

Cryopreservation of Protocorm-like Bodies of *Vanda lilacina* Teijsm. & Binn., a Thai Orchid Species, by V-cryo-plate and D-cryo-plate Methods

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Abstract

Protocorm-like bodies (PLBs) of *Vanda lilacina* Teijsm. & Binn. were used to study two new cryopreservation methods, the V-cryo-plate and D-cryo-plate methods, for survival score and percentage of survival. PLBs were dehydrated with PVS2 solution (0, 20, 40, 60, and 120 min) and silica gel (0, 0.5, 1, 1.5, 2, 2.5, and 3 h) for the V-cryo-plate and D-cryo-plate methods, respectively. The results showed that the V-cryo-plate method, dehydration with PVS2 solution for 20 min, was a suitable method. It gave a survival score of 0.31, higher than control (+LN; 0 min) and gave a percentage of survival of 33.33 %. For the D-cryo-plate method, the suitable method for cryopreserved PLBs of *V. lilacina* was dehydration with silica gel for 1 h. It gave the highest survival score of 0.78 and percentage of survival was up to 83.78 %. Then, an unpaired t-test was used to compare data of survival scores of cryopreserved *V. lilacina* PLBs between the V-cryo-plate and D-cryo-plate methods. The best suitable cryopreservation method of *V. lilacina* PLBs is the D-cryo-plate method using silica gel for 1 h in dehydration step.

Keywords: Plant cryopreservation, Cryo-plate, Dehydration, Vitrification, PVS2, Orchidaceae

Introduction

Orchidaceae is the largest family of flowering plants. About 190 genera and 1,300 species of orchids have an origin in Thailand [1,2]. *Vanda* orchids are the most horticulturally important in the orchid genera, with amazing shapes, colors, and patterns of flowers especially found in the *Vanda* genus. These support hybridizers or growers to produce hybrid species or flowers for flower markets. Normally, *Vanda* orchids are mostly epiphytic. Flowers usually bloom every few months for two to three weeks and have a variety of colors, such as blue-purple (the colors which are the most recognizable), dark red, yellow, white, pink, etc. Wild populations of this genus in forests are decreasing. At present, *Vanda* orchids are currently listed as endangered species in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) [3]. *Vanda lilacina* Teijsm. & Binn. (Figure 1A) is one of the important *Vanda* orchids in Thailand. It is one of the orchids which have been widely propagated as ornamental plants and as cut flowers traded within the country and exported to foreign countries. It has 10 - 15 white flowers with pale pink spots at the lip in axillary inflorescence. Flower size is about 1.5 cm across. It can be found in semi-evergreen and mixed-deciduous forests in most parts of Thailand, except in central and southern Thailand [4].

Cryopreservation is an important method for conserving genetic resources for long-term storage using little space and maintenance [5,6]. This technique has been continually developed through many methods and protocols in many species in the past, such as the simple freezing method for orange [7], the vitrification and dehydration method for *Asparagus officinalis* [8,9], the encapsulation-vitrification

method for wasabi (*Wasabia japonica*) [10], the droplet method for potato [11], the encapsulation-dehydration method for potato [12], etc. For the orchid family, cryopreservation has been used like other plant families, such as the vitrification method for *Doritis pulcherrima* [13], encapsulation-vitrification for *Dendrobium nobile* [14], encapsulation-dehydration for *Phalaenopsis bellina* [15], etc. Genus *Vanda* has had a few reports on it, such as *Vanda pumila* by the vitrification method [16], and *Vanda coerulea* by the vitrification method [17] and the encapsulation-dehydration method [18]. Although many papers reported successful cryopreservation, these methods require a high technical skill, and some cases may inhibit regrowth [19]. A new tool has been developed, known as the cryo-plate. The cryo-plate method was developed by Yamamoto *et al.* [20] for use with large explants that are sensitive to physical damage and cryoprotectant toxicity [21]. The cryo-plate methods have high efficiency, ease of handling, and operational simplicity, and a high regrowth rate [22,23]. Cryo-plate is an aluminum plate that has high cooling ($4,000 - 5,000\text{ }^{\circ}\text{C min}^{-1}$) and warming rates ($3,000 - 4,500\text{ }^{\circ}\text{C min}^{-1}$) [22,24]. It is used for new cryopreservation methods, such as vitrification-cryo-plate (V-cryo-plate) and dehydration-cryo-plate (D-cryo-plate). The V-cryo-plate method [20] is based on the chemical dehydration of explants on a cryo-plate, such as PVS2 [25-28], PVS3 [26], but the D-cryo-plate method [25] is based on physical dehydration, such as the air current of a laminar air-flow cabinet [25,29-31], silica gel [24,31], or drying beads [24]. The D-cryo-plate method has the benefit of decreasing the amount of chemicals used, but requires longer periods of time [20,27]. The V-cryo-plate and D-cryo-plate methods were used in monocot, such as sugarcane [28] and mat rush [21]. In the family Orchidaceae, the cryo-plate method is the newest method for cryopreserving orchid plant materials, firstly seen in *Arundina graminifolia* protocorms in 2016 [24,32]. In this study, we used PVS2 solution and silica gel for dehydration in the V-cryo-plate and D-cryo-plate methods, respectively. This research is the first report using V-cryo-plate and D-cryo-plate methods for cryopreservation of *Vanda lilacina* PLBs.

Materials and methods

Plant materials

PLBs of *Vanda lilacina* Teijsm. & Binn. were induced from protocorms cultured on half-strength Murashige and Skoog agar medium ($\frac{1}{2}$ MS) for 12 weeks (**Figure 1B**).

Cryo-plate

Custom-made aluminum cryo-plates (cryo-plate) (Taiyo Nippon Sanso Corp., Japan) with 12 wells and a size $37 \times 7 \times 0.5\text{ mm}^3$ were used in all experiments. Wells in a cryo-plate were oval shaped and with a length of 2.5 mm, a width of 1.5 mm, and a depth of 0.75 mm [25] (**Figure 1C**). These plates were provided by Dr. Takao Niino and Dr. Shin-ichi Yamamoto (Tsukuba, Japan).

Effects of liquid nitrogen and exposure time to PVS2 solution in dehydration step of V-cryo-plate method

V-cryo-plate procedure

The successful steps of the V-cryo-plate procedure were as follows:

1. Three PLBs of *V. lilacina* put into a well of a cryo-plate filled with 2 % Na-alginate solution (2 % Na-alginate in $\frac{1}{2}$ MS medium solution).
2. Covering with 50 mM CaCl_2 solution for 30 min at room temperature ($25 \pm 2\text{ }^{\circ}\text{C}$) (**Figure 1D**) for bead forming (**Figure 1E**) and CaCl_2 solution removed by tapping the cryo-plate on filter paper.
3. Transferal to loading solution (LS) (2 M glycerol and 0.4 M sucrose in $\frac{1}{2}$ MS medium solution) for 30 min (**Figure 1F**).
4. Removal of the cryo-plate from LS and transferal to PVS2 solution (30 % (w/v) glycerol + 15 % (w/v) ethylene glycol + 15 % (w/v) DMSO in $\frac{1}{2}$ MS medium solution) for 0, 20, 40, 60, and 120 min for dehydration of PLBs of *V. lilacina* (**Figure 1G**).
5. The cryo-plate put into 1.8 ml uncapped cryotubes and then directly plunged into LN for 1 h (**Figure 1H**). For control, PLBs treated with the same steps as described above, but not plunged into liquid nitrogen (-LN).

6. After that, the cryo-plate taken out of the cryotube and immersed into unloading solution (1.2 M sucrose in ½ MS medium solution) and held for 15 min (**Figure 1J**).

7. PLBs attached to the cryo-plate removed and transferred to culture on ½ MS agar medium (**Figure 1K**). All treatments kept in light conditions (white fluorescent light at the intensity of 37 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 16 h per d, 25 ± 2 °C) for 4 weeks.

Effects of liquid nitrogen and duration of silica gel in dehydration step of D-cryo-plate method **D-cryo-plate procedure**

The successful steps for the D-cryo-plate procedure were basically the same as those described for the V-cryo-plate protocol, but with modification of step 4, as follows:

4. Removal of the cryo-plate from LS and transferal for dehydration with silica gel method (25 g silica gel in 100×15 mm² petri dish and covering with filter paper) for 0, 0.5, 1, 1.5, 2, 2.5, and 3 h (**Figure 1H**).

Moisture content determination

For the D-cryo-plate method, PLBs were in steps 1 - 3 of D-cryo-plate procedures before being transferred to 100×15 mm² petri dishes that were filled with 25 g of silica gel and covered with filter paper. Four replications of cryo-plate were weighed every 30 min for 3 h, then kept in a hot-air oven (60 - 80 °C) (**Figure 1L**) and weighed every day until stable (about 48 h) for dry weight record (**Figure 1M**). The moisture content was calculated by using the equation of moisture content wet basis (%) [24] following formula (1):

$$\text{Moisture content (\%)} = \frac{W_i - W_f}{W_i} \times 100 \quad (1)$$

W_i is initial weight and W_f is the final weight.

Determination of survival percentage and survival score

PLBs of *V. lilacina* in the V-cryo-plate and D-cryo-plate methods that had been kept in light conditions for 4 weeks on ½ MS agar medium were checked for survival by Tetrazolium (TZ) test or TTC test [33,34] with a stereo microscope via counting the score of survival in each PLB. Survived PLBs (PLBs showed a bright red color of triphenyl formazan) were scored 1. Dead PLBs (PLBs showed as colorless) were scored 0. Injured PLBs (showed a spot of bright red color on PLBs) were scored 0.5 (**Figure 2**).

Data of survival scores were calculated to average survival score (2) and percentage of survival (3); followed by,

$$\text{Average survival score} = \frac{\sum \text{the score of survival in each PLBs}}{\text{number of PLBs}} \quad (2)$$

$$\text{Percentage of survival} = \frac{\text{Number of PLBs that score} > 0}{\text{Number of all PLBs}} \times 100 \quad (3)$$

Experimental design and statistical analysis

Experimental design was conducted using split-plot design in a completely randomized design (CRD) with eight replications. Each replication included 3 pieces of *V. lilacina* PLBs. The factor of liquid nitrogen (LN) was the main plot and duration of silica gel was the subplot. The data of survival scores of explants were recorded after culturing for 4 weeks. The data were statistically analyzed with an Analysis of Variance (ANOVA) test and means were compared using the least significant difference (LSD) at a significance level of $P \leq 0.05$. The data of survival scores from suitable treatments of the V-cryo-plate and D-cryo-plate methods were compared by analyzing with an unpaired t Test at a significance level of $P \leq 0.05$.

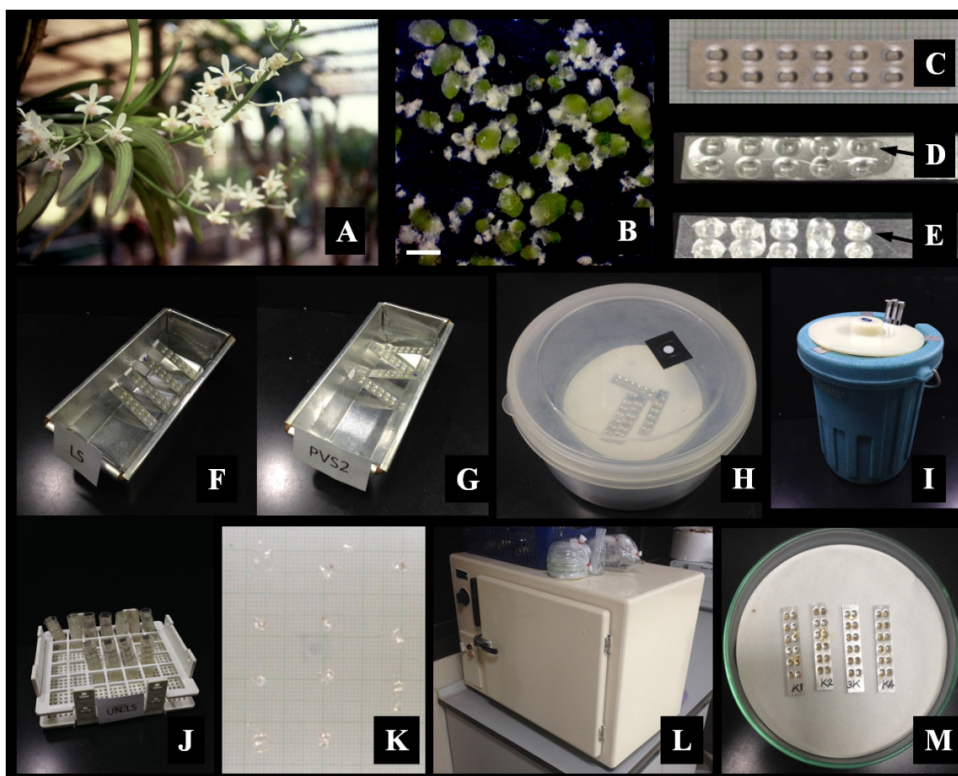


Figure 1 Cryopreservation protocol for cryopreserved PLBs of *V. lilacina*: (A) *V. lilacina* plant, (B) 12-week-old PLBs were induced from protocorms cultured on 1/2 MS (bar = 1 mm), (C) 37×7×0.5 mm³ custom-made aluminum cryo-plates (cryo-plate), (D-E) PLBs were encapsulated (black arrow) to the cryo-plate with Na-alginate solution and CaCl₂ solution for 30 min, (F) cryo-plates in loading solution for 30 min, dehydrated PLBs using (G) PVS2 solution for V-cryo-plate method and (H) silica gel for D-cryo-plate method, (I) cryo-plates immersion in liquid nitrogen for 1 h, (J) cryo-plates in unloading solution for 15 min, (K) cryopreserved PLBs were cultured on 1/2 MS medium, (L) cryo-plates with attached PLBs for determining moisture content by oven drying method at 60 - 80 °C for about 48 h in hot air oven, (M) cryo-plates were dried from hot air oven method.

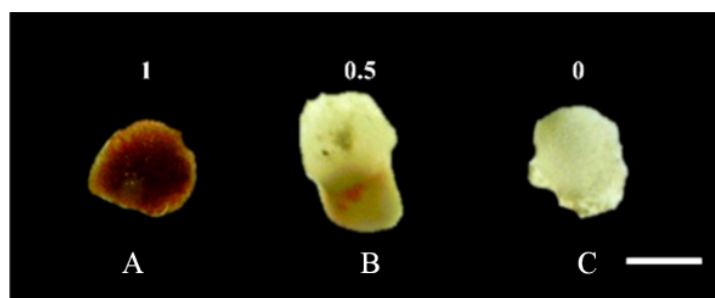


Figure 2 Survival score determination of PLBs of *Vanda lilacina* Teijsm. & Binn. checked with Tetrazolium (TZ) test or TTC test after being kept in light conditions for 4 weeks on 1/2 MS agar medium. (A) Survived PLBs (PLBs showed bright red color of triphenyl formazan) were scored 1. (B) Injured PLBs (showed spot of bright red color on PLBs) were scored 0.5. (C) Dead PLBs (PLBs showed colorless) were scored 0 (Bar = 0.5 mm).

Results and discussion

In the V-cryo-plate method, PLBs of *V. lilacina* were dehydrated with PVS2 solution for 0, 20, 40, 60, and 120 min. The results showed that, in LN, survival score was 0.16 and average percentage of survival was 16.81 % lower than control, which gave an average survival score of 0.36 and average percentage of survival of 38.65 %. Moreover, the results showed the effect of exposure time to PVS2 solution and interaction between effect of LN and effect of exposure time to PVS2 solution affecting survival score and percentage of survival. The exposure time to PVS2 solution at 0 and 120 min with LN gave both survival scores of 0.00 and percentage of survival of 0.00 %. These were lower than using PVS2 solution for 0 min without liquid nitrogen, which gave survival score of 0.76 and percentage of survival up to 76 %. When using PVS2 solution for 20 and 40 min with LN, the survival score and percentage of survival increased to 0.31 and 0.28, respectively, and both percentages of survival were up to 33.33 %. Exposure to PVS2 solution for 20 min was enough for cryopreserving the PLBs (**Table 1**). Moreover, when considering PLBs of *V. lilacina* that were recovered from the V-cryo-plate method on ½ MS agar medium at the 4th week (**Figure 3**), when using PVS2 solution for 20 min with LN, PLBs could recover and grow the same as in PVS2 solution for 20 min without LN. They showed green PLBs, but the size of the PLBs were smaller than control (PVS2 0 min, -LN), which showed growth with large green PLBs. In contrast, PLBs that have been cryopreserved without PVS2 solution in the dehydration step showed colorless PLBs, with the early size of PLBs.

The exposure time to PVS2 solution at 20 min for cryopreserved PLBs in V-cryo-plate method was lower than the encapsulation-vitrification method that cryopreserved orchid plant materials, such as 150 min for PLBs of *Dendrobium candidum* [35] and 115 min for PLBs of *D. nobile* [14], but was nearly similar to cryopreserved orchid plant materials in the droplet-vitrification method, such as 30 min for PLBs of *Vanda coerulea* [36], 30 min for protocorms of *Grammatophyllum speciosum* [37], and 30 min for shoot apices of *Vanilla planifolia* ‘Andrews’ [38]. This factor determined the suitable range of dehydration time in which PVS2 sufficiently diffused into the PLBs for the V-cryo-plate method. The insufficient or excessive dehydration of PVS2 solution caused low survival score and percentage of survival, because chemical toxicity of PVS2 solution can damage cells, or the remaining water in cells can damage the cells after cryopreservation [6,22,25].

For improving survival score and percentage of survival, many papers have reported about the preculturing orchid plant materials before cryopreservation, such as precultured PLBs of *Dendrobium* Bobby Messina on ½ MS agar medium with 0.6 M sucrose for 1 d [39], precultured protocorms of *Grammatophyllum speciosum* on ½ MS agar medium with 0.4 M sucrose for 2 d [37], and precultured shoot apices of *Vanilla planifolia* ‘Andrews’ on MS agar medium with 0.3 M sucrose for 1 d [38]. The addition of high sucrose concentration in preculture media helps osmoprotection by maintaining turgor, stabilizing cellular membranes, and inducing dehydration tolerance in plants [40,41]. Moreover, sucrose preculturing caused changes in total soluble protein and sugar levels in cell suspensions [42]. In this study, cryopreserved PLBs in the V-cryo-plate method showed the percentage of survival up to 33.33%, although they had not been precultured before cryopreservation. Thus, preculturing PLBs with high concentration of sucrose before the V-cryo-plate method with PVS2 solution for 20 min may help to obtain better results.

In the D-cryo-plate method, PLBs of *V. lilacina* were dehydrated with silica gel for 0, 0.5, 1, 1.5, 2, 2.5, and 3 h. The results showed that, in LN, survival score was 0.18 and average percentage of survival was 20.10 % lower than control (-LN), in which average survival score was 0.52 and average percentage of survival was 53.10 %. Moreover, the results showed the effect of duration of silica gel and interaction between effect of liquid nitrogen and effect of duration of silica gel affecting survival score and percentage of survival. The duration of silica gel dehydration at 1 h with LN gave the highest survival score of 0.78 and the highest percentage of survival of 83.78 % when compared with the duration of silica gel at 0, 0.5, 1.5, 2, 2.5, and 3 h with LN, which gave survival scores of 0.00, 0.02, 0.26, 0.17, 0.02, and 0.03, respectively, and percentages of survival of 0.00, 2.63, 27.78, 18.42, 2.70, and 5.41 %, respectively. The duration of silica gel dehydration at 1 h with LN was nearly the same as using silica gel for 1 h without LN, which gave survival score of 0.89 and percentage of survival of 87.50 %. The duration of

silica gel in dehydration step related to moisture content for cryopreservation in the D-cryo-plate method. The moisture content of PLBs of *V. lilacina* that were dehydrated with silica gel from 0 - 3 h decreased the moisture content from 76.16 % to 26.70 %. The PLBs dehydrated with silica gel at 1h and which had high survival score and percentage of survival gave optimal moisture content at 36.52 %. (**Table 2**) Moreover, when considering PLBs of *V. lilacina* that were recovered from the D-cryo-plate method on ½ MS agar medium at the 4th week, when using silica gel for 1 h with LN, PLBs could recover and grow the same as using silica gel for 0 and 1 h without LN. They showed green PLBs with the same size of PLBs that used silica gel for 0 and 1 h without LN. In contrast, PLBs that had been cryopreserved (+LN) without using silica gel in the dehydration step showed colorless PLBs, with the early size of PLBs (**Figure 4**).

In this study, silica gel was used for 1 h in the dehydration step and gave the optimal moisture content at 36.52 % by the D-cryo-plate method. The duration time in the D-cryo-plate method was faster than the encapsulation-dehydration method, which took longer times, such as 5 h for PLBs of *Dendrobium nobile* orchid [14], 5 h for PLBs of *Phalaenopsis bellina* orchid [15], or 6 h for shoot tips of apple (*Malus*) [43], because the size of beads in the encapsulation-dehydration method is larger than that of encapsulation beads that were controlled by the size of the wells on cryo-plate. The optimal moisture content depended on plants and explants. For example, optimal moisture content was about 28 - 35 % for shoot tip of sugarcane [30], optimal moisture content at 27 % for mat rush buds (*Juncus decipiens* Nakai) of line “Ohara 4 No.2” and “Kitakei 2” [25], and optimal moisture content at 26 % for mat rush buds of line “Hiroshima 4 gou(1)” [21]. Optimal moisture content is important for success in cryopreservation. When explants are not sufficiently dehydrated, intracellular ice will generate cryo-injury in liquid nitrogen. In contrast, lower moisture content will generate osmotic shock, osmotic stress, and cell damage when explants are over-dehydrated [44,45]. The ways to successful cryopreservation with the D-cryo-plate technique determined the optimal dehydration time, optimal moisture content, and control of moisture content of plant samples before freezing [14,22,46]. Other ways, such as preculturing, using antioxidant chemicals or using plant growth regulators for cryopreservation, may depend on plant species and explants [16,22,47-49].

Table 1 Effects of liquid nitrogen and exposure time to PVS2 solution in dehydration step of V-cryo-plate method on survival score and percentage of survival of PLBs of *Vanda lilacina* Teijsm. & Binn. The data is represented as average ± SE.

PVS2 solution (min)	-LN		+LN	
	Survival score ^{1/}	Survival (%)	Survival score ^{2/}	Survival (%)
0	0.76±0.08 a	76.00	0.00±0.00 b	0.00
20	0.63±0.11 a	70.83	0.31±0.13 a	33.33
40	0.25±0.08 b	23.81	0.28±0.09 a	33.33
60	0.16±0.08 b	18.75	0.21±0.13 ab	17.39
120	0.02±0.02 b	3.85	0.00±0.00 b	0.00
Mean	0.36±0.06	38.65	0.16±0.04	16.81

^{1/}The value followed by the same letters in the column is not significantly different at the 95 % confidence level by LSD (5 % LSD = 0.23).

^{2/}The value followed by the same letters in the column is not significantly different at the 95 % confidence level by LSD (5 % LSD = 0.26).

Lastly, an unpaired t-test was used to compare the survival scores between the V-cryo-plate and D-cryo-plate methods on cryopreserved *V. lilacina* PLBs. There was a significant difference in the survival scores for the V-cryo-plate and D-cryo-plate methods. These results suggested that the suitable method for cryopreservation of *V. lilacina* PLBs is the D-cryo-plate method using silica gel for 1 h in the

dehydration step (**Figure 5**). There are many advantages of the D-cryo-plate method, such as the samples held in cryo-plates being easy to handle and having a high rate of survival from the very high cooling and warming rates. Moreover, large specimens can be used, such as $1.0 \times 1.0 \text{ mm}^2$ (long*wide) in shoot tips of persimmon [47], $2.0 \times 1.5 \text{ mm}^2$ in buds of mat rush [25], and about $2.0\text{-}2.5 \times 1.5\text{-}2.0 \text{ mm}^2$ in lateral buds of mat rush [21].

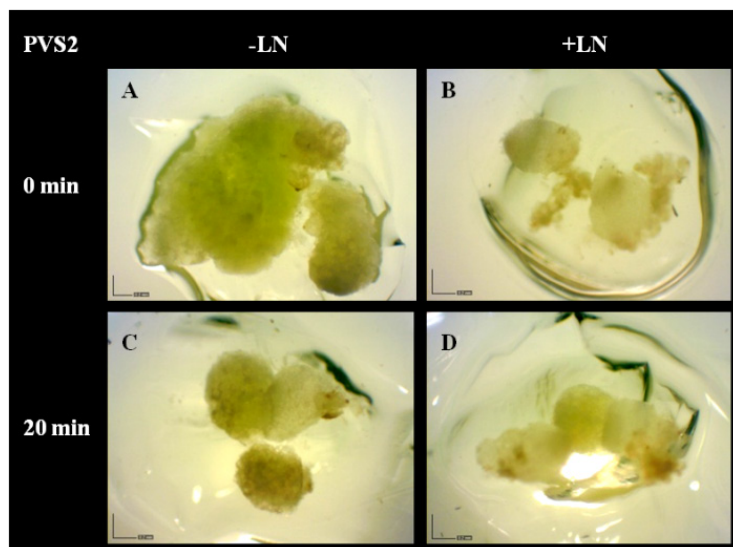


Figure 3 PLBs of *V. lilacina* recovered from V-cryo-plate method on $\frac{1}{2}$ MS agar medium at the 4th week. (A, C) non-cryopreserved (-LN) and (B, D) cryopreserved (+LN), (A, B) without PVS2 solution (0 min) and (C, D) with PVS2 solution (20 min) (bar = 0.2 mm).

Table 2 Effects of liquid nitrogen and duration of silica gel in dehydration step of D-cryo-plate method on survival score and percentage of survival of PLBs of *Vanda lilacina* Teijsm. & Binn. The data is represented as average $\pm$ SE.

Silica gel (h)	Moisture content (%)	-LN		+LN	
		Survival score ^{1/}	Survival (%)	Survival score ^{2/}	Survival (%)
0	76.16 $\pm$ 0.65	1.00 $\pm$ 0.00 a	100.00	0.00 $\pm$ 0.00 c	0.00
0.5	55.42 $\pm$ 1.56	0.99 $\pm$ 0.01 a	100.00	0.02 $\pm$ 0.02 c	2.63
1.0	36.52 $\pm$ 1.59	0.89 $\pm$ 0.06 a	87.50	0.78 $\pm$ 0.07 a	83.78
1.5	30.55 $\pm$ 1.28	0.63 $\pm$ 0.08 b	65.79	0.26 $\pm$ 0.08 b	27.78
2.0	27.74 $\pm$ 1.07	0.15 $\pm$ 0.07 c	18.42	0.17 $\pm$ 0.05 b	18.42
2.5	27.26 $\pm$ 0.90	0.00 $\pm$ 0.00 d	0.00	0.02 $\pm$ 0.02 c	2.70
3.0	26.70 $\pm$ 0.87	0.00 $\pm$ 0.00 d	0.00	0.03 $\pm$ 0.03 c	5.41
Mean		0.52 $\pm$ 0.05	53.10	0.18 $\pm$ 0.03	20.10

^{1/}The value followed by the same letters in the column is not significantly different at the 95 % confidence level by LSD (5 % LSD = 0.13).

^{2/}The value followed by the same letters in the column is not significantly different at the 95 % confidence level by LSD (5 % LSD = 0.13).

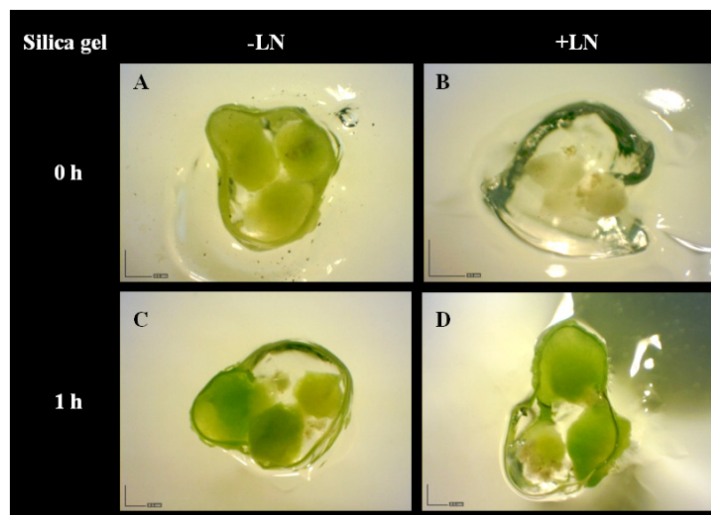


Figure 4 PLBs of *V. lilacina* recovered from D-cryo-plate method on $\frac{1}{2}$ MS agar medium at the 4th week. (A, C) non-cryopreserved (-LN) and (B, D) cryopreserved (+LN), (A, B) without silica gel (0 min) and (C, D) with silica gel (1 h) (bar = 0.5 mm).

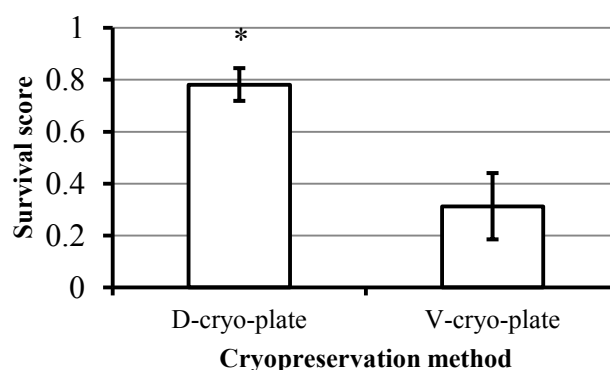


Figure 5 Survival scores between D-cryo-plate (dehydrated with silica gel for 1 h) and V-cryo-plate (dehydrated with PVS2 solution for 20 min) for cryopreserved *V. lilacina* (bar = $\pm$ SE).

Conclusions

It was concluded that cryopreservation of *V. lilacina* PLBs was successful by dehydrating with silica gel for 1 h for the D-cryo-plate method (83.78 % survival) and dehydrating with PVS2 solution for 20 min for the V-cryo-plate (33.33 % survival). The suitable medium for culture is $\frac{1}{2}$ MS agar medium. These two methods have high potential to be developed for cryopreserving other vandaceous orchids.

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