periodic acid	1 %	
Schiff's reagent		
Pararosaniline HCl	1 %	
Sodium metabisulfite	4 %	
(in Hydrochloric acid 0.25 mol L^{-1})		
Ninhydrin staining solution		
Ninhydrin	200	mg
Butanol	95	mL
Acetic acid	10 %	

APPENDIX 3

Flow cytometry investigation buffer

Otto's buffer (Otto, 1992)

Otto I

Citric acid	100	mM
Tween 20	0.5 %	(pH 2-3)

Otto II

$Na_2PO_4.12H_2O$	400	mM (pH 8-9)

APPENDIX 4

Viability measurement

2, 3, 5-triphenyltetrazolium chloride (TTC) solution

TTC	1 %	
Buffer		
K_2PO_4	0.05	M
Na_2HPO_4	0.05	M

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Publication

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Proceeding

Chaireok, S., Thammasiri, K. and Meesawat, U. 2014. The 2nd International Orchid Symposium (IOS2014). Golden Tulip Sovereign Hotel, Bangkok, Thailand. February 19-21.