In Vitro Propagation and Cryopreservation of Lady's Slipper Orchid (*Paphiopedilum niveum* (Rchb.f.) Stein)

Sutthinut Soonthornkalump

A Thesis Submitted in Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology
Prince of Songkla University
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Author	Mr. Sutthinut Soonthornkalump
Major Program	Biology

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Dean of Graduate School

This is to certify that the work here submitted is the results of candidate's own investigations. Due acknowledgement has been made of any assistance received.

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Candidate

I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

…….Signature

(Mr. Sutthinut Soonthornkalump)

Candidate

ชื่อวิทยานิพนธ์ การขยายพันธุ์ในสภาพหลอดทดลองและการเก็บรักษาพันธุกรรมในสภาพต่ำกว่าจุดเยือกแข็งของรองเท้านารีขาวสตูล (*Paphiopedilum niveum* (Rchb.f.) Stein)
ผู้เขียน นายศุทธิณัฏฐ์ สุนทรกลัมพ์
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บทคัดย่อ

รองเท้านารีขาวสตูล (*Paphiopedilum niveum* (Rchb.f.) Stein) เป็นกล้วยไม้หายากที่พบได้เฉพาะพื้นที่ภาคใต้ของประเทศไทยและทางตอนเหนือของประเทศมาเลเซีย ปัจจุบันประชากรในธรรมชาติมีแนวโน้มลดลงจากการลดลงของพื้นที่ป่าและการลักลอบเก็บเพื่อการค้าแบบผิดกฎหมาย ทำให้รองเท้านารีขาวสตูลถูกจัดเป็นพืชใกล้สูญพันธุ์ตามบัญชี 1 ของอนุสัญญาว่าด้วยการค้าระหว่างประเทศ ซึ่งชนิดสัตว์ป่าและพืชป่าที่ใกล้จะสูญพันธุ์ (CITES) และเนื่องจากการขยายพันธุ์ด้วยวิธีธรรมชาติและวิธีดั้งเดิมใช้ระยะเวลายาวนานและได้ต้นพันธุ์จำนวนน้อย จึงทำให้การพัฒนาวิธีการขยายพันธุ์ในสภาพหลอดทดลองและการเก็บรักษาพันธุกรรมในสภาพต่ำกว่าจุดเยือกแข็งมีความจำเป็นต่อการอนุรักษ์พันธุกรรมของรองเท้านารีขาวสตูลนอกสภาพถิ่นอาศัยในระยะยาว การศึกษานี้ประกอบด้วย 1) การศึกษาผลของสารควบคุมการเจริญเติบโตต่อการขยายพันธุ์รองเท้านารีขาวสตูลด้วยการชักนำและการเพิ่มจำนวนโพรโทคอร์มไลค์บอดี้หรือโซมาติกเอ็มบริโอจากโพรโทคอร์ม และ 2) การเก็บรักษาพันธุกรรมในสภาพต่ำกว่าจุดเยือกแข็งด้วยวิธี V cryo-plate จากการศึกษาการขยายพันธุ์รองเท้านารีขาวสตูลพบว่าโพรโทคอร์มอายุ 4 เดือนที่ได้จากการเพาะเมล็ดสามารถชักนำให้เกิดโซมาติกเอ็มบริโอได้โดยตรง เมื่อเพาะเลี้ยงบนอาหารสูตร Modified Vacin and Went (MVW) ที่มี 1-Naphthaleneacetic acid (NAA) ความเข้มข้น 0.1 มก./ล. ในสภาวะไม่มีแสงเป็นเวลา 3 เดือน และโซมาติกเอ็มบริโอรุ่นที่ 1 สามารถเพิ่มจำนวนเป็นโซมาติกเอ็มบริโอรุ่นที่ 2 เมื่อเพาะเลี้ยงบนอาหารสูตร MVW ที่ไม่มีสารควบคุมการเจริญเติบโตของพืชเป็นระยะเวลา 2 เดือน สภาวะมีแสง และสามารถชักนำโซมาติกเอ็มบริโอให้เป็นต้นอ่อนเมื่อเพาะเลี้ยงบนอาหารสูตรชักนำต้นอ่อนเป็นระยะเวลา 4 เดือน และต้นอ่อนที่เกิดจากโซมาติกเอ็มบริโอรุ่นที่ 2 (V_2) และ 3 (V_3) มีรูปแบบพันธุกรรมไม่แตกต่างจากต้นอ่อนที่เกิดจากโพรโทคอร์มเริ่มต้น (V_1) สำหรับวิธีการที่เหมาะสมในการเก็บรักษาโซมาติกเอ็มบริโอในสภาพต่ำกว่าจุดเยือกแข็งด้วยวิธี V cryo-plate พบว่าโซมาติกเอ็มบริโอ (ขนาดเส้นผ่านศูนย์กลาง 1-1.5 มม) สามารถมีชีวิตรอดได้ (20%) ภายหลังการเก็บรักษาในไนโตรเจนเหลว เมื่อผ่านการ Precondition โดยเพาะเลี้ยงบนอาหารสูตร MVW ที่มีน้ำตาลซูโครส 0.1 M เป็นเวลา 7 วัน ตามด้วยการ Preculture บนอาหารสูตร MVW ที่มีน้ำตาลซูโครส 0.2 M และ 0.6 M เป็นเวลา 1 วัน ตามลำดับ แล้วนำโซมาติกเอ็มบริโอวางติดลงบนแผ่น cryo-plate ด้วย alginate gel แล้วแช่ในสารละลาย loading solution (LS) ที่มีกลีเซอรอลเข้มข้น 2 M และ น้ำตาลซูโครสความเข้มข้น 1.2 M เป็นเวลา 30 นาที จากนั้นย้าย cryo-plate ที่มีโซมาติกเอ็มบริโอติดอยู่ลง

แช่ในสารละลาย Plant Vitrification Solution 2 (PVS2) เป็นเวลา 60 นาที และการเติมกรดแอสคอร์บิก (Ascorbic acid) ความเข้มข้น 0.1 mM ในอาหารเพาะเลี้ยงในวันที่ 7 ของการ Precondition สามารถลดปริมาณอนุมูลอิสระรวม (Total Reactive Oxygen Species, ROS) และสาร Malondialdehyde (MDA) ในโซมาติกเอ็มบริโอและช่วยเพิ่มอัตราการรอดชีวิตเป็น 39% อย่างมีนัยสำคัญทางสถิติ

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ABSTRACT

The snow white lady slipper orchid (*Paphiopedilum niveum* (Rchb.f.) Stein) is an endangered species that distributed in Southern Thailand and Northern Malaysia. The habitat destruction and over-collecting decreased the natural population. Even though *P. niveum* was protected by Appendix I of the CITES but the conventional propagation method provided low productivity and also take a long period of time which could not meet the commercial demand. Thus, the conservation of genetic resources of *P. niveum* has required the micropropagation and cryopreservation for long-term storage. This study was composed of 1) the study of the effect of plant growth regulators (PGRs) on the direct somatic embryogenesis and SEs proliferation from protocorm and 2) cryopreservation via V cryo-plate method with the application of ascorbic acid (AA). The results showed that SEs could be generated from four-month-old protocorm when culturing on modified Vacin and Went (MVW) containing 0.1 mgl^{-1} 1-Naphthaleneacetic acids (NAA) for 3 months under the dark condition. These primary SEs could be proliferated into secondary SEs after when culturing on free-PGRs MVW in light condition and continuously developed into plantlets after being transferred to culture on plantlet induction medium for 4 months. The uniformity of the genetic pattern between the mother plant (V_1) and regenerated plants (V_2 and V_3) was evaluated by RAPD analysis. The results of cryopreservation using V cryo-plate method showed that the cryopreserved *P. niveum* SEs could survive (~20%) after precondition on MVW containing 0.1 M sucrose (7 days) followed by two-step preculture on MVW containing 0.2 M and 0.6 M sucrose (each with 1 day). SEs were embedded on cryo-plate with alginate gel before put into loading solution containing 2 M glycerol and 1.2 M sucrose for 30 min. The SEs was dehydrated with plant vitrification solution 2 (PVS2) for 60 min. The application of ascorbic acid (AA) in the critical step could reduce total reactive oxygen species (ROS) and malondialdehyde (MDA) production which significantly improved the survival percentage to 39% of cryopreserved *P. niveum* SEs.

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Sutthinut Soonthornkalump

CONTENT

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LISTS OF TABLES

LISTS OF FIGURES

LISTS OF ABBREVIATION

AA	Ascorbic acid
ABA	Abscisic acid
AC	Activated charcoal
ANOVA	Analysis of variance
AFLP	Amplified Fragment Length Polymorphism
BA	6-Benzylaminopurine
BH	Banana homogenate
CAPS	Cleaved Amplified Polymorphic Sequence
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CRD	Completely randomized design
CW	Coconut water
D	Day
DCFDA	2,7-Dichlorodihydrofluorescein diacetate
DMRT	Duncan multiple range test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSE	Direct somatic embryogenesis
DV	Droplet vitrification
2,4-D	2,4-Dichlorophenoxyacetic acid
EV	Encapsulation vitrification
GS	Graduate school
H	Hour
ISSR	Inter Simple Sequence Repeat
LS	Loading solution
LN	Liquid nitrogen
MDA	Malondialdehyde
MS	Murashige and Skoog medium
MVW	Modified Vacin and Went medium
NAA	1-Naphthaleneacetic acid
NIAS	National Institute of Agrobiological Sciences
PCR	Polymerase chain reaction
PLBs	Protocorm-like bodies
PGRs	Plant growth regulators
PSU	Prince of Songkhla University
PUFA	Polyunsaturated fatty acid
PVS2	Plant vitrification solution 2
PVP-40	Polyvinylpyrrolidone
RAPD	Random Amplified of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive oxygen species
SCAR	Sequence Characterized Amplified Region
SCoT	Start Codon Targeted
SE	Somatic embryo
SSR	Simple Sequence Repeat
SV	Somaclonal variation
TBA	Thiobarbituric acid

TCA	Trichloroacetic acid
TDZ	Thidiazuron
V	Vitrification
VC	V cryo-plate
VW	Vacin and Went medium

CHAPTER 1
INTRODUCTION

1.1 Introduction

Paphiopedilum niveum (Rchb.f.) Stein is an endangered orchid which distributed in Southern Thailand and Northern Peninsular Malaysia (Pedersen et al., 2011). Its conservation status is a serious concern due to climate change, increases fragmentation of wild population from the decreasing of habitat (Seaton et al., 2010). Moreover, the increase of harmfully illegal collection pressured on natural populations of *Paphiopedilum* (Zeng et al., 2013). Thus, the efficient conservation procedure is an urgent requirement. In term of conservation, simultaneously of mass clonal propagation and long-term storage of germplasm of *P. niveum* using cryopreservation may achieve the goal of *P. niveum* conservation.

Plant tissue culture seems to be the suitable resolution to reduce the pressure of wild population of *P. niveum*. Because it was employed in mass production in short time and reasonable cost (Liao et al., 2011). Many previous experiments revealed that the application of single or a combination of classical plant growth regulators (PGRs) can affect plant multiplication. However, the use of PGRs may be a cause of genetic alteration in various types such as an alteration of cytological and chromosomal mutation, leading to somaclonal variation (Samarfard et al., 2013). The occurrence of somaclonal variation has been defined as a serious problem in the conservation process (Sarasan et al., 2006). There are many detection methods of somaclonal variation such as morphological observation, cytological and molecular techniques (Rao, 2004). The simplest and reasonable molecular method is Random amplification polymorphism DNA (RAPD) which very useful in the clonal fidelity assessment (Rao 2004). The previous study used RAPD to assess the genetic homogeneity of regenerated plantlets from seedlings of *Aerides vandarum* x *Vanda stangeana* cultured on a multiple shoot induction medium (½MS + 2 mgl^{-1} TDZ) (Kishor and Devi, 2009). While the genotypic alteration at 5.81% was found in the regenerant PLBs induced from *in vitro* pseudostem of *Cymbidium giganteum* which was detected the genotypic alteration by RAPD after cultured on MS contained 0.909 µM TDZ (Roy et al., 2012). It demonstrated the probability of somaclonal variation is linked to the type of explant, type of PGRs and PGR concentration.

V cryo-plate, a novel technique of cryopreservation, is the application of vitrification and the use of a small aluminum plate with wells. The V cryo-plate provided rapid thermal exchange rate which enables to reduce the damage from ice crystal formation (Vujović et al., 2011). Moreover, the use of V cryo-plate is easy to handle many pieces of explant in the process of cryopreservation and can reduce the loss of plant material (Yamamoto et al., 2011A). The V

cryo-plate technique has been investigated in economic crops such as mulberry (*Morus* sp.) (Yamamoto et al., 2011A), mint (*Mentha* spp.) (Yamamoto et al., 2012A), Dalmatian chrysanthemum (*Tanacetrum cinerariifolium*) (Yamamoto et al., 2011B), strawberry (*Fragaria* x *ananassa*) (Yamamoto et al., 2012B) and carnation (*Dianthus caryophyllus*) (Sekizawa et al., 2012). Recently, the literature review showed the limitation of orchids cryopreservation especially, *Paphiopedilum* species. Moreover, the use of V cryo-plate method in orchids cryopreservation is taken in the initiation step. Thus, this study focused on the investigation of PGRs induction of direct somatic embryogenesis, the proliferation of somatic embryo, histological observation and genetic stability assessment using RAPD. The optimized protocol of V cryo-plate method and the effect of ascorbic acid as an antioxidant to improve the survival rate of post-cryopreserved *P. niveum* PLBs were also determined.

1.2 Review of Literature

1.2.1 The genus *Paphiopedilum* and subgenus *Brachypetalum*

Paphiopedilum is a small to large orchids with the deeply slipper-shaped labellum which classified to subfamily Cypripedioideae of the family Orchidaceae (Pedersen et al., 2011). There are 3 subgenera were classified to this genus, *Brachypetalum, Pahiopedilum* and *Megastaminodium.* The subgenus *Brachypetalum* was comprised 3 sections, *Pavisepalum* (6 sp.), *Concoloria* (6 sp.) and *Emersoniana* (2 sp.) (Cribb, 2014; Górniak et al., 2014; Koopowitz et al., 2017). There are 14 species from subgenus *Brachypetalum* (*Concoloria* 5 sp.) and *Pahiopedilum* (*Pardalopetalum* 1 sp.; *Pahiopedilum* 5 sp. and *Barbata* 3 sp.) distributed in Thailand (Pedersen et al., 2011). The section of *Concoloria* was comprised of 6 sp. with 6 varieties (Fig. 1) distinguished by their markedly tessellated adaxial and densely maroon spotted at abaxial of leaves. The flower color is pale yellow-cream or white with scattered purple dot and tridentate apex of staminode (Cribb, 2014; Averyanov et al., 2017; Koopowitz et al., 2017). The center of distribution area of *Concoloria* is Thailand and Indochina, 5 species with 3 varieties were found in almost part of Thailand which composed of *P. bellatulum, P. concolor, P. niveum, P. thaianum* and 3 varieties of *P. godefroyae* (Fig. 1) (Pedersen et al., 2011; Cribb, 2014).

1.2.2 *Paphiopedilum niveum* (Rchb.f.) Stein

1.2.2.1 Distribution and botanical description

Paphiopedilum niveum is distributed in the shaded area of limestone of Southern Thailand to Northern Peninsular Malaysia at 0-200 m above sea level (Pedersen et al., 2011). The flowering season is April-July (summer) but also possible flowering in all year

round (Braem and Öhlund, 2016). According to Pedersen et al. (2011), the descriptive data of *P. niveum* characteristics and botanical description were shown (Fig. 2 and Table 1).

	Species	Distribution
	Paphiopedilum bellatulum (Rchb.f.) Stein	W Myanmar, SW China, Laos, Veitnam and Thailand (Chiang Mai, Lamphun and Tak)
	Paphiopedilum concolor var. *concolor* (Bateman) Pfitzer	SE Myanmar, SWChina, Loas, Cambodia, Veitnam and Thailand ((NE) Sakon Nakhon, Nakhon Ratchasima, (W) Kanchanaburi, (E) Sa Kaew, Trat, (S) Chumphon)
	Paphiopedilum concolor var. *longipetalum* (Rolfe) Pfitzer	S Myanmar (Tanintharyi)
	Paphiopedilum concolor var. *trungkienii* Aver., O.Gruss, C.X.Canh & N.H.Tuan	N. Veitnam (Thai Nguyen)
	**Paphiopedilum godefroyae* var. *godefroyae* (God.-Leb.) Stein	S Thailand (Chumphon, Surat thani and Krabi)
	**Paphiopedilum godefroyae* var. *ang-thong* (Fowlie) Bream	S Thailand (Surat thani)
	**Paphiopedilum godefroyae* var. *leucochilum* (Rolfe) Hallier	S Thailand (Chumphon, Nakhon Si Thammarat, Phangnga, Trang and Krabi)
	Paphiopedilum myanmaricum Koop., Iamwir. & S.Laohap.	S Myanmar (Tanintharyi)
	Paphiopedilum niveum (Rchb.f.) Stein	S Thailand (Karabi, Trang, Songkhla, Satun) and Northern Malaysia (Langkawi)
	**Paphiopedilum thaianum* Iamwir.	S Thailand (Phangnga)

Figure 1 Biodiversity and distribution of subgenus *Brachypetalum* of the world (constructed from Pedersen et al., 2011; Cribb, 2014; Averyanov et al., 2017; Koopowitz et al., 2017) *endemic to Thailand

Table 1 Description of morphological characteristics of *Paphiopedilum niveum* (adopted from Pedersen et al., 2011)

Plant part	Quantity	Length (cm)	Width (cm)	Shape	Color
Leaves	4-5	8-19	2.4-3.6	Narrowly, elliptic, rounded and minutely emarginated apex, ciliate at the base	Mottled very dark and pale green above, heavily dotted purple below
Peduncle	1	>20	-	-	Purple with shortly dense white pubescent
Bracts	1	1.1-1.4	1-1.2	Broadly ovate, obtuse	White to pale green, spotted purple
Flowers	1-3	-	Ø 6-8	-	White often highly dotted purple towards base of segment and front of labellum. Pubescent on outside and at base of petals
Dorsal sepal	1	2.7-4.2	3-5	Very broadly ovate, obtuse to emarginate	White often highly dotted purple towards base.
Synsepal	1	2-3	1.5-3	Concave, ovate, obtuse	White often highly dotted purple towards base.
Lateral petal	2	3.3-4.3	2.2-3.9	Elliptic, rounded with shortly ciliate on margins	White often highly dotted purple towards base.
Labellum	1	2.2-3.6	1.5-1.8	Ovoid to ellipsoidal with incurve margins	White often highly dotted purple towards base.
Column	1	-	-	Sessile or shortly stalked, glabrous Stigma stalked, tripartite less papillose	White
Ovary	1	4.5	-	Inferior, long with densely shortly pubescent	Green flushed with dark purple
Staminode	1	0.6-0.9	1-1.2	Broader than long, transversely subelliptic, one-to three-toothed at apex	White with yellow blotched at the center
Anther (pollinia)	2	0.15	0.15	Bilocular, borne on short filament	Yellow, pollen viscid

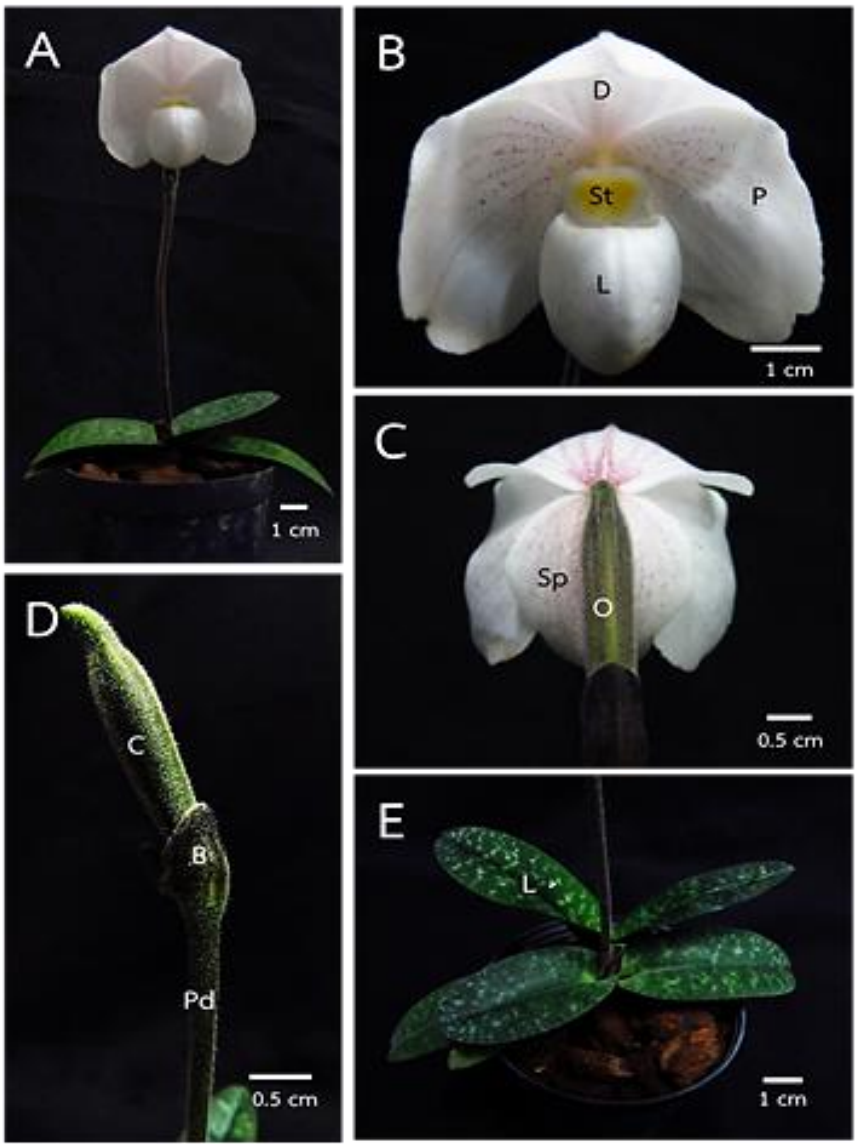

Figure 2 Morphology of *P. niveum*: A flowering plant; B Front view of flower showing dorsal sepal (D), lateral petal (P), labellum (L) and staminode (St); C Rear view of flower: synsepal (Sp), ovary (O), D Capsule (C), bract (B); and peduncle (Pd); E leaf (Lf)

1.2.2.2 Conservation situation and *ex situ* conservation

Paphiopedilum niveum has high value and very popular in the worldwide ornamental plant market (Kaewubon et al., 2010). Generally, orchid needs mycorrhizal fungi in root system for exchange essential phosphate and nitrate ions for seed germination and plant development (Athipunyakom et al., 2004). Thus, *Paphiopedilum* was determined as a slow grower compared with other orchid genera. Due to the situation of the wild orchid was threatened by global climate change and the over-collecting to sell in the domestic market and illegal international trading. Thus, all species of *Paphiopedilum* were protected by international wild-collected trading by listed in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I (McGough et al., 2006). CITES Appendix I is the most strictly prohibited wild-collected living or dead specimens. However, trading of plant or plant part from artificial propagation is allowed with permission which controlled by particularly strict regulation of CITES (CITES, 2014). Recently, the conventional method of *Paphiopedilum* propagation has unreliable to fulfill commercial demand due to low productivity and extremely time-consuming (Ng et al. 2010; Ng and Saleh 2011). For instance, the encouraging of artificial cultivation by *in vitro* culture can facilitate massive propagation leading to the reduced wild-collected pressure of medicinal orchid such as *Dendrobium catenatum* (Liu et al., 2014). The previous study reported that the transplanted seedling of *Paphiopedilum wardii* demonstrated the survivability in natural habitat in Yunnan and Guangdong at 49.67-60.33% after reintroduced for 2 years (Zeng et al., 2012). So the micropropagation of *Paphiopedilum* would be the efficient tool to supply the artificially propagated plant to the orchid collector and reduce wild-collecting pressure from the natural population.

1.2.3 Micropropagation and somatic embryogenesis of *Paphiopedilum*

The conventional propagation by bud division is unreliable and cannot meet market demand because of the extreme time-consuming (Huang et al., 2001). So, mass propagation of *Paphiopedilum* may reduce the pressure of wild population. Micropropagation is an effective tool to produce large-scale clonal propagation in many plants including orchids (Martin and Madassery, 2005). Since biotechnology was applied in plant tissue culture, somatic embryogenesis has become ideally method for large-scale cloning of valuable cultivar (Deo et al., 2010).

Large scale propagation via somatic embryogenesis is an efficient method which could be facilitated in many branches of plant biotechnology such as, massive clonal propagation, artificial seed production, gene transformation and also been an effective model in the molecular and morphogenetic event (Deo et al., 2010). The somatic embryogenesis is the

dedifferentiation of a somatic cell to somatic embryo (SE) without vascular tissue which connected to parental tissue (Jiménez, 2005). Orchid SE, known as protocorm like-bodies (PLBs), has special characters from the other higher plants cause of the distinctive morphology (Lee et al., 2013).

There are two regeneration pathways of somatic embryogenesis; direct somatic embryogenesis and indirect somatic embryogenesis. Direct somatic embryogenesis is the direct regeneration of SE from explant without callus formation (Chawla, 2000) while indirect somatic embryogenesis is the regeneration that intervening callus phase and subsequently forms the somatic embryo (Ji et al., 2011). Generally, the somatic embryogenesis induction composed of two stages including induction and expression. The proper modification of culture condition (medium and PGRs) could stimulate the reversibility of competence somatic cell (Jiménez, 2005). Expression stage might be activated by reduction of PGRs, particularly auxin (Jiménez, 2001). Auxin is known as a promoter of callus proliferation and an inhibition of cell differentiation. While cytokinin plays an important role in embryogenic cell formation and stimulates maturation of somatic embryo (Chawla, 2000; Deo et al., 2010). Supplementation of auxin and cytokinin in SE induction were presented in several studies which were summarized in Table 2. It demonstrated that the most popular auxin and cytokinin were 1-naphthaleneacetic acid (NAA) and thidiazuron (TDZ), respectively.

Table 2 Previous reports on somatic embryogenesis in *Paphiopedilum* spp.

Species	Explant	Basal medium	PGRs supplemented in basal medium				References
			Callus induction	Callus proliferation	SE induction	SE proliferation	
P. callosum x *lawrenceanum*	Protocorm	½ strength MS + 100 mg/l myo-inositol + 0.5 mgl^{-1} nicotinic acid + 0.5 mgl^{-1} pyridoxine HCl + 0.1 mgl^{-1} thiamine + 2 mgl^{-1} glycine + 1 gl^{-1} peptone + 170 mgl^{-1} NaH_2PO_4 + 2% sucrose + 0.22% gelrite	-	5 mgl^{-1} 2,4-D + 1 mgl^{-1} TDZ	-	-	Lin et al, 2000
P. Alma Gavaert	Seed-derived callus	½ MS (1/2 strength macronutrient + full-micronutrient) + 100 mgl^{-1} myo-inositol + 0.5 mgl^{-1} nicotinic acid + 0.5 mgl^{-1} pyridoxine HCl + 0.1 mgl^{-1} thiamine HCl + 2 mgl^{-1} glycine + 1 gl^{-1} peptone + 170 mgl^{-1} NaH_2PO_4 + 2% sucrose + 0.22% gelrite	5 mgl^{-1} 2,4-D + 1 mgl^{-1} TDZ in darkness	5 mgl^{-1} 2,4-D + 1 mgl^{-1} TDZ in darkness	0.5 mgl^{-1} NAA + 0.1 mgl^{-1} TDZ	-	Hong et al, 2008
P. niveum	Seed-derived callus	MVW (full strength macronutrient VW + ½ strength MS micronutrient) + 100 mgl^{-1} myo-inositol + 0.5 mgl^{-1} nicotinic acid + 0.5 mgl^{-1} pyridoxine HCl + 0.1 mgl^{-1} thiamine HCl + 2 gl^{-1} peptone + 2% sucrose + 0.2% gelrite	1 mgl^{-1} 2,4-D + 0.1 mgl^{-1} TDZ in darkness	-	0.1 mgl^{-1} NAA + 0.5 mgl^{-1} TDZ + 1% sucrose	-	Kaewubon et al., 2010
P. rothschildianum	Stem nodal	½ strength MS	0.86 mgl^{-1} kinetin	-	0.86 mgl^{-1} kinetin	Free-PGRs	Ng & Saleh, 2011
P. hangianum	Protocorm	½ strength MS	5 mgl^{-1} 2,4-D + 1 mgl^{-1} TDZ	-	5 mgl^{-1} kinetin + 2 mgl^{-1} BA	-	Zeng et al., 2013

Table 2 Previous reports on somatic embryogenesis in *Paphiopedilum* spp. (continued)

Species	Explant	Basal medium	PGRs supplemented in basal medium				References
			Callus induction	Callus proliferation	SE induction	SE proliferation	
P. niveum	Seed-derived callus	MVW (full strength macronutrient VW + ½ strength MS micronutrient) + 100 mgl^{-1} myo-inositol + 0.5 mgl^{-1} nicotinic acid + 0.5 mgl^{-1} pyridoxine HCl + 0.1 mgl^{-1} thiamine HCl + 2 gl^{-1} peptone + 2% sucrose + 0.2% gelrite	-	15 gl^{-1}sucrose + 10% Coconut water (CW)	0.5 mgl^{-1} 2,4-D + 0.1 mgl^{-1} TDZ	10 gl^{-1}sucrose + 0.2% AC + 10% CW	Chaireok et al., 2015

1.2.4 Cryopreservation based on vitrification and V cryo-plate technique

Cryopreservation is the storage of living materials of plant or animal at an extremely low temperature (lower than -130 °C) and the stored materials can still survive and regrowth after thawing (Day and Stacey, 2007). However, the sufficient dehydration of intracellular water during cryopreservation procedure is essential to investigate (Pegg, 2010). Recently, several cryopreservation procedures have been developed such as controlled rate cooling, dehydration, encapsulation dehydration, vitrification, encapsulation vitrification and droplet-vitrification (Kaczmarczyk et al., 2012).

Vitrification technique comprised of the step of preculture, osmoprotection, dehydration, rapid warming and regrowth (Sakai and Engelmann, 2007). Preculture is the step which stimulates an endogenous cryoprotectant accumulation in plant cells using high sugar concentration in medium with suitable incubation time (Sakai and Engelmann, 2007). The studies on preculture conditions on the vitrification based protocol were shown in Table 3. After preculture, loading solution (LS) is used as osmoprotectant for protecting plant material from the excessive osmotic stress which could be modified based on a combination of glycerol and sucrose at different concentrations (Table 4). Dehydration using plant vitrification solution 2 (PVS2) is the most common use in this crucial step (Kami, 2012). PVS2 containing glycerol, dimethyl sulfoxide (DMSO), ethylene glycol and sugars in high level has toxic and excessive osmotic stress (Kaczmarczyk et al., 2012). So, the determination of optimal incubation time of PSV2 is needed. The optimal use of PVS2 in previous studies were shown in Table 5. After immersion in liquid nitrogen (LN), rewarming is an essential step to avoid damaging devitrification and prevent ice-formation during rewarming (Sakai and Engelmann, 2007). Cryopreserved tube need to warm in hot water for a few minutes (Kami, 2012) or using 1 M sucrose solution at room temperature for V cryo-plate technique (Sekizawa et al., 2011). The successful of growth recovery after exposure to LN can be determined by green and resumed growth of plant material (Sakai and Engelmann, 2007). Occasionally callus tissue may be regenerated during the regrowth step and possibly use as plant material (Kaczmarczyk, 2012). However, the risk of occurrence of genetic variation, an undesirable in plant germplasm storage, may be a high frequency (Sakai and Engelmann, 2007). For instance, the isolated *Citrus* cells from embryogenic callus culture presented the significant change in DNA methylation profile after cryopreservation via vitrification method (Hao et al., 2002).

V cryo-plate is one of the most novel technique which has been developed by Japanese scientist team from the National Agriculture and Food Research Organization (NARO) who applied for a small aluminum plate with ten wells on the upper side called V cryo-plate (Yamamoto et al., 2012A). This V cryo-plate has facilitated as plant material holder which

reduce the injury and loss of plant material during the cryopreservation process (Yamamoto et al., 2011). The first successful of V cryo-plate method was reported by Yamamoto et al. (2011). This method combines the advantage of vitrification and droplet vitrification (Yamamoto et al., 2011). Therefore, the vitrification has low complexity in the process and no need to use special equipment (Reed, 2008) and the use of aluminum foil of droplet vitrification is facilitated uniformly rapid thermal exchange (Vujović et al., 2011). Moreover, V cryo-plate is useful in transferring of plant material in the steps of LS and PVS2 (Sekizawa et al., 2011). The V cryo-plate technique was used in some crop plants such as mulberry (*Morus* spp.) (Yamamoto et al., 2012C), carnation (*Dianthus caryophyllus*) (Sekizawa et al., 2011) and several varieties of potato (*Solanum tuberosum*) (Yamamoto et al., 2012D; Yamamoto et al., 2015). The V cryo-plate method was expected to be a new method assisting the *ex situ* conservation of endangered wild orchids.

Table 3 Summarized preculture conditions of the vitrification-based protocol

Plant species	Explant	Method	Preculture time (d)	Sucrose (M)	References
Doriteanopsis New Toyohashi*	Cell suspension	V	7	0.1 +1 mgl^{-1} ABA	Tsukazaki et al., 2000
Fragaria x *ananassa* cv. Donner	Shoot	V	1	0.3	Niino et al., 2003
Rosa spp.	Shoot	DV	1 and 2	0.5	Halmagyi and Pinker, 2006
Leontopodium hayachinense	Shoot	V	1	0.3	Tanaka et al., 2008
*Brassia rex**	PLBs	V	2	0.5	Shuhaida et al., 2009
Dianthus caryophyllus	Shoot	VC	2	0.3	Sekizawa et al., 2011
Mentha spp.	Shoot	VC	2	0.3	Yamamoto et al., 2011A
*Dendrobium nobile**	PLBs	EV	2	0.4	Mohanty et al., 2012
Anemarrhena asphodeloides	Callus	V	2	0.5	Hong and Yin, 2012
Dendrobium sonia-28*	PLBs	V	2	0.4	Poobathy et al., 2012
Solanum tuberosum	Shoot	VC	overnight	0.3	Yamamoto et al., 2015
*Paphiopedilum niveum**	PLBs clump	V	5	0.75	Chaireok et al., 2016

V = Vitrification; DV = Droplet vitrification; EV = Encapsulation vitrification; VC= V cryo-plate

* orchid species

Table 4 Summarized osmoprotectant treatment of the vitrification-based protocol

Plant species	Explant	Method	Loading solution	Incubation time (min)	References
*Vanilla planifolia**	Shoot tip	DV	2 M glycerol + 0.4 M sucrose	20-30	Gonzalez-Arnoa et al., 2008
*Dendrobium candidum**	PLBs	EV	2 M glycerol + 1 M sucrose	80	Yin and Hong, 2009
Dianthus caryophyllus	Shoot	VC	2 M glycerol + 1.4 M sucrose	90	Sekizawa et al., 2011
*Dendrobium nobile**	PLBs	EV	2 M glycerol + 0.4 M sucrose	60	Mohanty et al., 2012
Mentha spp.	Shoot	VC	2 M glycerol + 0.8 M sucrose	30	Yamamoto et al., 2012A
*Oncidium flexuosum**	Seed	V	2 M glycerol + 0.4 M sucrose	30	Galdiano et al., 2013
*Bletilla formosana**	Seed	V	2 M glycerol + 0.4 M sucrose	10-30	Hu et al., 2013
*Dendrobium chrysanthum**	PLBs	EV	2 M glycerol + 0.4 M sucrose	80	Mohanty et al., 2012
Solanum tuberosum	Shoot	VC	2 M glycerol + 0.8 M sucrose	30	Yamamoto et al., 2015
*Paphiopedilum niveum**	PLBs clump	V	2 M glycerol + 0.5 M sucrose	30	Chaireok et al., 2016

V = Vitrification; DV = Droplet vitrification; EV = Encapsulation vitrification; VC= V cryo-plate

* orchid species

Table 5 Summarized incubation time for dehydration using PSV2

Plant species	Explant	Method	Incubation time (min)	References
*Vanda coerulea**	Seed	V	20	Thammasiri and Soamkul, 2007
*Phaius tankervilleae**	Seed	V	60	Hirano et al., 2009
*Dendrobium candidum**	PLBs	EV	150	Yin and Hong, 2009
*Cymbidium finlaysonianum**, *C. goeringii** and *C. macrorhizon**	Seed	V	30 and 60	Hirano et al., 2011
Vanda Kaseem's Delight*	PLBs	V	20	Poobathy et al., 2012
*Bletilla formosana**	Seed	V	30	Hu et al., 2013
Garcinia mangostana	Shoot	V	25	Ibrahim and Normah, 2013
*Paphiopedilum niveum**	PLBs clump	V	90	Chaireok et al., 2016

V = Vitrification; DV = Droplet vitrification; EV = Encapsulation vitrification; VC = V cryo-plate

* orchid species

1.2.5 The uses of ascorbic acid as an antioxidant in cryopreservation

Reactive oxygen species (ROS) is a by-product of the photosynthesis and cellular respiration which was regulated by ascorbate-glutathione cycle which prevents the immoderate reduction or oxidation (Foyer and Noctor, 2011). In general, the high level of ascorbate could be found in the apoplastic channel which acts as a cell membrane defender against ROS (Pereira, 2004). Therefore, the cell membrane is a sensitive organelle which can be damaged by ROS (Shalata and Neumann, 2001). In addition, the damaged polyunsaturated fatty acid (PUFA) from the plasma membrane can generate malondialdehyde (MDA) which can interrupt biological function when it binding with protein (Uchendu et al., 2010).

Ascorbic acid (AA) or vitamin C is a well-known antioxidant and very popular use (Arrigoni and De Tullio, 2002). The previous report showed that a few concentration of ascorbic acid (0.005%) was reported to control browning of Cavendish banana (Ko et al., 2009). However, there are a few reports of AA supplementation in plant cryopreservation. The low concentration at 0.5 mgl^{-1} AA showed the decreased of MDA level leading to improved regrowth rate of blackberry shoot tip when added in any step of vitrification namely, preculture, loading, rinsing, and regrowth (Uchendu et al., 2010). The addition of AA (0.28 mM) in loading solution could improve the survival rate in cryopreserved *Nephelium ramboutan-ake* shoot tip because AA could decrease the oxidative stress (Chua and Normah, 2011). Hence, these reports revealed the potential of AA as an antioxidant in the reduction of oxidative stress leading to improve the survivability of explant in cryopreservation.

1.2.6 Detection of genetic alteration by molecular marker

Somaclonal variation (SV) is any genetic variation types which are detected in cells or tissues cultured *in vitro* (Evans et al., 1984). This SV may occur permanently or temporarily change in different level such as morphological changes, physiological responses and epigenetic (Bairu et al., 2011). The SV may use as a tool for crop improvement which important in the mutant selection (Lestari, 2006). However, in term of conservation, the uniform of genetic in clonal propagation is an ideal expectation to maintain genetic resources (Bairu et al., 2011). There are many factors affecting SV such as the type of explants, PGRs, culture period, proliferation rate and culture condition lead to a genetic change in the *in vitro* variants (Skirvin, 1994). Thus the monitoring of genetic stability in plant tissue culture is necessary. The basic molecular marker techniques can divide into 2 categories; polymerase chain reaction (PCR) based technique and non-PCR-based technique (Table 6). In the latter case, this technique detects the DNA polymorphism by the hybridizing digested DNA with the radioactive probe such as the Southern blot. Meanwhile, the PCR-based technique uses the PCR technology and random primers to detect the polymorphism of DNA (Agarwal et al., 2008). Currently, several

molecular marker techniques are available as tool to indicate the genetic variation of *in vitro* cultures (Table 7). For instance, amplified fragment length polymorphism (AFLP) was used to detect the somaclonal variation in the primary regenerant of *Echinacea purpurea* derived from leaf organogenesis (Chuang et al., 2009). The Random amplification polymorphism DNA (RAPD) was successfully applied to the genetic stability assessment in various clonal micropropagated plants such as *Musa* spp. (Ray et al., 2006), *Clivia miniata* (Wang et al., 2012), and *Dendrobium nobile* (Bhattacharyya et al., 2014). The inter-simple sequence repeat (ISSR) demonstrated the genetic fidelity of regenerant clones from three different explants of *Gerbera jamesonii*. It was found that the genetic stability was observed in the clone derived from capitulum and shoot tip whereas the SV was found in leaf-derived clones (Bhatia et al., 2009).

Table 6 Techniques in molecular marker (adopted from Agarwal et al., 2008)

Techniques	RFLP	RAPD	SSR	SCAR	ISSR	AFLP
Type	Non-PCR	PCR	PCR	PCR	PCR	PCR
Dominance	Co-dominant	Dominant	Co-dominant	Dominance	Dominance	Co-dominant
Abundance	High	High	Medium	Low	High	High
Reproducibility	High	Low	Medium	High	High	High
Degree of polymorphism	Medium	Medium	Medium	Medium	High	Medium
Technical requirement	High	Low	Low	Medium	Low-high	Medium
The quantity of DNA required	High	Low	Low	Low	Low	Medium

AFLP: Amplified Fragment Length Polymorphism; ISSR: Inter Simple Sequence Repeat; RAPD: Random Amplified Polymorphism DNA; RFLP: Restriction Fragment Length Polymorphism; SCAR: Sequence Characterized Amplified Region; SSR: Simple Sequence Repeat

Table 7 Detection of somaclonal variation-derived of *in vitro* culture using various molecular marker techniques (adopted from Bairu et al., 2011)

Plant species	Explant and PGRs	Molecular marker techniques	References
Saussurea involucrata	Callus, NAA and BA	RAPD	Yuan et al., 2009
Freesia hybrida	Callus, 2,4-D	AFLP, MSAP	Gao et al., 2010
Theobroma cacao	Embryo, TDZ	CAPS	Rodríqeuz López et al., 2010
Lilium tsingtauense	Embryo, 2,4-D	RAPD, ISSR	Yang et al., 2010
Cymbidium giganteum	pseudobulb-derived PLB, TDZ	RAPD, SSR	Roy et al., 2012
Dendrobium nobile	pseudobulb-derived PLB, TDZ	RAPD, SCoT	Bhattacharyya et al., 2014

AFLP: Amplified Fragment Length Polymorphism; RAPD: Random Amplified Polymorphism DNA; CAPS: Cleaved Amplified Polymorphic Sequence; ISSR: Inter Simple Sequence Repeat; MSAP: Methylation Sensitive Amplification Polymorphism; SCoT: Start Codon Targeted; SSR: Simple Sequence Repeat

1.3 Objectives

1. To examine the effects of auxin (NAA) and cytokinin (TDZ) on somatic embryogenesis and somatic embryo proliferation of *P. niveum*
2. To determine the genetic fidelity in *P. niveum* after PGR treatment
3. To optimize the conditions for cryopreservation of *P. niveum* SEs using the V cryo-plate technique
4. To examine the contents of ROS and MDA during the optimized V cryo-plate protocol after ascorbic acid supplementation

CHAPTER 2
RESEARCH METHODOLOGY

This study composed 2 main parts which was direct somatic embryogenesis and cryopreservation using V cryo-plate method (Fig. 3)

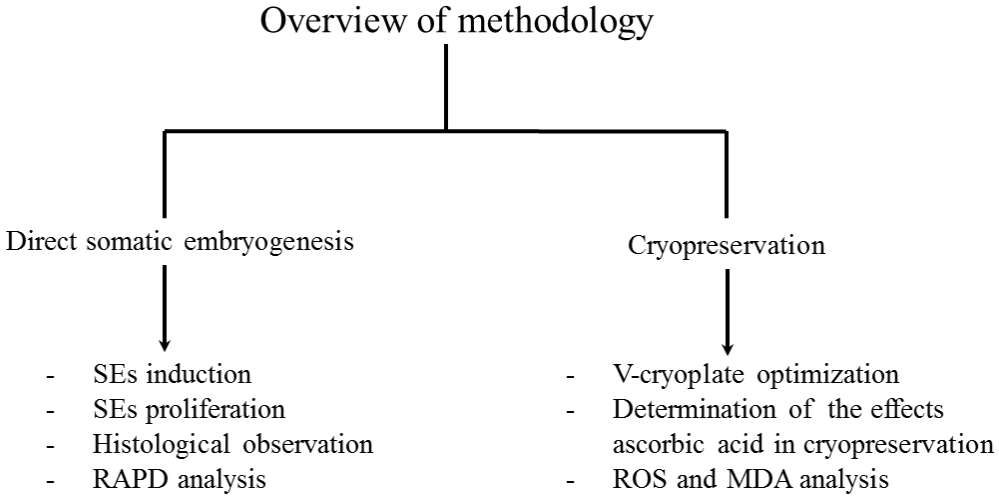

Figure 3 Overview of methodology of this study

2.1 Plant material and culture medium

Five-month-old capsule from a hand-pollinated plant of *P. niveum* was used as plant material. Mother plants were cultured in the shaded greenhouse. The modified Vacin and Went medium (MVW) contained full-strength macronutrient of VW basal medium (Vacin and Went 1949) and half-strength micronutrient of MS (Murashige and Skoog, 1962), 5 mgl^{-1} of 1000 ppm chitosan (Olizac Technologies, Pathumwan, Bangkok, Thailand), 2 gl^{-1} Bacto™ peptone (Becton, Dickinson and Co., Sparks, MD, USA) and 20 gl^{-1} sucrose. The pH of medium adjusts to 5.3-5.4 with 1 N NaOH or HCl prior to sterile with autoclave at 121 °C for 20 min. The basal medium for SE induction was solidified with 2 gl^{-1} phytagel™ (Sigma-Aldrich Co., St. Louis, MO, USA). Addition of 0.72% agar in the medium was used for plantlet regeneration.

2.2 Seed pretreatment

The collected capsule was thoroughly washed with tap water and surface-disinfected in 70% ethanol for 30 sec and then flame. The flamed capsule was cut longitudinally and the seeds were scooped out and soaked in 1% (v/v) clorox® (Clorox Company, Oakland, CA, USA) containing a few drops of Tween 20 and occasionally shake for 60 min (Shimura and Koda 2004; Lee, 2007). Then, these seeds were rinsed with distilled water for 3 times. Pretreated seeds (approx. 400 seeds) were inoculated into a 125 ml Erlenmeyer flask containing 40 ml of liquid MVW medium. The cultures were placed on agitator at 120 rpm in darkness at

25±2 ˚C and subcultured at monthly interval for 4 months. Four-month-old protocorm obtained in this step was used as plant material for SEs induction experiment (Fig. 4).

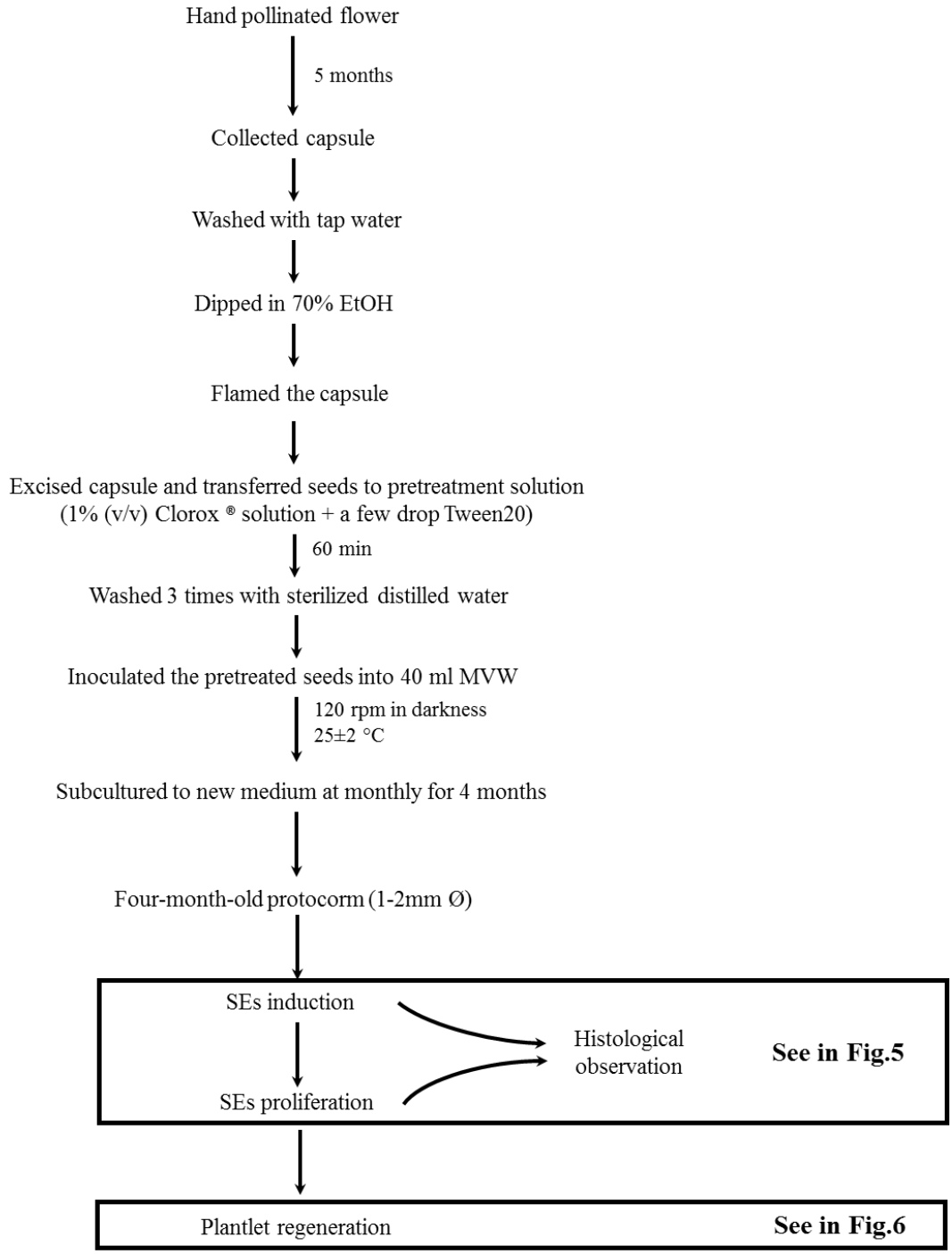

Figure 4 Schematic diagram of plant material preparation and the experiment of *Paphiopedilum niveum* micropropagation

2.3 Somatic embryo (SEs) induction, proliferation and genetic fidelity assessment

2.3.1 SEs induction

Four-month-old protocorms (approximately 1-2 mm in diameter) were cultured on solid MVW supplemented with single and combination of NAA (0, 0.1, 0.3 and 0.5 mgl^{-1}) (Fluka Chemie GmbH, Buchs, Switzerland) and TDZ (Sigma-Aldrich Co., St. Louis, MO, USA) (0, 0.1, 0.5 and 1 mgl^{-1}). The cultures were maintained in the darkness and subsequently transferred to fresh medium for 3 months at monthly interval. After 3 months, these treated protocorms were transferred to culture under 16 h of photoperiod at the intensity of 23 $\mu mol^{-1}m^{-2}s^{-1}$ using Philips cool white fluorescent lights at 25±2 °C for a month (Fig. 5). Ten replicate bottles, each with 4 protocorms, were performed for each treatment. The survival percentage (browning protocorm was determined as dead), the percentage of SEs formation and number of SEs per explant was recorded after culture for 3 months.

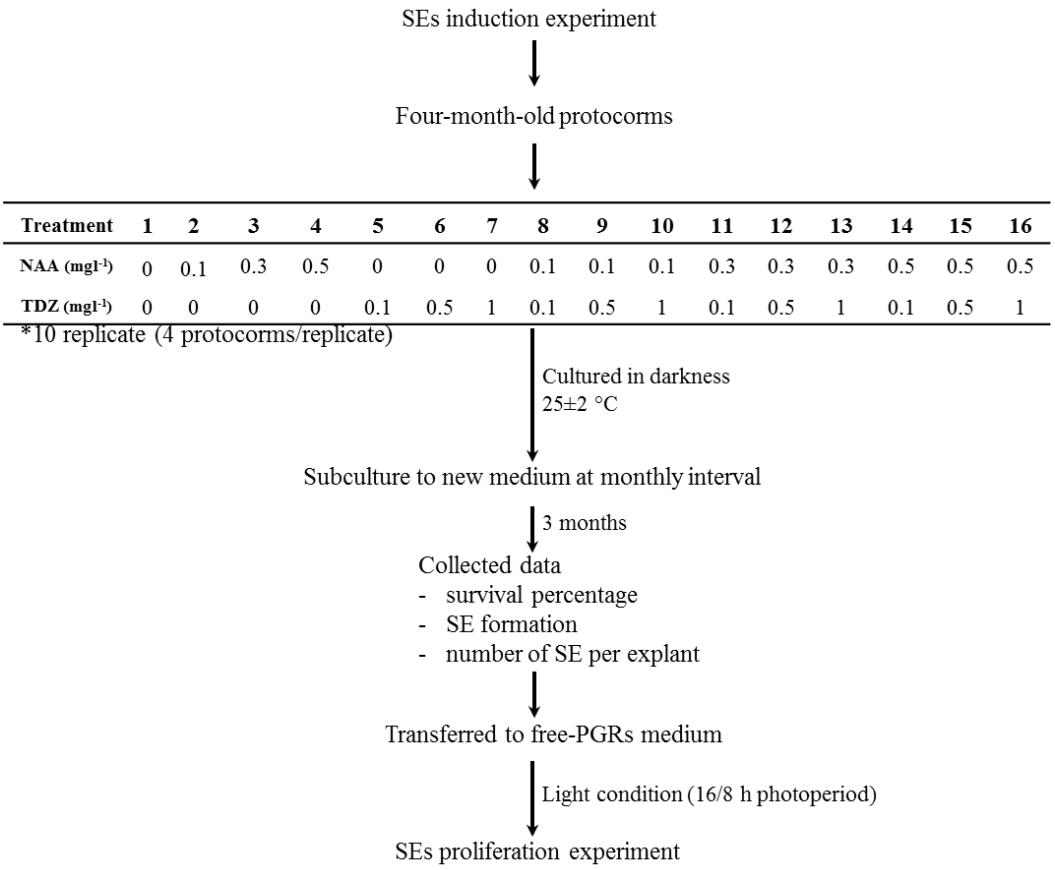

Figure 5 Summarized procedure of direct SEs induction

2.3.2 SEs proliferation

Protocorm-derived SEs were acclimatized on free hormone solid MVW medium for a month. Then, the SEs clump (approximately 100 mg/clump) were cultured on solid MVW containing NAA (0, 0.1, 0.3 and 0.5 mgl^{-1}) in combination with kinetin (Fluka Chemie GmbH, Buchs, Switzerland) (0, 1, 5 and 10 mgl^{-1}). All treatments, each with 10 replicates (clumps), were subsequently transferred to fresh medium for monthly interval (2 months) and the cultures were maintained under the light conditions as described above. Percentages of the increased fresh weight, rowning, and SE visualization (i.e. vigor and color) were determined after culture for 2 months (Fig. 6).

SEs proliferation experiment

↓

Four-month-old SEs clump

↓

Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NAA (mgl^{-1})	0	0.1	0.3	0.5	0	0	0	0.1	0.1	0.1	0.3	0.3	0.3	0.5	0.5	0.5
Kinetin (mgl^{-1})	0	0	0	0	1	5	10	1	5	10	1	5	10	1	5	10

Approx. 100 mg of SEs with 10 replicates

↓ Light condition (16/8 h photoperiod)
25±2 °C

Subculture to new medium at monthly interval

↓ 2 months

Collected data
- The increased fresh weight percentage
- Survival percentage
- SE visualization (color)

↓

Transferred to free-PGRs medium

↓ Light condition (16/8 photoperiod)
1 month

Transferred to plantlet regeneration medium

↓ Light condition (16/8 photoperiod)
4 months

Growth in green house condition

Figure 6 Summarized diagram of SEs proliferation and plantlet regeneration

2.3.3 Plantlet regeneration

Regenerated SEs with small leaves induced plantlet regeneration by cultured on solid MVW supplemented with 20 gl^{-1} sucrose, 2 gl^{-1} peptone, 2 gl^{-1} activated charcoal (AC) and 50 gl^{-1} banana homogenate (BH) for 4 months in light condition as described above. After that, the

5 - cm height of vigorous plantlets with well-developed root and shoot were *ex vitro* and transferred to greenhouse condition. Plantlets were transplanted into 2 inch-nursery-pot containing dried sphagnum moss and growth in the greenhouse at Department of Biology, Faculty of Science, Prince of Songkla University, Hatyai campus, Songkhla, Thailand.

2.3.4 Histological observation

Intact protocorm (the control) and regenerant SEs from SEs induction and SEs proliferation were collected and fixed in FAAII (5:5:90 v/v of formaldehyde : glacial acetic acid: 70% ethyl alcohol) for at least 48 h. Fixed specimens were dehydrated through tertiary-butyl-alcohol series, infiltrated and embedded in paraplast (Histoplast PE; Richard-Allan Scientific, Kalamazoo, MI, USA). Embedded samples were cut at 6 µm in thickness with a rotary microtome (AO, 820 SPENCER) and affixed to a glass slide. Sections were stained with hematoxylin and safranin (Johansen, 1940) to observe the general structures and to indicate the stage of SE formation. All samples were observed under an Olympus BX 51 TRF light microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and photographed by Olympus DP72 digital camera (Olympus Optical Co. Ltd., Tokyo, Japan).

2.4 RAPD assessment

A comparison of the genetic banding patterns using RAPD between the mother plant (V_1 generation) and *in vitro* regenerated plants (V_2 and V_3 generation) was performed.

2.4.1 Genomic DNA extraction

Fresh leaf tissue (approximately 10 mg) was ground to a fine powder in a mortar with liquid nitrogen. Then, DNA was extracted using the DNA extraction kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) DNA was redissolved with TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and stored at -20 ˚C in a refrigerator. The concentration and purity of DNA were determined using BioDrop µLITE (BioDrop Ltd., Cambridge, UK) by measuring the absorbance at 260/280 nm. The quantity test was performed using 1% agarose gel electrophoresis and automated gel imagining using Gel Doc™ EZ Gel Documentation System (Bio-Rad, California, USA).

2.4.2 Primer screening, RAPD amplification, and analysis

A total of 48 primers in operon and UBC series were used for primer screening. Among this primer, ten primers were selected to determine the comparison of genetic pattern among V_1-V_3 plants (Table 8). RAPD amplification procedure was followed by Chung et al. (2006). A mixture for PCR reaction contained 1 µl of 20 ng of template DNA, 2 µl of dNTP (5 mM each of dATP, dGTP, dCTP and dTTP), 1.5 µl of 50 pM of selected primer, 2.5 µl of 10X ThermoPol™ buffer (containing 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Tris®X-100, pH 8.8 and 2 mM $MgSO_4$), 0.25 µl (1 unit) of Taq DNA polymerase (New

England BioLabs, Massachusetts, USA), and 17.5 µl of deionized water (24.5 µl in total volume). The PCR reaction composed of the precycling denaturation at 94 °C for 3 min. A cycling protocol was initiated at 94 °C for 40 s, 37 °C for 1 min and 72 °C for 1 min with total cycling at 40 cycles. Then the termination of the cycling was done with a final extension at 72 °C for 10 min using Biometra thermocycler model T-1 (Biometra GmbH, Göttingen, Germany). To determine genetic homogeneity, three clones of *in vitro* plantlets were randomly selected for used in the assessment. The result of DNA banding patterns of each RAPD primer was compared between mother plant (V_1 generation) and regenerant plants (V_2-V_3 generation).

Table 8 RAPD primers used for the genetic fidelity assessment of *in vitro Paphiopedilum niveum* among the mother (V_1) and the regenerant plants (V_2-V_3)

Primer	Primer nucleotide (5′-3′)	Band size (bp)	Number of scorable bands/primer/clone			Total number of bands/primer/clone		
			Clone1	Clone2	Clone3	Clone1	Clone2	Clone3
OPA-11	CAATCGCCGT	250-2,000	7	4	10	21	12	30
OPA-18	AGGTGACCGT	150-2,200	13	10	11	39	30	33
OPAA-16	GGAACCCACA	250-2,800	6	8	10	18	24	30
OPAB-2	GGAAACCCCT	240-2,000	10	12	10	30	36	30
OPAB-8	GTTACGGACC	320-2,000	10	8	13	30	24	39
OPAD-8	GGCAGGCAAG	300-2,100	14	14	10	42	42	30
OPAD-11	CAATCGGGTC	200-1,900	8	8	11	24	24	33
OPZ-3	CAGCACCGCA	200-1,500	12	7	8	36	21	24
OPZ-11	CTCAGTCGCA	200-2,500	13	9	11	39	27	33
UBC-719	GGTGGTTGGG	200-1,500	9	11	4	27	33	12
Total			102	91	98	306	273	294

2.5 V cryo-plate protocol, total ROS and MDA determination

This optimized V cryo-plate protocol was partially modified based on the protocol presented by Sekizawa et al. (2011). All treatments employed in this study composed of 3 replicates, each with 10 samples (a single SE) (Fig. 7).

2.5.1 Two-step preculture

The SEs (~1-1.5 mm in diameter) were preconditioned on solid MVW with 0.1 M sucrose (7 d) were firstly precultured on the same medium supplemented with 0.2 M sucrose for 1 d (the 1st preculture). The 2nd preculture with the same medium containing different sucrose concentrations (0.4 and 0.6 M sucrose) for a day was tested.

2.5.2 SE embedding on the cryo-plate

Precultured SEs, one by one, were placed into wells of aluminum cryo-plate (7 mm x 37 mm x 0.5 mm with Ø 1.5 mm, depth 0.75 mm of ten wells) filled with 2 µl mixture of 2% (w/v) Na-alginate and 0.4 M sucrose in calcium-free liquid MVW. The CaCl2 solution (0.1 M $CaCl_2$ in liquid MVW with 0.4 M sucrose) was added onto cryo-plate and covered completely. After complete polymerization (~15 min at room temperature), the CaCl2 solution was removed by autopipette and the residual solution was absorbed with a piece of filter paper.

2.5.3 Osmoprotection and dehydration

The cryo-plate with embedded SEs was incubated in the pipetting reservoir containing the amount of 50 ml LS; 2 M glycerol supplemented with various concentrations of sucrose (0.4, 0.8 and 1.2 M) in MVW for 30 min at 25°C. Next, SEs attached to the cryo-plates were dehydrated by placing cryo-plates in the pipetting reservoir filled with 50 ml of PVS2 (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose) for 30, 45 and 60 min at 25°C (Sakai and Engelmann, 2007).

2.5.4 Cryopreservation in liquid nitrogen (LN), thawing and regrowth

After that, cryo-plate was put into 2-ml cryotube, uncapped and rapidly plunged into LN for at least 1 h. After thawing in liquid MVW containing 1 M sucrose for 15 min at room temperature, cryo-plate was rinsed with liquid MVW supplemented 2% sucrose. The SE was gently transferred from the alginate, and then the naked SE was cultured on the regrowth medium (Fe-free solid MVW medium containing 0.1 mgl-1 NAA, 0.2% (w/v) PVP-40 and 0.2 % (w/v) AC). These post-cryopreserved SEs were maintained in the darkness for 7 d before being transferred to light condition. The percentage of survival rate and visual observation were evaluated after culture under light condition for 7 d.

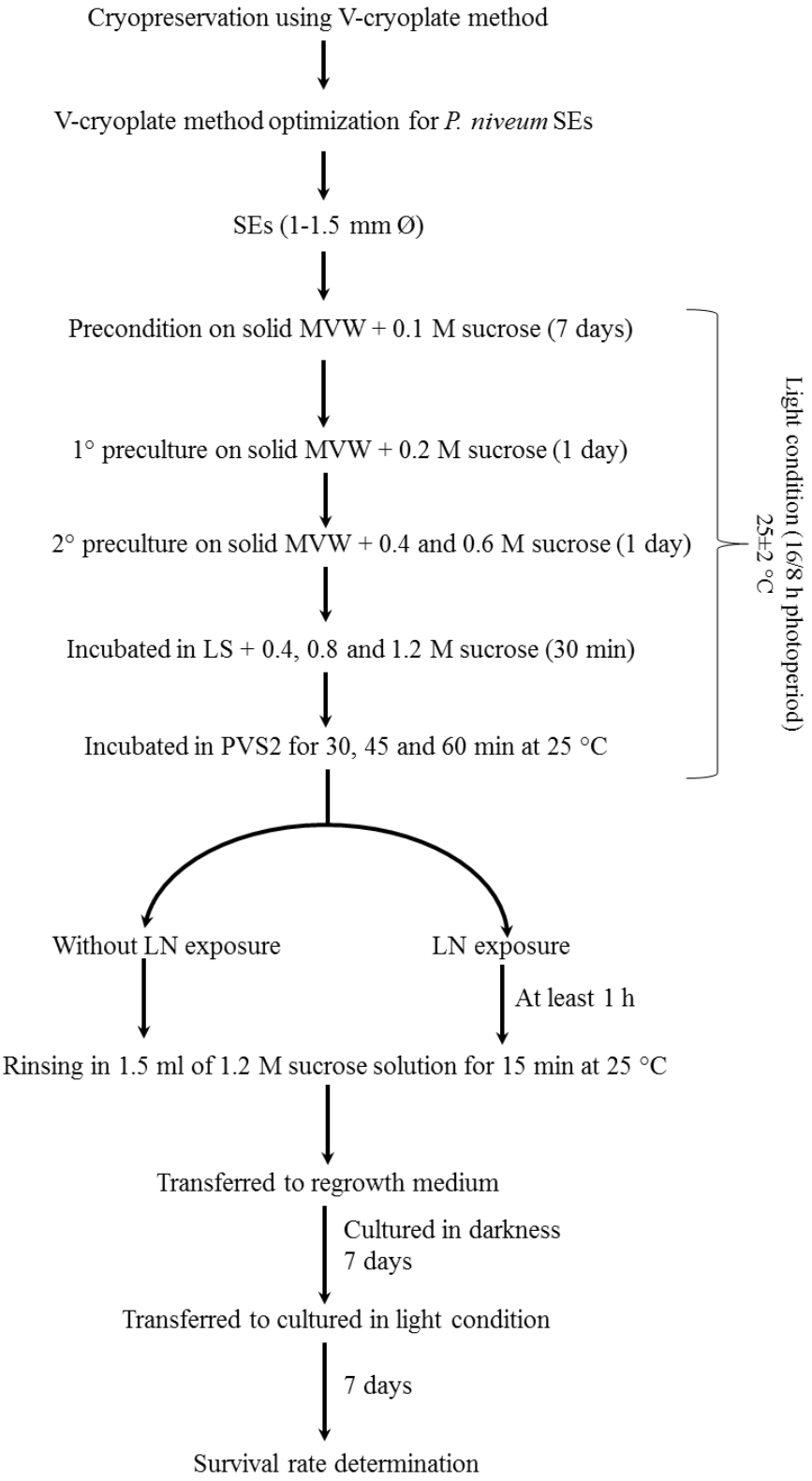

Figure 7 Summarized diagram of the cryopreservation optimization by V cryo-plate

2.5.5 Water content determination (WC)

The WC of SEs from the best condition was examined followed by Khoddamzadeh et al. (2011). Three replicates of six SEs from intact SEs (the control), precondition, preculture, osmoprotection and various dehydration period (30, 45 and 60 min) were weighed and then dried in the hot air oven (130 °C/ 24 h), and then reweighed. The percentage of WC was calculated using the equation below

$$WC\ \% = \frac{[FW - DW]}{FW}\ X\ 100$$

FW = Fresh weight of SEs

DW = Dry weight of SEs

2.5.6 Ascorbic acid (AA) supplementation in the optimized V cryo-plate method

The AA (0.1 mM) supplementation was conducted on day 7 of culture for 1 d before the beginning of the 1st preculture. The total ROS and MDA content were measured during V cryo-plate method at the 1st preculture, 2nd preculture, osmoprotection and dehydration steps. The intact SE was used as the control. The survival percentage of the AA-treated and the non AA-treated (from both -LN and +LN) was determined. The treatment was performed with 3 replicates, each with 6 samples (SEs).

2.5.7 Determination of total ROS

The determining total ROS was measured according to the protocol as described by Jambunathan (2010). The three replications of six SEs were ground in LN. A ground powder of SEs was homogenized with 1 ml of 10 mM Tris buffer (pH 7.2) and centrifuged at 12,000X g for 20 min at 4°C. The 1 ml of sample mixture (100 µl supernatant and 900 µl Tris-buffer) was added with 10 µl of 1 mM DCFDA and then vortexed. The sample mixture was incubated in darkness for 10 min prior to measurement. The sample mixture with DCFDA, the sample mixture without DCFDA (the control) and 1 ml Tris-buffer (the blank) were measured using a spectrofluorometer (FP-8200, JASCO). The absorbance was read at 504 nm and 524 nm. The total ROS content was calculated by standard curve of protein which determined by Bradford reagent. The data was reported as the relative of the total ROS unit per mg of protein.

2.5.8 MDA analysis

The lipid peroxidation was measured by MDA assay (Verleysen et al., 2004). The three replications of of SEs were weighed (ca. 100 mg) and added with the reaction mixture containing 700 µl of deionized water and 750 µl of TBA reagent (0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA)). And then, the samples mixture was boiled at 95 °C for 25 min, rapidly cooled on ice (5 min) and then centrifuged at 1000X g (10 min).

The absorbance was measured at 532 and 600 nm against TBA reagent (the blank). The concentration of MDA is calculated using the extinction coefficient of MDA (155 mM^{-1} cm^{-1}) from Beer–Lambert's equation (Health and Packer, 1968). The MDA contents was compared between the AA-treated and the non AA-treated SEs.

2.6 Statistical analysis

The experiment of somatic embryogenesis and cryopreservation were designed using a completely randomized design (CRD). Ten replicates were prepared for somatic embryogenesis and three replicates were used in each treatment of cryopreservation. The mean values were subjected to analysis of variance (ANOVA) and separated using Duncan's multiple range tests (DMRT) at $P \leq 0.05$. A three-way ANOVA was used for a comparison effect between the variables during the interaction of the step in cryopreservation. Statistical analysis was performed using SPSS statistics software.

CHAPTER 3
RESULTS

3.1 *In vitro* cloning via direct somatic embryogenesis

3.1.1 SEs induction

SE were formed on solid MVW medium supplemented with NAA only (0.1 and 0.3 mgl^{-1}) and 0.1 mgl^{-1} NAA in combination with TDZ (0.1, 0.5 and 1 mgl^{-1}) after a culture period of 3 months. Among these treatments, the percentage of SE formation of 50-68.33% (Table 9) showed no statistically significant difference. Maximum number of SE per explant (5.19 SEs/explant) was obtained on this basal medium supplemented with 0.1 mgl^{-1} NAA. However, number of SE per explant tended to decrease after culturing on media containing 0.1 mgl^{-1} NAA combined with higher concentrations (0.1-0.5 mgl^{-1}) of TDZ. Moreover, the treatment of 0.1 mgl^{-1} NAA also provided survival rate of 87.5%, which was on par with the control (95%), and were greater than those of other treatments.

Table 9 Effects of NAA and TDZ on SE induction of *Paphiopedilum niveum*. Data derived from four-month-old protocorms cultured on MVW medium supplemented with various concentrations of PGRs and grown in darkness for 3 months.

PGR (mgl^{-1})		Survival rate (%)	SE formation (%)	Number of SEs per explant
NAA	TDZ			
0	0	95.00±3.33^{a}	0.00±0.00^{b}	0.00±0.00^{c}
0	0.1	52.50±7.86^{bcd}	0.00±0.00^{b}	0.00±0.00^{c}
0	0.5	45.00±6.23^{bcdef}	0.00±0.00^{b}	0.00±0.00^{c}
0	1	50.00±7.45^{bcde}	0.00±0.00^{b}	0.00±0.00^{c}
0.1	0	87.50±4.16^{a}	68.33±11.77^{a}	5.19±0.67^{a}
0.1	0.1	55.00±8.98^{bcd}	50.00±15.11^{a}	4.60±0.78^{ab}
0.1	0.5	60.00±5.53^{bc}	61.67±13.62^{a}	3.38±0.78^{ab}
0.1	1	40.00±4.08^{def}	53.33±11.86^{a}	2.67±0.75^{b}
0.3	0	62.50±4.17^{b}	60.00±12.72^{a}	4.52±0.82^{ab}
0.3	0.1	57.50±3.82^{bcd}	0.00±0.00^{b}	0.00±0.00^{c}
0.3	0.5	30.00±6.24^{f}	0.00±0.00^{b}	0.00±0.00^{c}
0.3	1	27.50±4.49^{f}	0.00±0.00^{b}	0.00±0.00^{c}
0.5	0	42.50±3.82^{cdef}	0.00±0.00^{b}	0.00±0.00^{c}
0.5	0.1	30.00±3.33^{f}	0.00±0.00^{b}	0.00±0.00^{c}
0.5	0.5	35.00±4.08^{ef}	0.00±0.00^{b}	0.00±0.00^{c}
0.5	1	55.00±6.24^{bcd}	0.00±0.00^{b}	0.00±0.00^{c}

Values shown above represent the mean±standard error (S.E.). Comparison of the mean values was analyzed using the Duncan's Multiple Range Test (DMRT). Values with different letters indicate significant differences at $P \leq 0.05$.

3.1.2 SEs proliferation

One-month old protocorm-derived SEs cultured on PGR-free medium in light conditions were grown on solid MVW supplemented with NAA (0, 0.1, 0.3 and 0.5 mgl^{-1}), or in combination with kinetin (0, 1, 5 and 10 mgl^{-1}). The largest increase in fresh weight (FW) of SE (183.33 mg/100 mg of initial fresh weight) and maximum survival rate (88.33%) were obtained on the PGR-free control media (Table 10). The combination of NAA and kinetin appeared to lower the increased of FW in comparison to the control, and some treatments exhibited undesirable effects. For instance, the use of 0.3 mgl^{-1} NAA combined with 1 or 5 mgl^{-1} kinetin showed a decrease in percentage of survival, and the deterioration of color of the SE.

Table 10 Effects of NAA and kinetin on SE proliferation, survival, and color of the regenerated SEs of *Paphiopedilum niveum.* Data derived from SE clumps cultured on MVW supplemented with single and combination of NAA and kinetin after culture for 2 months

PGR (mgl^{-1})		Increased FW (mg)/ 100 mg initial FW	Survival (%)	Visual observation (Color of SEs)
NAA	kinetin			
0	0	183.33±28.93^{a}	88.33±0.77^{a}	Green
0	1	62.32±23.49^{cd}	80.00±1.02^{ab}	Green
0	5	93.44±29.79^{abcd}	78.33±1.03^{ab}	Green
0	10	93.55±34.96^{abcd}	55.00±1.35^{ab}	Yellow
0.1	0	158.05±34.76^{abc}	83.33±1.11^{ab}	Green
0.1	1	128.85±20.87^{abcd}	80.00±0.83^{ab}	Green
0.1	5	82.25±27.94^{bcd}	80.00±0.83^{ab}	Green
0.1	10	168.96±36.40^{ab}	86.67±0.99^{a}	Green
0.3	0	56.90±20.51^{d}	80.00±1.10^{ab}	Green
0.3	1	147.18±34.36^{abcd}	50.00±1.18^{ab}	Yellow
0.3	5	93.42±25.80^{abcd}	46.67±1.38^{b}	Yellow
0.3	10	122.38±19.43^{abcd}	73.33±1.16^{ab}	Green
0.5	0	95.78±17.38^{abcd}	60.00±1.43^{ab}	Yellow
0.5	1	140.48±42.27^{abcd}	76.67±1.19^{ab}	Green
0.5	5	127.70±35.97^{abcd}	76.67±1.19^{ab}	Green
0.5	10	109.00±23.92^{abcd}	76.67±1.00^{ab}	Green

Values shown above represent the mean±standard error (S.E.). Comparison of the mean values was analyzed using the Duncan's Multiple Range Test (DMRT). Values with different letters indicate significant differences at $P \leq 0.05$, FW: fresh weight

3.1.3 Developmental pattern via direct somatic embryogenesis (DSE)

Small protruding shoots of SEs (the primary SEs) were observed on four-month-old protocorms cultured on MVW containing 0.1 mgl^{-1} NAA in the darkness for 2 months (Fig. 8C). These SEs eventually developed to form the obvious character of SEs, with distinctive shoots showing within 3 months of culture (Fig. 8D). The clusters of primary SEs also exhibited healthy green shoots after being transferred onto PGR-free MVW under light for a month (Fig. 8E). Two months after the transfer, secondary SEs were observed on the primary SEs (Fig. 8F). The primary and secondary SEs with well-developed shoots were then excised into single SE for use as explants, and were subsequently transferred to the plantlet regeneration medium (MVW containing 2 gl^{-1} activated charcoal and 50 gl^{-1} banana homogenate). The shoots, followed by autonomous rooting without any PGRs, were obtained 4 months after the sub-culturing. The plantlets grew well under the greenhouse condition (Fig. 8G). Histological observations revealed that four-month-old protocorms were comprised of two portions of parenchymatous tissues, where the cells of the upper part of protocorm were smaller meristematic cells with large nuclei compared to those at the basal part (Fig. 9A). At the initial stage of culture on SE induction medium, cell division of proembryogenic cells were observed at the surface of the protocorm without an intervening callus phase. This is represented by the densely stained cells with large nuclei (Fig. 9B). The division of subepidermal and epidermal cells at basal layer were limited, and enabled the unequal expansion of cell layers which gave rise to the embryo proper (Fig. 9C). Moreover, the histodifferentiation indicated that the regeneration process was direct somatic embryogenesis (DSE), which had occurred within 1 month of culture, with the embryogenic masses subsequently developing into the ‘globular’ stage in the second month of culture (Fig. 9D). At the third month, the enlargement of multiple primary SE was observed at the basal or lateral part of the original protocorm (Fig. 9E). After these primary SEs were transferred to the media for SE proliferation, , the secondary SE originating on the surface of the primary SE was then observed after culturing on PGR-free MVW for 2 months (Fig. 9F). After that, the *ex vitro* plantlet presented the well developed of root system under green house condition (Fig. 9G).

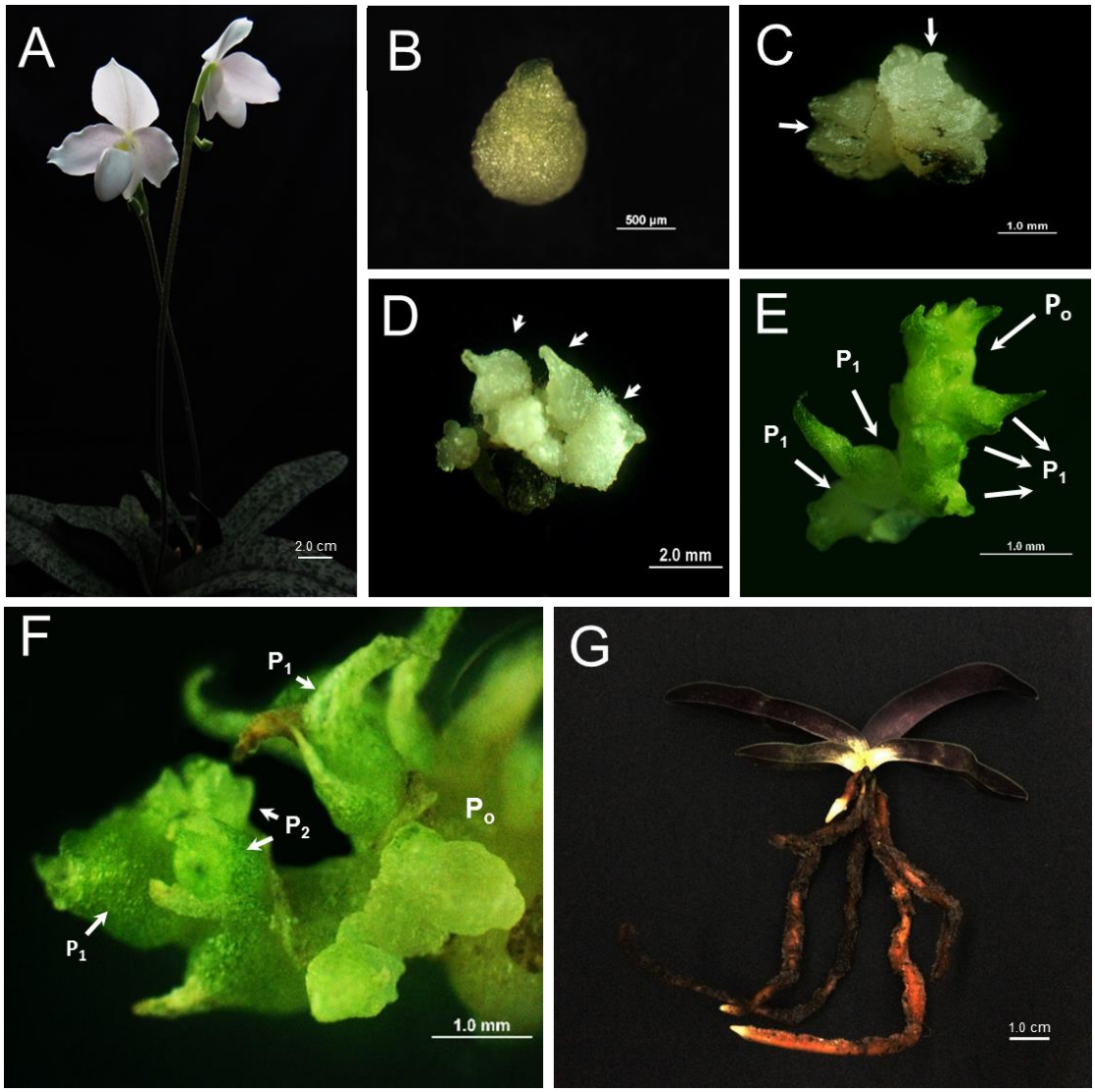

Figure 8 Morphological characteristics during SE formation and proliferation of *Paphiopedilum niveum*. **(A)** Flowering plant. **(B)** Four-month-old protocorm with a small shoot. **(C)** Early stages of primary SEs (arrows) developing directly from protocorms cultured on MVW + 0.1 mgl^{-1} NAA for 2 months in darkness. **(D)** Cluster of 3 month old SEs with distinctive shoots (arrows) cultured on MVW + 0.1 mgl^{-1} NAA **(E)** Original protocorm (P_0) and primary SEs (P_1) exhibiting healthy green shoots after culture on PGR-free MVW for 1 month under light conditions. **(F)** Secondary SEs (P_2) proliferating from the tissue of primary SEs (P_1) growing on the original protocorm (P_0) after a further 2 months of culture on PGR-free MVW. **(G)** Vigorous regenerated plantlets after removal from flasks and grown under greenhouse conditions for 4 months

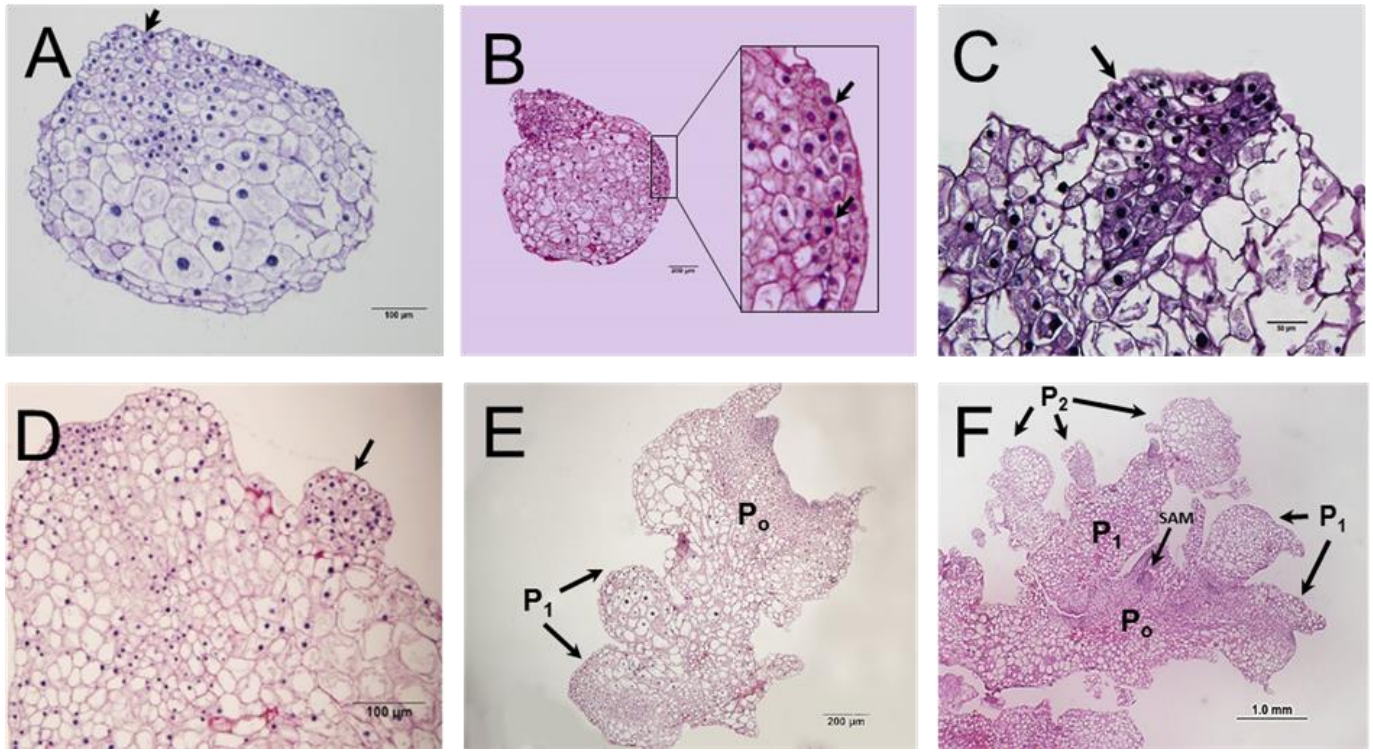

Figure 9 Histological observation on direct somatic embryogenesis (DSE) of *Paphiopedilum niveum*. **(A)** Four-month-old protocorm exhibiting single meristem region at shoot pole (arrow). **(B)** Protocorm showing the initiation of new meristematic tissue, indicated by active dividing cells with densely stained cytoplasm (inset and arrow) after one month of culture on MVW + 0.1 mgl^{-1} NAA. **(C)** Developing SE (globular in shape) represented by the meristematic cells at the epidermal and subepidermal layers (arrows) with heavily stained cytoplasm. **(D)** Primary SE (arrow) exhibiting unequal division of meristemoids emerging from the protocorm surface after culturing for 2 months on MVW + 0.1 mgl^{-1} NAA. **(E)** Primary SEs (P_1) (arrows) developing at the lateral zone of basal part of the original protocorm (P_0) 3 months after culturing on MVW + 0.1 mgl^{-1} NAA. **(F)** Cluster of secondary SEs (P_2) developing on the primary SEs (P_1) which were formed on the original protocorm (P_0) after a further 2 months of culture on PGR-free MVW.

3.1.4 Genetic variation assessment using RAPD

Ten selected RAPD primers were used in the genomic DNA amplification. The assessment revealed that there was no variation between the *in vit*ro mother plant (V_1) and the regenerant clones (V_2 and V_3), as indicated by the identical banding patterns in all the samples (Fig. 10). The results showed a number of scored reproducible bands in clones 1, 2 and 3 of 102, 91 and 98 bands, respectively (Table 1). The total numbers of bands in the V_1–V_3 plantlets of clone 1 (306 bands), clone 2 (273 bands) and clone 3 (294 bands) were analyzed. The sizes of the amplified bands, which the primers chosen could be separated distinctively, were in the range of 150 to 2,500 bp.

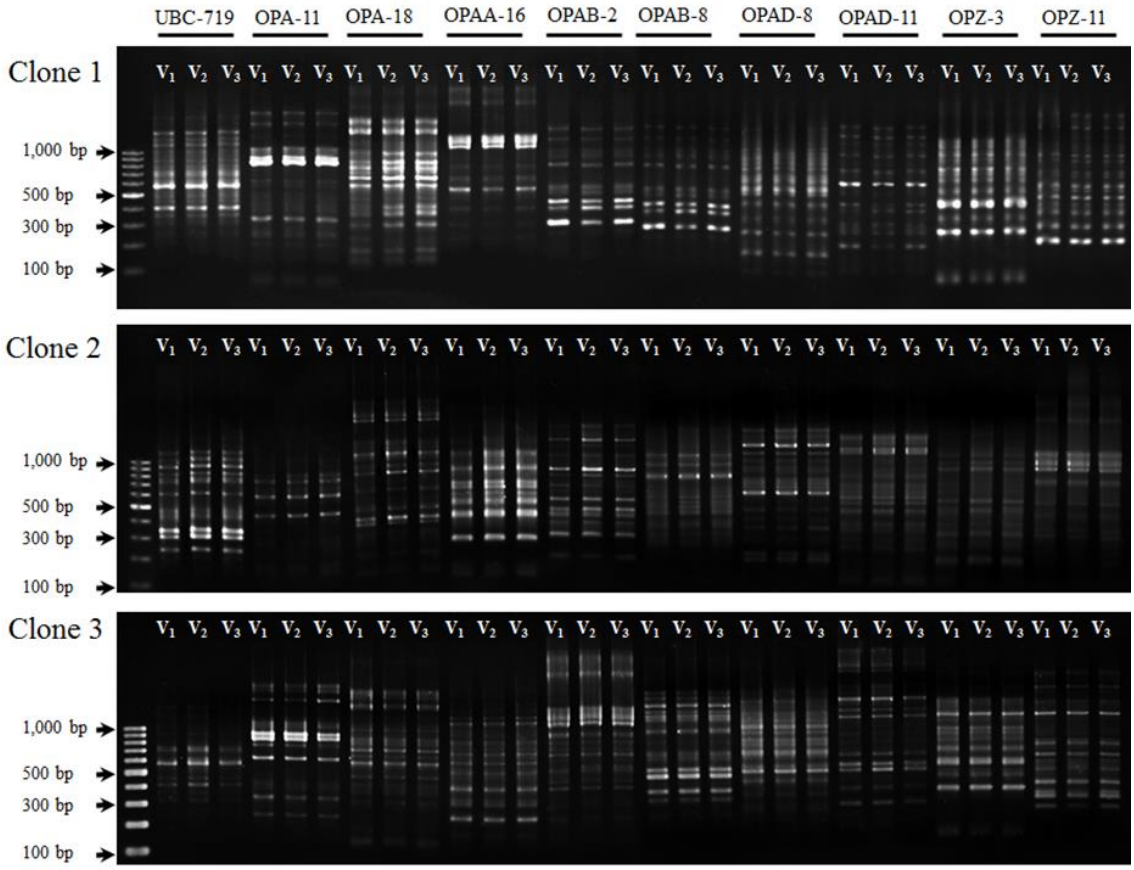

Figure 10 Gel electrophoresis of RAPD from clone 1–3 of *Paphiopedilum niveum* showing monomorphic banding patterns generated using ten selected primers. The mother plant is represented by (V_1), and the regenerant plants by (V_2 and V_3)

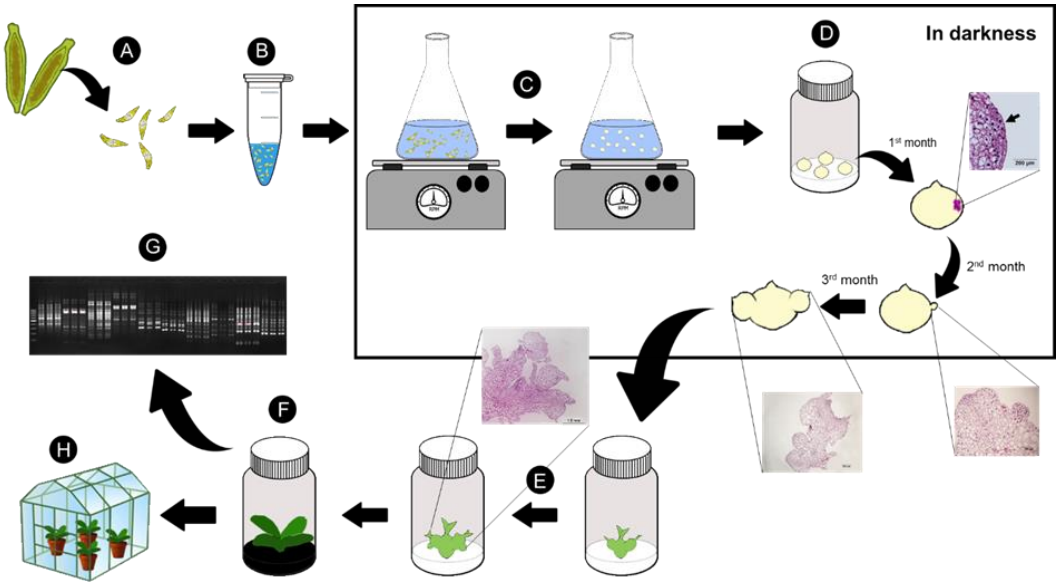

Figure 11 Diagrammatic summary of *in vitro* cloning of genetically uniform *Paphiopedilum niveum* via direct somatic embryogenesis (DSE). (**A**) Seeds were collected from five-month-old capsule. (**B**) Pretreatment with 1% (*v/v*) clorox solution for 60 min. (**C**) Pretreated seeds were cultured in liquid PGR-free MVW in the darkness for 4 months. (**D**) Four-month-old protocorms were cultured on SE induction medium (MVW containing 0.1 mgl^{-1} NAA) for 3 months to induce DSE, (**E**) SE clump proliferated after culture on SE proliferation medium (PGR-free MVW) for 2 month. (**F**) SE has formed a plantlet after culture on MVW containing 0.2% activated charcoal and 50 mgl^{-1} banana homogenate for 4 months. (**G**) Genetic fidelity of *in vitro* plantlets (V_2 and V_3) was examined by RAPD analysis in comparison with the mother plant (V_1). (**H**) Four-month-old plantlets have acclimatized and grew well under greenhouse conditions.

3.2 Cryopreservation by V cryo-plate method

3.2.1 The optimization of the V cryo-plate method and water content determination

According to the highest survival percentage of non-cryopreserved (46.67±23.09%) and cryopreserved SEs (20.00±10.33%), the suitable conditions (treatment no. 18, Table 11) for V cryo-plate method of this orchid species were suggested. The appropiate conditions were as follows: samples were in 0.6 M sucrose (1 d) for the 2nd preculture followed by 1.2 M sucrose in LS for 30 min, and then being transferred to exposure time with PVS2 for 60 min. Based on 3-way ANOVA with no significant interaction between/among these mean values (preculture, osmoprotection and dehydration), PVS2 with exposure time' (dehydration period) was the crucial factor affecting the survival percentage of SEs (Table 12). Thus, a shorter period of time in PVS2 (dehydration period) for 30 and 45 min showed lower survival percentage in comparison to the suitable treatment (60 min). The WC after 60 min PVS2 dehydration provided the significant decrease to 19.44±2.29% which was lower than that after 30 min (26.94±0.76%) and 45 min (24.71±1.60%) PVS2 dehydration (Fig. 12).

Table 11 Factors (2nd preculture, osmoprotection and dehydration) affecting the survival percentage of *Paphiopedilum niveum* SEs. Data were taken after culture on regrowth medium for 14 days

Steps in V cryo-plate						
Treatment No.	Preculture		Osmoprotection	Dehydration	Survival (%)	
	Sucrose (M)		Sucrose (M)			
	1st*	2nd*	LS containing 2 M glycerol + sucrose**	PVS2 exposure time (min)	-LN	+LN
1	0.2	0.4	0.4	30	13.33±6.67^{abc}	0.00±0.00^{b}
2				45	6.67±6.67^{bc}	0.00±0.00^{b}
3				60	0.00±0.00^{c}	0.00±0.00^{b}
4			0.8	30	33.33±6.67^{abc}	0.00±0.00^{b}
5				45	16.67±16.67^{abc}	0.00±0.00^{b}
6				60	6.67±6.67^{bc}	0.00±0.00^{b}
7			1.2	30	20.00±0.00^{abc}	0.00±0.00^{b}
8				45	0.00±0.00^{c}	0.00±0.00^{b}
9				60	6.67±6.67^{bc}	0.00±0.00^{b}
10		0.6	0.4	30	25.39±12.99^{abc}	0.00±0.00^{b}
11				45	5.57±5.57^{bc}	0.00±0.00^{b}
12				60	35.56±9.87^{ab}	0.00±0.00^{b}
13			0.8	30	6.67±6.67^{bc}	0.00±0.00^{b}
14				45	6.67±6.67^{bc}	0.00±0.00^{b}
15				60	0.00±0.00^{c}	0.00±0.00^{b}
16			1.2	30	20.00±11.55^{abc}	0.00±0.00^{b}
17				45	0.00±0.00^{c}	0.00±0.00^{b}
18				60	46.67±23.09^{a}	20.00±10.33^{a}

The means (mean±S.E.) in column followed by different letters are significantly different at $P \leq 0.05$ with DMRT

*: preculture for a day, **: osmoprotection for 30 min; -LN: non-cryopreserved SEs; +LN: cryopreserved SEs via V cryo-plate method

Table 12 Three-way ANOVA with interaction between all combinations of three main factors (preculture; P, osmoprotection; O and dehydration; D) exhibiting in non cryopreserved *Paphiopedilum niveum* SEs via V cryo-plate method

Factors	DF	MS	F	*P*
Preculture (P)	1	600.00	1.898	ns
Osmoprotection (O)	2	7.41	0.023	ns
Dehydration with PVS2 (D)	2	1258.91	3.983	*
Interaction P X O	2	822.22	2.606	ns
Interaction P X D	2	921.50	2.916	ns
Interaction O X D	4	590.30	1.868	ns
Interaction P X O X D	4	201.22	0.637	ns
Error	36			
Total	53			

DF: Degree of freedom; MS: Mean square; F: F-ratio

* indicated significant difference at $P \leq 0.05$, ns: non-significant difference

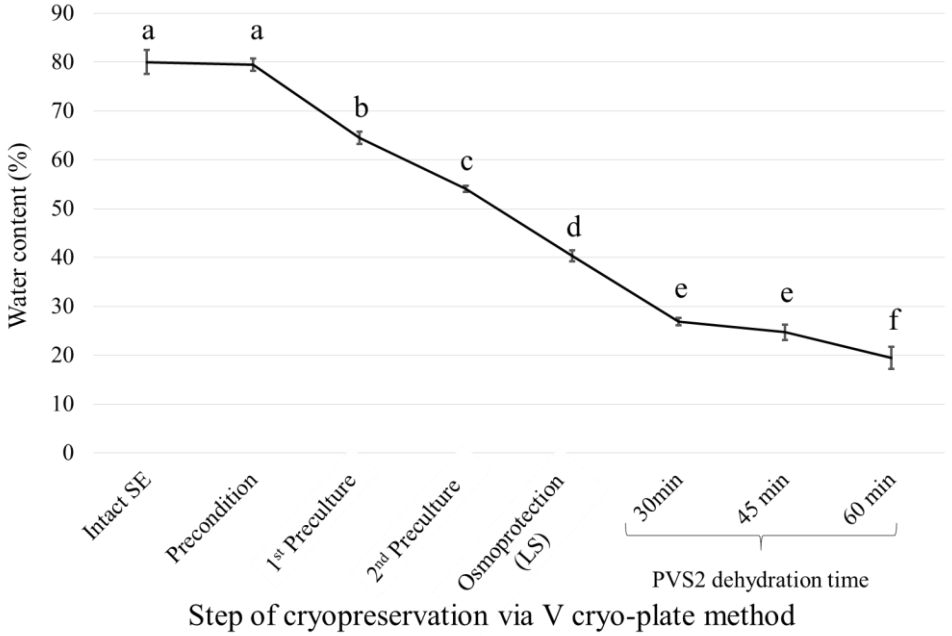

Figure 12 Percentage of water content in *Paphiopedilum niveum* SEs during cryopreservation using V cryo-plate method. Data were taken from the optimized protocol (treatment 18). Means with the same letter are not significantly different at $P \leq 0.05$ as determined by DMRT.

3.2.2 Effect of ascorbic acid (AA) supplementation on total ROS, MDA level, and survival of AA treated-SEs during the optimized V cryo-plate method

In our study, the AA supplementation was performed on day 7 of the culture (before the start of the 1st preculture), the total ROS and MDA levels were determined during the steps of the optimized V cryo-plate protocol, namely; precondition, the 1st preculture, the 2nd preculture, osmoprotection and dehydration. The non AA-treated SEs was used as a control. The survival percentage and visual observation were taken after 14 d of culture on regrowth medium.

The normal level of total ROS (8.09±1.12 ROS unit/ µg protein) was obtained from the intact SE. In the case of non AA-treated SEs, the increase of total ROS level was initially detected at precondition step (11.54±1.02 unit/ µg protein) and reached to the maximum level at the 1st preculture (17.18±0.52 ROS unit/ µg protein) (Fig. 13A). Thereafter, the ROS level, which was high at the 1st preculture, was gradually decreased to normal level at osmoprotection step and then markedly increased again at dehydration step. This result indicated that the arising of oxidative stress occured in the precondition step. In contrast, AA-treated SEs showed the low level of ROS production through 1st preculture to dehydration step. (Fig. 13A).

The result of MDA analysis showed that the MDA level was not significantly different in intact SEs (1.13±0.09 µM MDA/ 100 mg fresh weight) and SEs at the precondition step (1.08±0.07 µM MDA/ 100 mg fresh weight). Meanwhile, the continuous increase of MDA level was found from the 1st preculture to dehydration step which showed the highest MDA level in osmoprotection (8.34±0.11 µM MDA/ 100 mg fresh weight) (Fig. 13B). In contrast, the efficiency of AA treatment could efficiently reduce and stabilize MDA level from the 1st preculture to dehydration in AA-treated SEs. The reduction of total ROS and MDA level enabled improvement the survival rate in AA-treated non-cryopreserved SE (42.86±6.15%) and AA-treated cryopreserved SE (39.04±8.50%) which was significantly higher than non-cryopreserved (25.71±6.85%) and cryopreserved (8.57±3.40%) of non AA-treated SEs (Fig. 14). From visual observation, non AA-treated cryopreserved SEs exhibited the browning (Fig. 14A) while AA-treated cryopreserved SEs presented white color of the viable shoot after culture on regrowth medium for 14 days (Fig. 14B). The optimal V cryo-plate protocol for *P. niveum* SEs was illustrated in Figure 15.

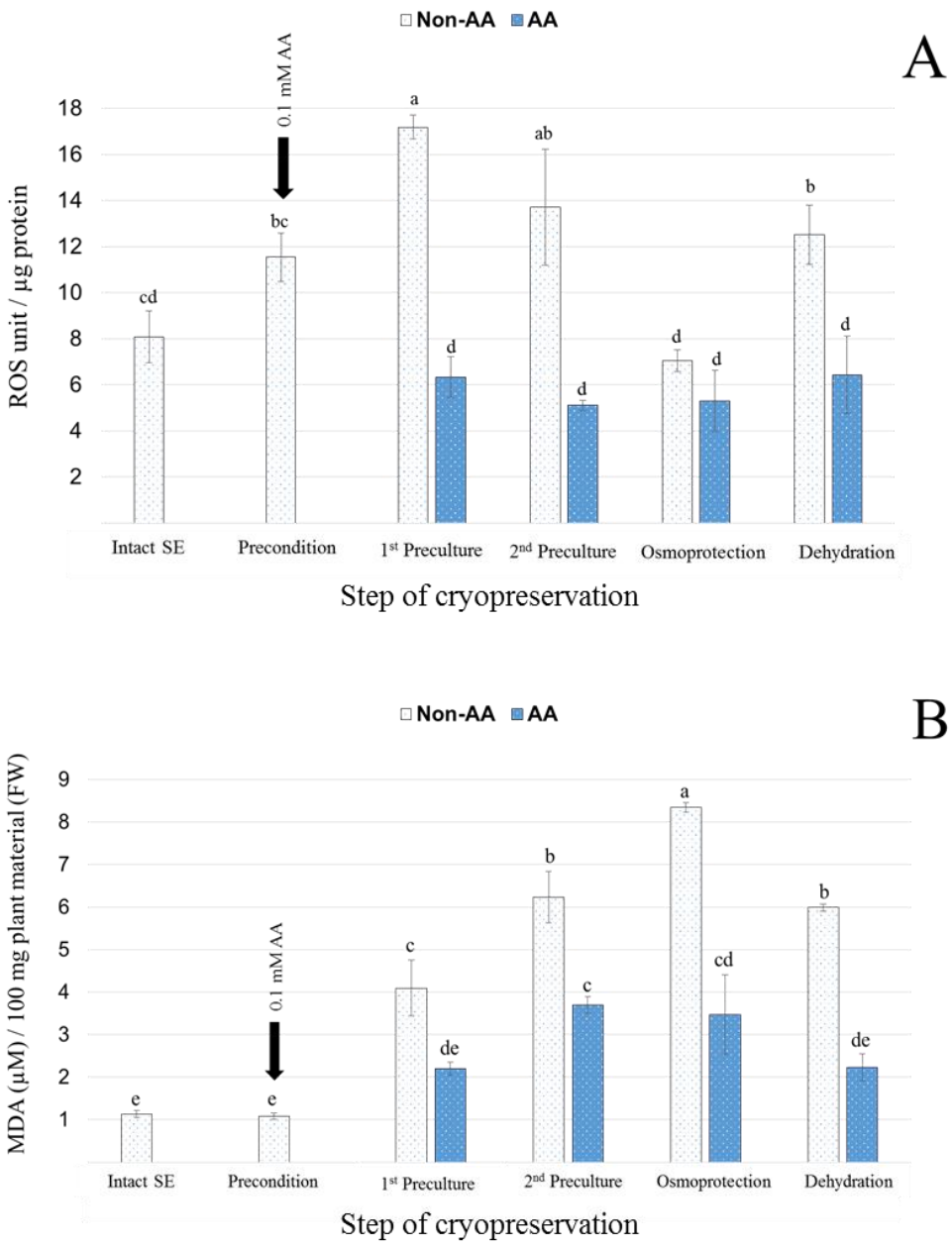

Figure 13 Determination of (A) total ROS and (B) MDA level during the optimized V cryo-plate method of non AA-treated and AA-treated cryopreserved *Paphiopedilum niveum* SEs. Application of ascorbic acid (AA) at 0.1 mM was done at the 7th day precondition (arrow). The optimized V cryo-plate method comprised of (1) precondition (MVW containing 0.1 M sucrose for 7 d), (2) two steps of preculture (MVW containing 0.2 M (1st preculture) and 0.6 M (2nd preculture) for 1 d each), (3) osmoprotection (loading solution containing 1.2 M sucrose for 30 min) and (4) dehydration (exposure time to PVS2 for 60 min at 25 °C). Means±S.E. with the same letter are not significantly different at $P \leq 0.05$ as determined by DMRT.

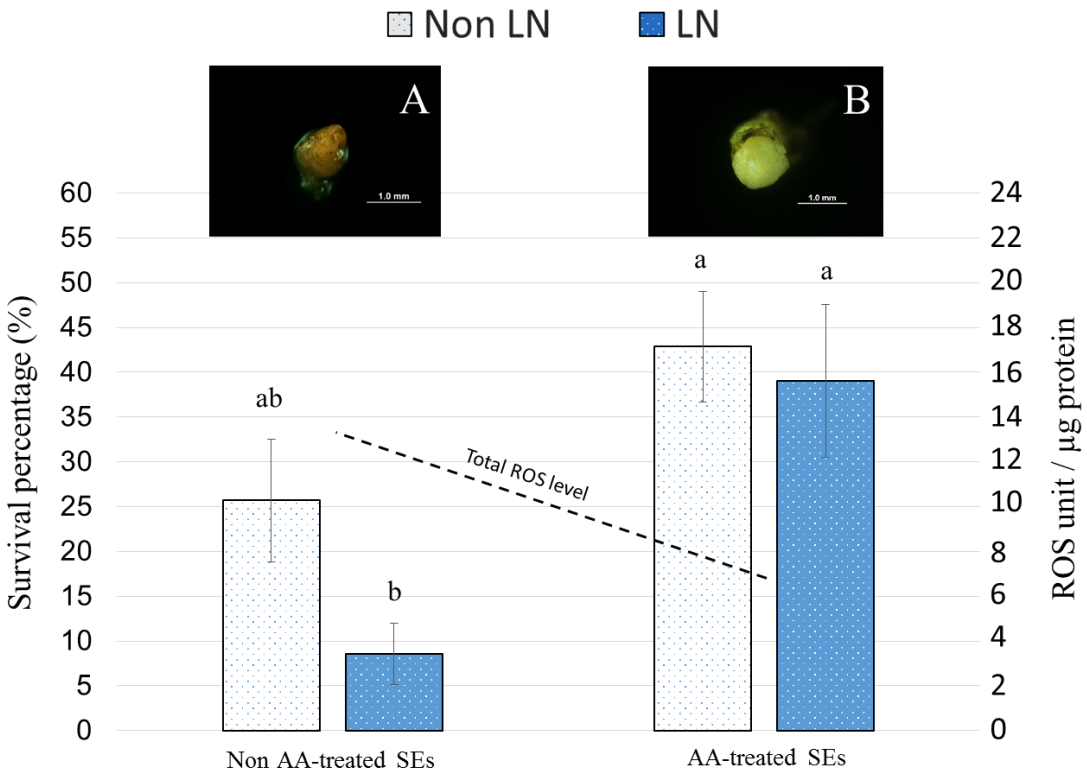

Figure 14 Survival percentage of non AA-treated and AA-treated *Paphiopedilum niveum* SEs compared to non-cryopreserved control SEs. Visual observation presenting (A) the browning of non AA-treated cryopreserved SE in comparison with (B) a lutescent shoot of viable AA-treated cryopreserved SE after culture on regrowth medium for 14 days. Means±S.E. with the same letter are not significantly different at $P \leq 0.05$ as determined by DMRT. AA: ascorbic acid (0.1 mM) application, Non LN: Non cryopreservation, LN: cryopreservation in liquid nitrogen.

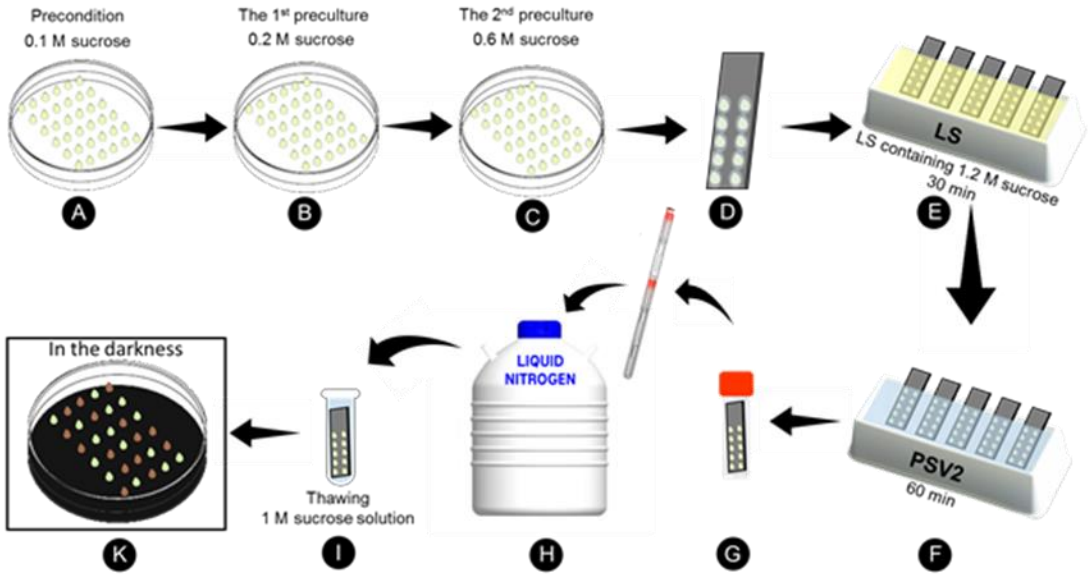

Figure 15 Schematic diagram of cryopreservation of *Paphiopedilum niveum* SEs using V cryo-plate method. (A) SEs were preconditioned on PGR-free MVW containing 0.1 M sucrose (7 d), followed by (B) the 1st preculture in MVW containing 0.2 M sucrose (1 d) and (C) the 2nd preculture in MVW containing 0.6 M sucrose for 1 d. (D) Precultured SEs were embedded onto cryo-plate using 2% alginate gel. (E) Cryo-plates were immersed into LS containing 1.2 M sucrose for 30 min, (F) dehydrated by PVS2 for 60 min (G) Cryotube containing cryo-plate was affixed on cryocane and then (H) plunged into LN. (I) Cryopreserved SEs were thawed in 1 M sucrose solution and (K) cultured on regrowth medium (Fe-free MVW medium containing 0.1 mgl^{-1} NAA, 0.2% (w/v) PVP-40 and 0.2% (w/v) AC) for 7 d in the darkness and then transferred to light condition.

CHAPTER 4
DISSCUSSION

4.1 *In vitro* cloning by direct somatic embryogenesis

4.1.1 SEs induction

The application of NAA at lower concentration (<0.1 mgl^{-1}), either singly or in combination with BA, has reportedly been able to induce organogenesis in many plant species. For instance, direct organogenesis has been reported after the leaf explant of *Hydrangea macrophylla* was cultured on B5 medium containing 0.05 mgl^{-1} NAA with 2.25 mgl^{-1} BA for 50 days (Liu et al., 2011). Gupta et al., (2013) also reported that the direct rhizogenesis (derived from leaf explant) and multiple shoot regeneration (originated from stolon) of *Glycyrrhiza glabra* could be induced on liquid and solid MS supplemented with 0.01 mgl^{-1} NAA only. In orchid species, direct somatic embryogenesis of *Oncidium flexuosum* (15-80%) could be induced from leaf explant after culture on ½ MS containing lower concentration (0.25 µM or 0.05 mgl^{-1}) of NAA in combination with 1.5-13.5 µM (0.33-2.97 mgl^{-1}) TDZ under the darkness (Mayer et al., 2010). Parthibhan et al., (2018) also reported that tTCL stem of *Dendrobium aqueum* culturing on ½ MS containing very low (0.01 mgl^{-1}) NAA combined with 0.5 mgl^{-1} BA could stimulate both indirect somatic embryogenesis (11.67%) and direct somatic embryogenesis (10%). Ahamed Sherif et al., (2018), however, revealed that the single use of 0.5 mgl^{-1} NAA promoted the potential for induction of nodal-derived direct somatic embryogenesis (36.5%) of *Anoectochilus elatus*. No evidence on SE induction of *Paphiopedilum niveum* using NAA at lower concentration has been reported prior to our current study, where we now report that the supplementation of NAA at low concentration (0.1 mgl^{-1}) could promote the SE formation in this species.

Various types of orchid explants have previously been reported to form SEs in response to the exogenous auxin and cytokinin. Chen and Chang (2001) reported that the use of ½ MS supplemented with 0.3 and 1 mgl^{-1} IAA, or only 0.3 mgl^{-1} NAA, was sufficient to induce the direct somatic embryogenesis (DSE) from the leaf of *Oncidium* 'Gower Ramsey', while a single cytokinin application (TDZ, BA, zeatin, and BA) showed greater results in terms of percentage of somatic embryogenesis formation, and number of SEs per explant. In addition, *Spathoglottis plicata* SEs could be induced from the rhizome-like structures after culture on Phytamax™ orchid maintenance medium containing 2 µM 2-4,D or 0.75 µM IAA for 35 days (Novak and Whitehouse, 2013). Some orchids required the combination of auxin and cytokinin to promote DSE induction. For instance, the DSE of *Cymbidium bicolor* (Mahendran and Bai, 2012) and

Anoectochilus elatus (Ahamed Sherif et al., 2018) could be induced from nodal segments cultured on modified Mitra medium supplemented with 4.54 µM TDZ and 2.69 µM NAA, and also from protocorms cultured on ½ MS containing 1.0 mgl^{-1} BAP and 2.0 mgl^{-1} 2-4,D. Our results show that low concentrations of NAA (0.1 mgl^{-1}) had a potential to induce DSE in *P. niveum*, while the combination of NAA and TDZ reduced the number of SEs as TDZ concentration increases (Table 9). The latter is consistent with the results of Vogel and Macedo (2011), who reported that the number of *Cyrtopodium glutiniferum* SEs decreased significantly after culturing on media containing high TDZ (5 µM) when compared with low TDZ (1 µM) treatments. Malabadi et al., (2004) also demonstrated similar results that higher concentrations of TDZ (13.62-22.71 µM) in VW could decrease SEs numbers of *Vanda coerulea* compared to those treated with 11.35 µM TDZ. Guo et al., (2011) revealed that the possibility of TDZ being an inhibitor of cytokinin oxidase leads to stimulate the accumulation of endogenous purine-based cytokinin (BA). This event may be responsible for the suppression of embryogenesis in some species such as orchard grass (*Dactylis glomerata*) and wheat (*Triticum aestivum*) (Van Staden et al., 2008), and in Cassava (*Manihot esculenta*) (Wongtiem et al., 2011). However, Zeng et al., (2013) revealed that seed-derived protocorms of *Paphiopedilum hangianum* could be induced to form the DSE after culture on modified ½ MS supplemented with 5 mgl^{-1} kinetin and 2 mgl^{-1} BA for 90 days. These reports demonstrated that the different types of explant sources, as well as the type and concentration of PGRs used, were essential factors for SE formation that involves the dedifferentiation of the somatic cells (Ji et al., 2011). Our results has now demonstrated that DSE induction from young protocorm of *P. niveum* can also be triggered even with low auxin concentrations.

4.1.2 SEs proliferation

According to our study, the maximum increased FW was found in the control (free-PRGs medium) but the combination of NAA and kinetin did not presented the suitable result. The combination of NAA and kinetin provided positive results in SE proliferation of *Dendrobium officinale* when cultured on MS medium supplemented with 1 mgl^{-1} kinetin, 0.2 mgl^{-1} NAA and 10% coconut water (Zhang et al., 2011). However, our results demonstrated the single application of high NAA (0.5 mgl^{-1}) and high kinetin (10 mgl^{-1}) reduced the survival rate of the SE of *P. niveum*, and resulted in a change of SE color from green to yellow compared to those cultured on lower concentrations of both NAA and kinetin. Our results were also consistent with Chen et al., (2002), who reported that the use of high kinetin concentrations (4.65 µM) could decrease the number of SEs in *Epidendrum radicans* as compared with lower

kinetin concentrations (1.39 µM). In addition, Ng and Saleh (2011) reported that the SE proliferation pattern of *P. rothschildianum* exhibited autonomous proliferation of secondary SEs from primary SEs, which occurred after a transfer from ½ MS medium containing low kinetin (0.86 mgl^{-1}) onto PGR-free ½ MS medium supplemented with 60 gl^{-1} banana homogenate. Our results showed that SEs could be proliferated from young protocorms cultured on MVW containing 0.1 mgl^{-1} NAA and subsequently transferred to PGR-free MVW. The increase of endogenous auxin accumulation via exogenous auxin supplementation has been reported to be necessary for the early stage of SE formation (Su and Zhang, 2009). Decreasing of auxin level could then allow further proliferation and differentiation of the SE (Yang and Zhang, 2010), and stimulate shoot formation (Novak et al., 2014).

4.1.3 Histological observation

The recent study exhibited that proembryogenic cells originated from epidermal and subepidermal layers of *P. niveum* protocorm without an intervening callus. This histological evidence presented here concerning the newly regenerated SE also coincides with the findings on the investigation of *Phalaenopsis* orchid somatic embryogenesis by Lee et al., (2013).These regenerated cells then formed a globular-shaped mass and differentiated to the SE, which was similar to SE observed in *Cymbidium bicolor* (Mahendran and Bai 2012), *Tolumnia* Louise Elmore 'Elsa' (Shen et al., 2018), *Phalaenopsis amabilis* and *Phalaenopsis* 'Nebula' (Gow et al., 2010).

4.1.4 RAPD analysis

RAPD-PCR analysis have been successfully applied to determine the genetic fidelity assessment in various clonal micropropagated plant species, for instance, in three-month-old regenerated plantlets of *Dendrobium* Second Love cultured *ex vitro* (Ferreira et al., 2006), and six-month-old *in vitro Alhagi maurorum* (Agarwal et al., 2015). In our study, RAPD analysis exhibited identical banding patterns, confirming that genetic homogeneity between the mother/original plant (V_1) and four-month-old *in vitro* regenerated plantlets (V_2-V_3) of *P. niveum* has been conserved. Krishna et al., (2016) also revaled that *in vitro* plant regeneration through embryogenesis with lower DNA methylation levels provided better chance of obtaining plants with genetic uniformity than organogenesis pathway. Other important factors that contributed to an increase of somaclonal variations include the type of PGRs selected (Bairu et al., 2011), as well as prolonged culture periods (Martin et al., 2004; Krishna et al., 2016). In the present study, NAA application showed no genetic alteration as compared to strong synthetic PGRs (TDZ and 2,4-D) as revealed by Bairu et al., (2011), as the absorbed NAA can be often

converted to the inactivated form, mainly glucosyl ester by enzymatic conjugation with glucose (Machakova et al., 2008; Sauer et al., 2013). However, increased somaclonal variation and epigenetic change have been observed when callus of cocoa (*Theobroma cacao*) was cultured for an extended period of time (Rodríguez López et al., 2010).

4.2 Cryopreservation by V cryo-plate method

In our study, the crucial factor affecting the survival of *P. niveum* SEs was the exposure time to PVS2 for 60 min (dehydration step) by V cryo-plate method. This condition also revealed that the suitable WC (~20%) was obtained from this PVS2 dehydration for 60 min while short dehydration time (30 and 45 min) gave the unfitting WC (>20%). It was consistent with Engelmann-Sylvestre and Engelmann (2015) who reported that the PVS2 dehydration for 60 min provided 71% survival of cryopreserved *Clinopodium odorum* shoot tip via V cryo-plate method and low survival percentage (>40%) was found after shorter PVS2 dehydration period (10-50 min). It was possible that insufficient dehydration time provided high intracellular WC leading to ice crystallization during cryopreservation at ultra-low temperature (Quain et al., 2009). The high residual WC (>25%), making cryopreserved *P. niveum* SEs difficult to survive, should be considered for cryopreservation (Chaireok et al., 2016). Sakai (2000) also mentioned that determination of WC was required for minimizing the cryoinjury by the balancing of intracellular WC.

. Our results demonstrated that the potential of the single treatment of low (0.1 mM) AA concentration in the critical step (before the arising of oxidative stress) could improve the survival of cryopreserved SEs of *P. niveum*. No research in determining the total ROS level on survival of any cryopreserved plant material has been reported. Uchendu et al. (2010), however, revealed that MDA level was decreased after 0.28 mM AA supplemented in almost step, namely; preculture, LS and thawing, during cryopreservation of blackberry shoot tip (*Rubus hybrida* cv. Chehalem and Hull Thornless). In addition, this AA application (multiple treatment) could enhance the survival percentage of these cryopreserved blackberry shoot tips. Meanwhile, the AA treatment without oxidative stress determination by applying 0.28 mM AA in LS step could enhance a few survival percentage (3.3%) of pulasan shoot tip (*Nephelium rumboutan-ake*) (Chua and Normah, 2011). Ibrahim and Normah (2013) also reported that there were no significant difference between non AA-treated and AA-treated (0.28 mM) cryopreserved mangosteen (*Garcinia mangostana*) shoot tip at different steps (preculture, LS, PVS2, unloading and recovery). Therefore, this is the first study to indicate that total ROS and

MDA level could be considered as indicators required for selection of the appropriate step of AA application during cryopreservation protocol.

CHAPTER 5
CONCLUSION

This study presented the simple protocol of somatic embryo (SE) induction and proliferation without genetic alteration. The primary SEs could be induced from four-month-old protocorm after culture on solid MVW containing 0.1 mgl^{-1} NAA for three months (with monthly subculture interval) in the darkness. The culture condition provided 68% of SE formation with the maximum number of SEs around 5 SEs per explant. These primary SEs presented the highest increase in fresh weight of proliferated SEs (183 mg/100 mg initial FW) when cultured on solid PGRs-free MVW for two months. These obtained SEs exhibiting the vigorous character developed to plantlet after being transferred to MVW supplemented with 0.2% AC and 50 mgl^{-1} BH for four months. The result of RAPD analysis proved that the plantlets which were developed from mother explant (V_1) and regenerated plantlets (V_2 and V_3) showed the uniform genetic.

The research also presented the first successful cryopreservation and the improvement in survival of cryopreserved of *P. niveum* SEs using V cryo-plate method. The optimized V cryo-plate method consisted of 1) SEs (1-1.5 mm Ø) were preconditioned in solid MVW containing 0.1 M sucrose for seven days, 2) and then SEs were treated with the two-step preculture in 0.2 M and 0.6 M sucrose, each with one day, 3) precultured SEs were incubated in LS supplemented with 1.2 M sucrose for 30 min, followed by 4) immersed in PVS2 for 60 min at room tempereture (25 °C). This optimized condition provided ~20% of survival cryopreserved *P. niveum* SEs. This protocol was improved to increase the survival of cryopreserved SEs by applied 0.1 mM AA on the 7^{th} day of precondition step (before the extreme arising of total ROS and MDA). The AA-treatment could decrease and stabilize total ROS and MDA level which improved the survival rate (up to 39%) of cryopreserved *P. niveum* SEs.

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APPENDICES

APPENDIX A

Culture medium

Modified Vacin and Went (1949)

Stock solution		Components	mgl^{-1}
Macronutrient	Stock 1	$(NH_4)_2SO_4$	500
		KH_2PO_4	250
		KNO_3	525
		$MgSO_4 \cdot 7H_2O$	250
	Calcium*	$Ca_3(PO_4)_2$	200
Micronutrient	Stock 2	$MnSO_4 \cdot 5H_2O$	22.3
		$ZnSO_4 \cdot 7H_20$	8.6
		H_3BO_3	6.2
		KI	0.83
		$Na_2MoO_4 \cdot 2H_2O$	0.25
		$CuSO_4 \cdot 5H_2O$	0.25
		$CoCl_2 \cdot 6H_2O$	0.25
	FeEDTA	Na_2EDTA	37.25
		$FeSO_4 \cdot 7H_2O$	27.85
Vitamins	Stock 3	Glycine	2
		Thiamine HCl (B1)	0.1
		Nicotinic acid	0.5
		Pyridoxine HCl (B6)	0.1
		Myo-inositol	100
Additives	Others	Bacto-peptone	2,000
		sucrose	20,000
		phytagel/gelrite	3,000
		activated charcoal	2,000
		1000 mgl^{-1} chitosan (MW=80)	5 mll^{-1}

The pH of medium adjusts to 5.3-5.4
*Dissolved in 1 N HCl

APPENDIX B

Histological studies

Formalin-aceto-alocohol II (FAA II)

Components	
70% EtOH	90 ml
Glacial acetic acid	5 ml
Formalin	5 ml

Fixation period is 48 h

Ethanol-tert-butyl alcohol series (EtOH/TBA series)

No.	Total alocohol (%)	Component (ml)				
		TBA	95% EtOH	100% EtOH	DW	Other
1	5	-	5	-	95	-
2	10	-	10	-	90	-
3	20	-	20	-	80	-
4	30	-	30	-	70	-
5	50	10	40	-	50	-
6	70	20	50	-	30	-
7	85	35	50	-	15	-
8	95	55	40	-	5	-
9	100	75	-	25	-	-
10	-	100	-	-	-	eosin
11	-	100	-	-	-	-
12	-	50	-	-	-	paraffin oil (50 ml)

DW: distilled water, TBA: tert-butyl alcohol

Delafield hematoxylin

Stock solution	Components	
Solution A*	Hematoxylin	4 g
	95% EtOH	125 ml
Solution B	Aluminium alam $(NH_4)Al(SO_4)_2$	8 g
	Distilled water	400 ml
Potassium permanganate solution	$KMnO_4$	0.2 g
	Distilled water	5 ml
Delafield Hematoxylin staining solution	Solution A	200 ml
	Solution B	200 ml
	Glycerine	200 ml
	Total volume	600 ml

Add $KMnO_4$ solution 5 ml in 600 ml of staining solution and incubate for 2 days
*dissolve in hot plate and filter

Safranin

Components	
Safranin O	2 g
Methyl cellosolve (ethylene glycol monoethyl ether)	100 ml
95% EtOH	50 ml
Sodium acetate	2 g
formalin	4 ml
Total volume	145 ml

Deparafifinization and rehydrateion process (Ruzin, 1999)

Immerse slide with paraffinized section in the following protocol

1. Xylene substitute I	10 min
2. Xylene substitute II	10 min
3. Absolute ethanol : Xylene substitute	5 min
4. Absolute ethanol I	2 min
5. Absolute ethanol II	2 min
6. 95% ethanol I	2 min
7. 95% ethanol II	2 min
8. 70% ethanol I	2 min
9. 70% ethanol II	2 min
10. Distilled water	

Delafield's hematoxylin and safranin O staining (Ruzin, 1999)

1.Stain in Delafield's hematoxylin	20-30 min
2. Rinse in distilled water	2 min
3. Acidulated water (1-2 drops of HCl / 100 ml distilled water)	10 sec
4. Rinse in distilled water	10 dips
5. Immerse into 0.1% (w/v) Li_2CO_3	2 min
6. Counterstain with safranin O	>3 min
7. Rinse in acidulated water	2 dips
8. Place into 0.1% (w/v) Li_2CO_3	2 min
9. Rinse with tap water	2 dips
10. Pass through 50% EtOH I, II	A few minutes in each strength
12. Pass through 70% EtOH I, II	A few minutes in each strength
13. Pass through 95% EtOH I, II	A few minutes in each strength
14. Pass through absolute EtOH I, II	A few minutes in each strength
15. Pass through absolute EtOH : Xylene substitute I, II (1:1)	A few minutes in each strength
16. Pass through Xylene I, II	A few minutes in each strength
17. Mount	

Result: Nucleus = Blue; Cytoplasm = Pink

APPENDIX C

RAPD analysis

dNTP

Components	
Each of 100 mM deoxynucleotide (dATP, dGTP, dCTP and dTTP)	50 µl
Deionized water	950 µl

Mix 100 µl of each deoxynucleotide to prepare 5 mM dNTP

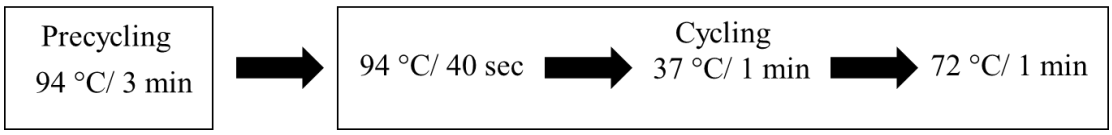
Primer (10 µM)

Components	
Each of 100 mM primer	5 µl
Deionized water	45µl

Mixture for PCR reaction

Components	
20 ng of template DNA	1 µl
dNTP	2 µl
Primer	1.5 µl
ThermoPol™ buffer	2.5 µl
Taq DNA polymerase	0.25 µl
deionized water	17.5 µl
Total volume	24.5 µl

The PCR reaction



total cycling at 40 cycles

APPENDIX D

V cryo-plate method

2% (w/v) Na-alginate solution

Components	
Stock 1 of MVW	10 ml
$Ca_3(PO_4)_2$	20 mg
2% (w/v) Na-alginate	2 g
0.4 M sucrose	13.662 g
Distilled water	Adjust to the final volume to 100 ml

The pH of solution adjusts to 5.3-5.4

$CaCl_2$ solution

Components	
Stock 1 of MVW	100 ml
$Ca_3(PO_4)_2$	200 mg
0.1 M $CaCl_2$	11.098 g
0.4 M sucrose	136.62 g
Distilled water	Adjust to the final volume to 1000 ml

The pH of solution adjusts to 5.3-5.4

Loading solution (LS)

Components	Sucrose concentration in LS		
	0.4 M LS	0.8 M LS	1.2 M LS
Stock 1 of MVW	100 ml	100 ml	100 ml
$Ca_3(PO_4)_2$	200 mg	200 mg	200 mg
Sucrose	136.92 g	273.84 g	410.76 g
2.0 M glycerol	184.18 g	184.18 g	184.18 g
Distilled water	Adjust to the final volume to 1000 ml		

The pH of solution adjusts to 5.3-5.4

Plant Vitrification Solution 2 (PVS2)

Components	
Stock 1 of MVW	100 ml
$Ca_3(PO_4)_2$	200 mg
0.4 M Sucrose	136.62 g
15% (w/v) ethylene glycol	150 g
15% (w/v) DMSO	150 g
30% (w/v) glycerol	300 g
Distilled water	Adjust to the final volume to 1000 ml

The pH of solution adjusts to 5.3-5.4
DMSO: Dimethyl sulfoxide

Unloading solution (1 M sucrose solution)

Components	
Stock 1 of MVW	100 ml
$Ca_3(PO_4)_2$	200 mg
1.0 M sucrose	342.30 g
Distilled water	Adjust to the final volume to 1000 ml

The pH of solution adjusts to 5.3-5.4

Rinsing solution

Components	
Stock 1 of MVW	100 ml
$Ca_3(PO_4)_2$	200 mg
Stock 2 of MVW	0.5 ml
Stock 3 of MVW	1 ml
Sucrose	20 g
PVP-40	2 g
Distilled water	Adjust to the final volume to 1000 ml

The pH of solution adjusts to 5.3-5.4
PVP-40: Polyvinylpyrrolidone-40

Precondition medium

Components	
Stock 1 of MVW	100 ml
$Ca_3(PO_4)_2$	200 mg
Stock 2 of MVW	0.5 ml
Stock 3 of MVW	1 ml
FeEDTA	10 ml
0.1 M Sucrose	34.23 g
phytagel/gelrite	3 g
1000 mgl^{-1} chitosan (MW=80)	5 ml
Distilled water	Adjust to the final volume to 1000 ml

The pH of solution adjusts to 5.3-5.4

Preculture medium

Components	Sucrose concentration	
	0.4 M sucrose	0.6 M sucrose
Stock 1 of MVW	100 ml	100 ml
$Ca_3(PO_4)_2$	200 mg	200 mg
Stock 2 of MVW	0.5 ml	0.5 ml
Stock 3 of MVW	1 ml	1 ml
FeEDTA	10 ml	10 ml
Sucrose	136.92 g	205.38 g
1000 mgl^{-1} chitosan (MW=80)	5 ml	5 ml
phytagel/gelrite	3 g	3 g
Distilled water	Adjust to the final volume to 1000 ml	

The pH of solution adjusts to 5.3-5.4

Regrowth medium (Fe-free)

Components	
Stock 1 of MVW	100 ml
$Ca_3(PO_4)_2$	200 mg
Stock 2 of MVW	0.5 ml
Stock 3 of MVW	1 ml
2 % Sucrose	20 g
PVP-40	2 g
activated charcoal	2 g
1000 mgl^{-1} chitosan (MW=80)	5 ml
phytagel/gelrite	3 g
Distilled water	Adjust to the final volume to 1000 ml

The pH of solution adjusts to 5.3-5.4

PVP-40: Polyvinylpyrrolidone-40

APPENDIX E

Oxidative stress determination

ROS determination

10 mM Tris-buffer

List of chemical	
Tris-HCl	15.76 g
Deionized water	100 ml

The pH of solution adjusts to 7.2

10 mM 2',7' –dichlorofluorescin diacetate (DCFDA)

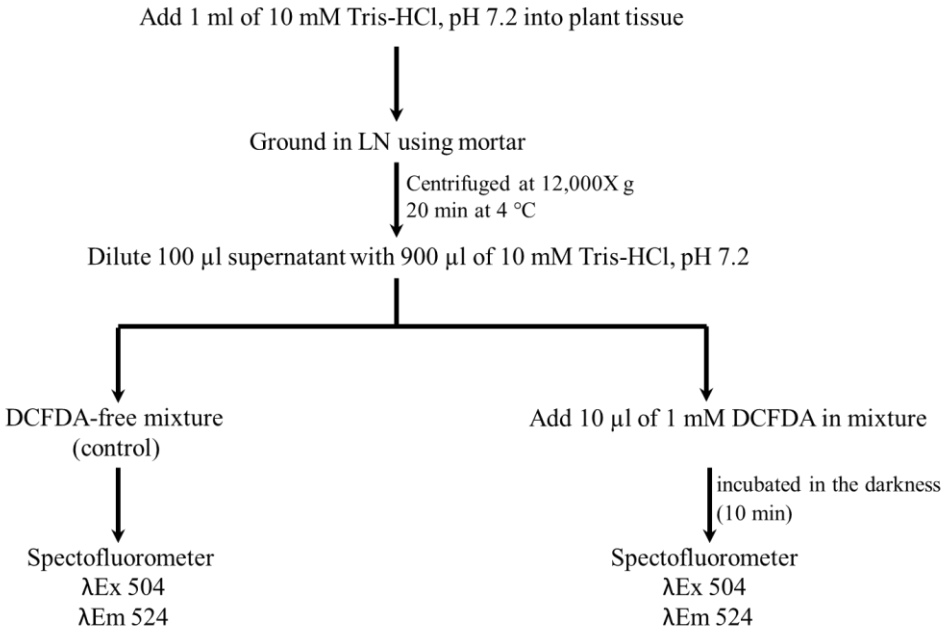
List of chemical	
DCFDA	4.8527 mg
DMSO	1 ml

DMSO: Dimethyl sulfoxide

Dilute to 1 mM DCFDA for working solution

ROS determination method

The fluorescence value of sample against control is compare with a total protein concentration of the sample which determined by Bradford reagent.



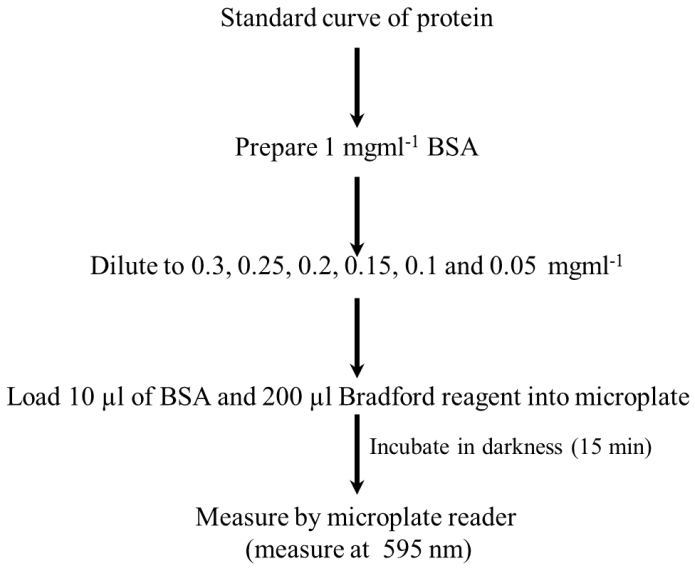
*The blank is 1 ml of 10 mM Tris-HCl, pH 7.2

1 mgml^{-1} BSA

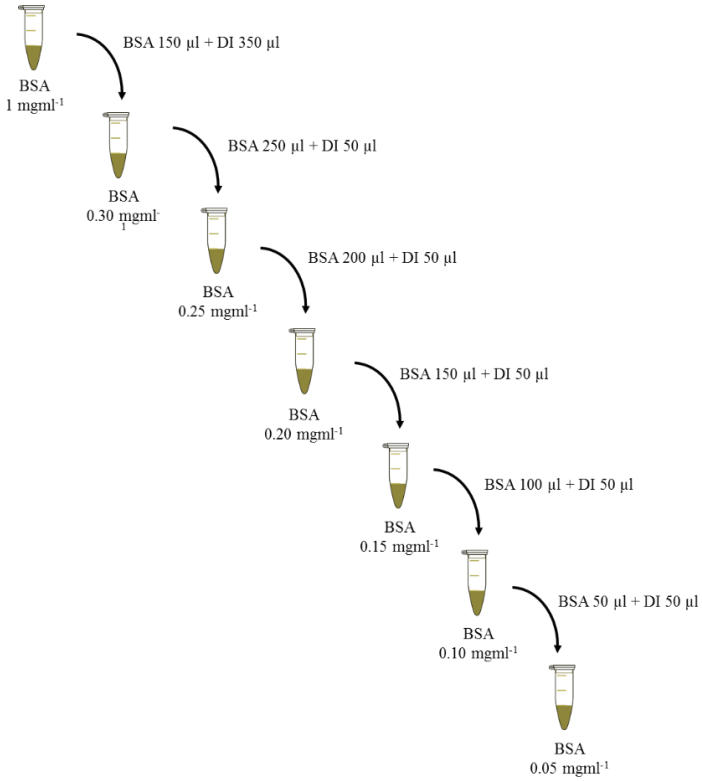
List of chemical	
BSA	5 mg
Deionized water	5 ml

BSA: Bovine serum albumin

Construction method of standard curve of protein concentration



Serial dilution of BSA



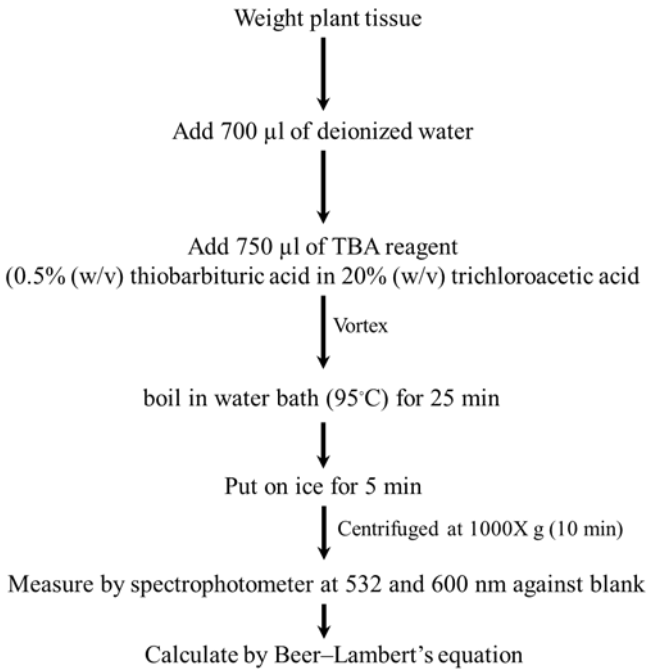
BSA: Bovine serum albumin, DI: Deionized water

MDA determination

TBA reagent (Verleysen et al., 2004)

List of chemical	
Thiobarbituric acid (TBA)	0.5 g
Trichloric acid (TCA)	20 g
Deionized water	100 ml

MDA analysis Method



*The blank is 1 ml of TBA reagent

Beer Lambert's equation (Heath and Packer, 1968)

$$\mathbf{C}=\frac{A}{L\varepsilon}$$

A = absorbance

C = Concentration ($moll^{-1}$)

L = path length

ε = extinction coefficient ($mM^{-1}cm^{-1}$)

*extinction coefficient of MDA = 155 $mM^{-1}cm^{-1}$

APPENDIX F

Data from statistical analysis

SEs induction

Survival percentage

Duncan[a]

VAR00001	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
13.00	10	27.5000					
12.00	10	30.0000					
14.00	10	30.0000					
15.00	10	35.0000	35.0000				
10.00	10	40.0000	40.0000	40.0000			
4.00	10	42.5000	42.5000	42.5000	42.5000		
6.00	10	45.0000	45.0000	45.0000	45.0000	45.0000	
7.00	10		50.0000	50.0000	50.0000	50.0000	
5.00	10			52.5000	52.5000	52.5000	
8.00	10			55.0000	55.0000	55.0000	
16.00	10			55.0000	55.0000	55.0000	
11.00	10			57.5000	57.5000	57.5000	
9.00	10				60.0000	60.0000	
3.00	10					62.5000	
2.00	10						87.5000
1.00	10						95.0000
Sig.		.053	.088	.056	.056	.056	.337

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

SE formation (%)

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
1.00	10	.0000	
4.00	10	.0000	
5.00	10	.0000	
6.00	10	.0000	
7.00	10	.0000	
11.00	10	.0000	
12.00	10	.0000	
13.00	10	.0000	
14.00	10	.0000	
15.00	10	.0000	
16.00	10	.0000	
8.00	10		50.0000
10.00	10		53.3330
3.00	10		60.0000
9.00	10		61.6660
2.00	10		68.3330
Sig.		1.000	.117

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Number of SEs per explant

Duncan[a]

VAR00001	N	Subset for alpha = 0.05		
		1	2	3
1.00	10	.0000		
6.00	10		2.6667	
5.00	10		3.3750	3.3750
3.00	10		4.5238	4.5238
4.00	10		4.6000	4.6000
2.00	10			5.1905
Sig.		1.000	.060	.078

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

SEs proliferation

Increased fresh weight (mg)/ 100 mg initial fresh weight

Duncan[a]

VAR00001	N	Subset for alpha = 0.05			
		1	2	3	4
9.000	10	56.89600			
2.000	10	62.31700	62.31700		
7.000	10	82.25200	82.25200	82.25200	
11.000	10	93.42000	93.42000	93.42000	93.42000
3.000	10	93.43500	93.43500	93.43500	93.43500
4.000	10	93.54900	93.54900	93.54900	93.54900
13.000	10	95.77900	95.77900	95.77900	95.77900
16.000	10	109.00000	109.00000	109.00000	109.00000
12.000	10	122.37900	122.37900	122.37900	122.37900
15.000	10	127.69700	127.69700	127.69700	127.69700
6.000	10	128.85200	128.85200	128.85200	128.85200
14.000	10	140.48400	140.48400	140.48400	140.48400
10.000	10	147.18300	147.18300	147.18300	147.18300
5.000	10		158.05300	158.05300	158.05300
8.000	10			168.96400	168.96400
1.000	10				183.33300
Sig.		.076	.059	.089	.078

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Survival percentage

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
11.000	10	46.66667	
10.000	10	50.00000	50.00000
4.000	10	55.00000	55.00000
13.000	10	60.00000	60.00000
12.000	10	73.33333	73.33333
14.000	10	76.66667	76.66667
15.000	10	76.66667	76.66667
16.000	10	76.66667	76.66667
3.000	10	78.33333	78.33333
2.000	10	80.00000	80.00000
6.000	10	80.00000	80.00000
7.000	10	80.00000	80.00000
9.000	10	80.00000	80.00000
5.000	10	83.33333	83.33333
8.000	10		86.66667
1.000	10		88.33333
Sig.		.065	.054

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

Cryopreservation via V cryo-plate

Survival rate of -LN SEs

Duncan[a]

VAR00001	N	Subset for alpha = 0.05		
		1	2	3
3.000	3	.00000		
8.000	3	.00000		
15.000	3	.00000		
17.000	3	.00000		
11.000	3	5.56667	5.56667	
2.000	3	6.66667	6.66667	
6.000	3	6.66667	6.66667	
9.000	3	6.66667	6.66667	
14.000	3	6.66667	6.66667	
1.000	3	13.33333	13.33333	13.33333
5.000	3	16.66667	16.66667	16.66667
7.000	3	20.00000	20.00000	20.00000
16.000	3	20.00000	20.00000	20.00000
13.000	3	23.33333	23.33333	23.33333
10.000	3	25.39683	25.39683	25.39683
4.000	3	33.33333	33.33333	33.33333
12.000	3		35.55556	35.55556
18.000	3			46.66667
Sig.		.064	.092	.056

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Survival rate of +LN SEs

Duncan[a]

VAR00001	N	Subset for alpha = 0.05	
		1	2
1.00	3	.0000	
4.00	3	.0000	
5.00	3	.0000	
6.00	3	.0000	
7.00	3	.0000	
11.00	3	.0000	
12.00	3	.0000	
13.00	3	.0000	
14.00	3	.0000	
15.00	3	.0000	
16.00	3	.0000	
8.00	3	.0000	
10.00	3	.0000	
3.00	3	.0000	
9.00	3	.0000	
2.00	3	.0000	
17.00	3	.0000	
18.00	3		20.0000
Sig.		1.000	.117

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Three-way ANOVA with interaction between all combinations of three main factors (preculture; P, osmoprotection; O and dehydration; D)

Factors	SS	DF	MS	F	*P*
Preculture (P)	600.00	1	600.00	1.898	0.000000
Osmoprotection (O)	14.81	2	7.41	0.023	0.176763
Dehydration with PVS2 (D)	2517.81	2	1258.91	3.983	0.976850
Interaction P X O	1644.44	2	822.22	2.606	0.027370
Interaction P X D	1843.00	2	921.50	2.916	0.088048
Interaction O X D	2361.19	4	590.30	1.868	0.067055
Interaction P X O X D	804.89	4	201.22	0.637	0.137397
Error	11378.00	36	316.06		
Total		53			

Water content

Duncan[a]

VAR00001	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
8.000	4	19.44362					
7.000	4		24.70703				
6.000	4		26.93827				
5.000	4			40.31189			
4.000	4				54.08435		
3.000	4					64.48554	
2.000	4						79.48554
1.000	4						79.63203
Sig.		1.000	.322	1.000	1.000	1.000	.948

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

ROS content of AA-treated and non AA-treated SEs

Duncan[a]

VAR00001	N	Subset for alpha = 0.05			
		1	2	3	4
8.000	3	5.11041			
9.000	3	5.29697			
7.000	3	6.33470			
10.000	3	6.42727			
5.000	3	7.05513			
1.000	3	8.08767	8.08767		
2.000	3		11.54429	11.54429	
6.000	3			12.50886	
4.000	3			13.70408	13.70408
3.000	3				17.18397
Sig.		.157	.069	.270	.068

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ROS content of AA-treated and non AA-treated SEs

Duncan[a]

VAR00001	N	Subset for alpha = 0.05				
		1	2	3	4	5
2.000	4	1.08279				
1.000	4	1.13457				
7.000	4	2.19885	2.19885			
10.000	4	2.23213	2.23213			
9.000	4		3.46838	3.46838		
8.000	4			3.69621		
3.000	4			4.09692		
6.000	4				5.98819	
4.000	4				6.23047	
5.000	4					8.34320
Sig.		.094	.057	.339	.694	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

VAR00002

Duncana

VAR00001	N	Subset for alpha = 0.05	
		1	2
2	7	8.57143	
1	7	25.71429	25.71429
4	7		39.04762
3	7		42.85714
Sig.		.074	.089

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.000.

VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
Master of Science (Plant Science) International program	Mahidol University	2012
Bachelor of Science (Fisheries)	Kasetsart University	2007

Scholarship and Awards during Enrollment

- Graduate Studies Scholarship (95000201) of Prince of Songkla University Graduate school, Prince of Songkla University, Thailand
- Scholarship for Support Exchange Students and International Credit Transferred through ASEAN Community, Graduate School, Prince of Songkla University, Thailand
- International Society of Horticultural Science (ISHS) Student award for the best poster presentation from The First Symposium on Tropical and Subtropical Ornamentals (TSO2016) held in Krabi, Thailand
- STEM workforce project
  National Science and Technology Development Agency (NSTDA), Thailand

List of Publication and Proceedings

- Soonthornkalump, S. 2016. *In vitro* conservation: confronting in the age of climate change. *Chronica Horticuturae*. 56(4): 17-23.
- Soonthornkalump, S., Nakkanong, K. and Meesawat, U. *In vitro* cloning via direct somatic embryogenesis and genetic stability assessment of *Paphiopedilum niveum* (Rchb.f.) Stein, the endangered Venus's slipper orchid (Submitted to *In Vitro* Cellular & Developmental Biology – Plant)
- Soonthornkalump, S., Yamamoto, S. and Meesawat, U. The optimization of V cryo-plate protocol and survival improvement for cryopreserved somatic embryos of *Paphiopedilum niveum* (Rchb.f.) Stein: Influence of ascorbic acid (Submitted to CryoLetters)