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CONSERVATION OF SOME ENDANGERED THAI ORCHID SPECIES USING CRYOPRESERVATION METHODS

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Abstract

Thailand is the origin of about 1,300 tropical orchid species in 178 genera. Deforestation and over-collection of wild Thai orchids for trade has placed these species at a risk of extinction. Therefore, the conservation, as well as sustainable use is urgently needed to conserve these by various means. The genus *Paphiopedilum* and *Dendrobium cruentum* are listed in Appendix I of CITES. Presently, various methods of cryopreservation of Thai orchid species were implemented. For cryopreservation, recent methods were used, namely vitrification (dehydration in PVS2 solution, consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide, prepared in modified Vacin and Went liquid medium), encapsulation-dehydration (encapsulation in calcium alginate beads followed by air-drying in a laminar air-flow cabinet), encapsulation-vitrification (encapsulation in calcium alginate beads followed by dehydration in PVS2 solution), droplet-vitrification (fast freezing from small drops of PVS2 solution on aluminium strip), and cryo-plate (a combination of encapsulation and droplet on very fast freezing aluminium plate) dehydrated with silica gel and drying beads. Application of these methods in seeds was successful in *Dendrobium chrysotoxum* (99% vitrification), *D. cruentum* (32% vitrification; 58% D cryo-plate), *D. draconis* (95% vitrification), *D. hercoglossum* (80% encapsulation-vitrification), *Doritis pulcherrima* (62% vitrification), *Paphiopedilum exul* (30% encapsulation-vitrification; 14% encapsulation-dehydration), *Rhynchostylis coelestis* (85% vitrification), *Vanda coerulea* (67% vitrification) as well as in protocorms of *Acampe rigida* (17% V cryo-plate; 74% D cryo-plate), *Arundina graminifolia* (76% and 74% cryo-plate dehydrated with drying beads and silica gel, respectively; 33% droplet-vitrification; 64% encapsulation-dehydration with drying beads or silica gel), *Dendrobium cariniferum* (15%, encapsulation-vitrification), *D. cruentum* (33% vitrification; 27% encapsulation-dehydration), *Grammaytophyllum speciosum* (14% encapsulation-vitrification), *Rhynchostylis gigantea* (19% vitrification), *Seidenfadenia mitrata* (67% vitrification), *Vanda coerulea* (40% encapsulation-dehydration), and in pollinia of *Dendrobium signatum* (56% and 50% V cryo-plate and D cryo-plate, respectively). Cryopreserved seeds, protocorms, and pollinia were able to develop into normal seedlings/plantlets. These methods appear to be promising techniques for cryopreservation of some Thai orchid species.

Introduction

THAILAND IS the origin of about 1,300 tropical orchid species and 178 genera (Thammasiri, 2008). Many Thai orchid species have good horticultural characteristics and are hence used as parents for breeding, making Thailand the leader in orchid exports. Climate change, deforestation (habitat destruction), and over-collection of wild Thai orchids for trade have placed Thai orchid species at a risk of extinction. Therefore, conservation, social awareness, and consciousness, as well as sustainable use are urgently needed to conserve orchids by various means (De and Pathak, 2018; Janakiram and Baskaran, 2018; Thammasiri, 2008, 2020). During the present investigation, various methods of *ex situ* conservation of Thai orchid species were implemented, namely cryopreservation, seed storage under OSSSU (Orchid Seed Stores for Sustainable Use) project, and micropropagation.

Cryopreservation

Thammasiri (2002) reported preservation of seeds of some Thai orchid species by vitrification; *Dendrobium chrysotoxum, D. draconis, Doritis pulcherrima,* and *Rhynchostylis coelestis* showed 99%, 95%, 62%, and 85% germination, respectively after seed cryopreservation by vitrification. Other Thai orchid seeds in *Dendrobium cruentum* (32% by vitrification) (Kagawa, 2006), *D. hercoglossum* (80% by encapsulation-vitrification) (Sheranaravenich, 2002), *Vanda coerulea* (67% by vitrification) (Thammasiri and Soamkul, 2007) and protocorms in *Dendrobium cariniferum* (15% by encapsulation-vitrification) (Pornchuti and Thammasiri, 2008), *D. cruentum* (33% by vitrification and 27% by encapsulation-dehydration) (Kagawa, 2006), *Seidenfadenia mitrata* (67% by vitrification) (Moonpen, 2006), and *Vanda coerulea* (40% by encapsulation-dehydration) (Jitsopakul *et al*., 2008) were later successfully cryopreserved. Sopalun *et al.* (2010) studied three vitrification-based methods, namely droplet-vitrification, encapsulation-dehydration, and encapsulation-vitrification, for cryopreservation of protocorms of *Grammatophyllum speciosum*, known as *Tiger orchid* or *Giant orchid*. Protocorms, 0.1 cm in diameter, obtained from 2-month-old seed cultures were used. For droplet-vitrification, protocorms were precultured on filter paper soaked in half strength Murashige and Skoog medium (½MS) containing 0.4 M sucrose at 25±2°C for 2 days, followed by soaking in loading solution (2M glycerol and 0.4 M sucrose in ½MS

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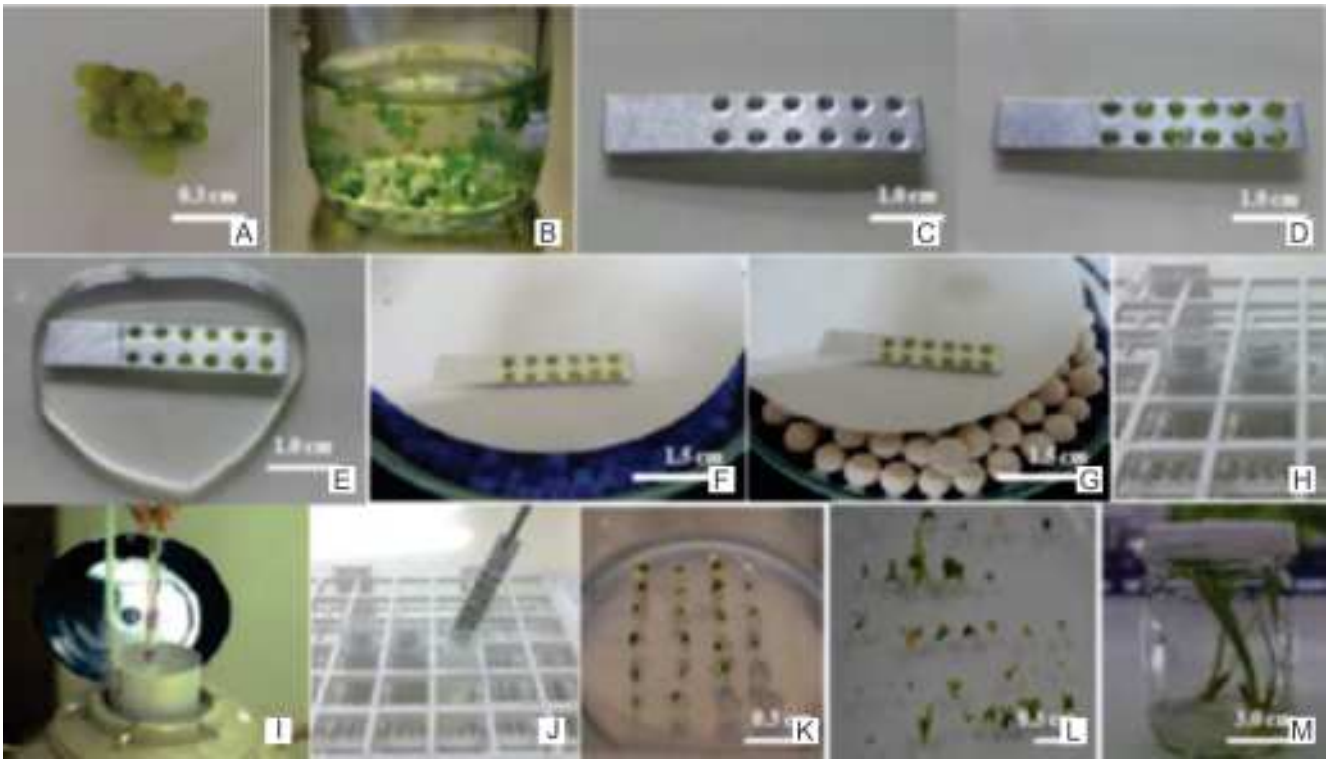

Fig. 1. A-M. Cryo-plate method dehydrated with silica gel or drying beads: A, Protocorm development; B, Preculture of protocorms in ½MS liquid medium with 0.7M sucrose for 1 day; C, Pouring the alginate solution containing 2% (w/v) sodium alginate in calcium-free ½MS basal medium with 0.4M sucrose in the wells; D, Placing the pre-cultured protocorms in the wells one by one; E, Pouring the calcium chloride solution containing 0.1M calcium chloride in ½MS basal medium with 0.4M sucrose; F, Dehydration with 50 g silica gel; G, Dehydration with 30 g drying beads; H, Putting each cryo-plate in a 2 ml cryotube; I, Plunging 2 ml cryotubes into liquid nitrogen for 1 day; J, Warming in 1.2M sucrose solution for 20 min; K, Plate on ½MS agar medium; L, Re-growth; M, Re-growth after 60 days.

liquid medium) for 20 min and then dehydration with PVS2 solution in ½MS liquid medium containing 0.4M sucrose at pH 5.7 for 30 min. For encapsulation-dehydration, encapsulated protocorms were precultured on ½MS liquid medium containing 0.4M sucrose on a shaker (110 rpm) at 25±2°C for 2 days, followed by soaking in the same loading solution for 20 min and then exposing these to a sterile air-flow at 2.5 inches/ water column from a laminar air-flow cabinet for 8 hrs. For encapsulation-vitrification, encapsulated protocorms were precultured on ½MS liquid medium containing 0.4M sucrose for 1 or 2 days, followed by soaking in the same loading solution for 20 min and then dehydration with PVS2 solution for 60 min. For all three methods, preculturing with 0.4M sucrose for 2 days resulted in a significant induction of dehydration and freezing tolerance. The cryopreservation results showed the highest protocorm re-growth after droplet-vitrification (38%), followed by encapsulation-dehydration (24%) and encapsulation-vitrification (14%). Plantlets developed from these three methods did not show any abnormal characteristics or ploidy level change when investigated by flow cytometry.

Fig. 2. A-C. *Arundina graminifolia*: A, Flower; B, Fruit; C, Plants.

Cordova II and Thammasiri (2016) developed the cryo-plate method using silica gel or drying beads for dehydration (Fig. 1). Protocorms were placed in the preculture solution consisting of 0.7 M sucrose on a shaker (110 rpm) at 25±3°C for 1 day. After that, protocorms were placed one by one in the wells which filled before with the alginate solution containing 2% (w/v) sodium alginate in calcium-free ½MS basal medium with 0.4M sucrose. The cryo-plates were hardened for 20 min by slowly dispensing the calcium chloride solution containing 0.1M calcium chloride in ½MS basal medium with 0.4M sucrose. Then the cryo-plates

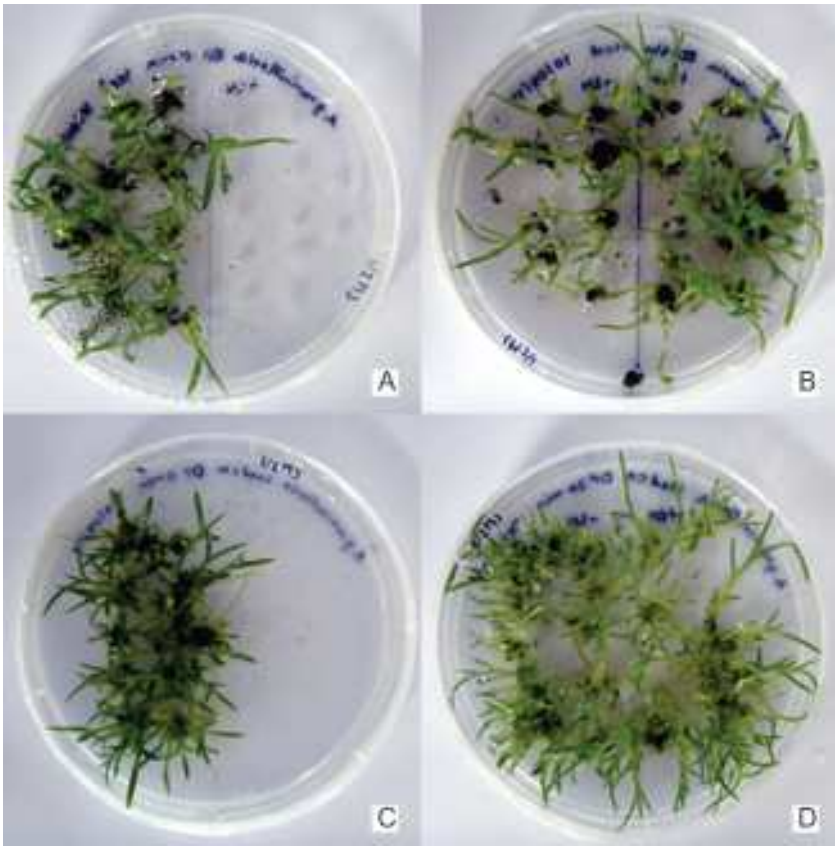

Fig. 3. A-D. Germination of *Arundina graminifolia* seeds in response to air-drying and immersion in liquid nitrogen (LN) by encapsulation-dehydration and D cryo-plate techniques: A, Encapsulation-dehydration at 0 min, germination percentage: non-cryopreserved (76%) and cryopreserved (0%); B, Encapsulation dehydration at 240 min, germination percentage: non-cryopreserved (77%) and cryopreserved (74%); C, D cryo-plate at 0 min, germination percentage: non-cryopreserved (95%) and cryopreserved (0%); D, D cryo-plate at 0 min, germination percentage: non-cryopreserved (93%) and cryopreserved (82%), n= 20 full seeds for each replicate.

were surface dried using sterile filter paper, placed in Petri dishes containing silica gel or drying beads in a laminar air-flow cabinet. Cryo-plates were dehydrated for 5 hrs until 25% moisture content was achieved. Dehydrated cryo-plates were placed in 2 ml cryotubes and plunged directly into liquid nitrogen for 1 day. Cryo-plates were removed from cryotubes and warmed in unloading solution (1.2M sucrose solution) for 20 min. Protocorms were then removed from the cryo-plate and placed on ½MS agar medium for re-growth. Growth conditions were conducted using 16 hrs light at 25±3°C.

For effect of the cryo-plate method, re-growth of control treatments dehydrated using silica gel (90%), drying beads (92.1%), silica gel (73.8%), and drying beads (76.5%) was observed. Re-growth was observed at the 2nd wk of transfer to ½MS medium. Dehydration using silica gel or drying beads did not significantly affect re-growth rate. Protocorms dehydrated using silica gel or drying beads developed into normal plantlets.

Earlier, Thammasiri *et al*. (2019) studied cryopreservation of *Arundina graminifolia* seeds (Fig. 2) by D cryo-plate and encapsulation-dehydration technique. The results (Tables 1-2; Fig. 3) showed that the D cryo-plate method gave the highest re-growth of 82%, followed by encapsulation-dehydration (74%). The D cryo-plate protocol is as follows: Pour the alginate solution containing 2% (w/v) sodium alginate in calcium-free ½ MS basal medium with 0.4M sucrose in the wells of the cryo-plate. For encapsulation-dehydration, protocol is the same as D cryo-plate without using cryo-plate. Place 20 to 50 seeds from 3-month-old selfing fruit in each well. Pour calcium chloride solution containing 0.1M calcium chloride in ½ MS basal medium with 0.4M sucrose. Subsequent exposure to 0.4M sucrose + 2M glycerol (loading solution) for 30 min was followed by dehydration under a laminar air-flow cabinet for 3 hrs. Put each cryo-plate in a 2 ml cryotube and plunge these into liquid nitrogen for 1 day. Warming was done in 1.2M sucrose solution (unloading solution) for 15 min, removed the solution and cryopreserved beads were cultured on ½MS agar medium.

Fig. 4. A-D. *Dendrobium signatum*: A, Blooming flowers; B, Pollinia embedded with 3% sodium alginate gel on aluminium cryo-plates; C, No survival of pollinia; D, Survival of pollinia after cryopreservation using V cryo-plate method.

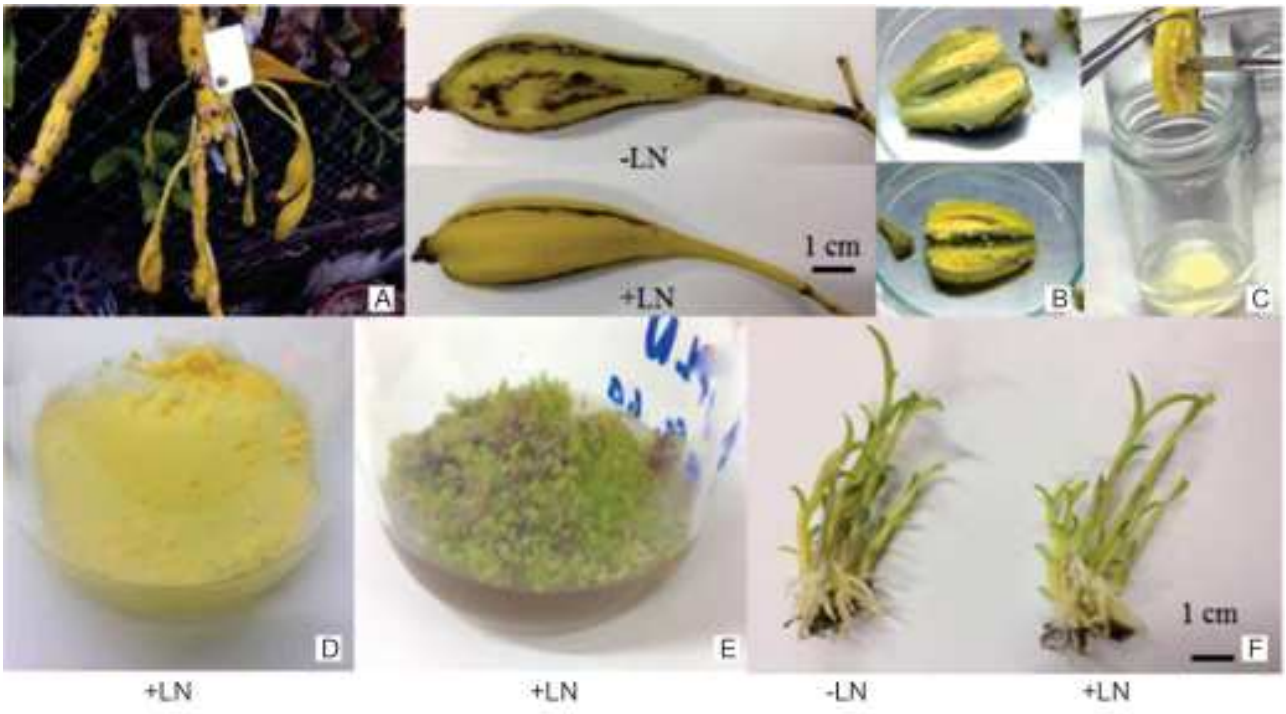

Fig. 5. A-F. Fruit formation after pollination with cryopreserved pollinia by V cryo-plate method and germination, in *Dendrobium signatum*: A, Fruit formation after 1 month; B, Fruit formation after 8 months; C-D, Yellow seeds sown on modified VW agar medium; E, Protocorms; F, Multiple shoot development after 4 months of culture.

Jitsopakul *et al.* (2019) studied the efficiency of V cryo-plate and D cryo-plate methods for cryopreservation of pollinia in *Dendrobium signatum*- a Thai orchid. Pollinia were collected from flowers and then placed on the aluminium cryo-plate containing 12 wells embedded with 3% sodium alginate gel (Fig. 4). In V cryo-plate method, cryo-plates with pollinia were immersed in 0.6M sucrose + 2M glycerol loading solution (LS) for 15 min, then dehydrated with PVS2 solution for 40 min at room temperature (29±2°C). In D cryo-plate method, cryo-plates with pollinia were treated with LS for 15 min, then dehydrated in a laminar air-flow cabinet for 3 hrs at room temperature (29±2°C). In both methods, cryo-plates were directly plunged into liquid nitrogen for 40 min, and rapidly warmed in 1.2M sucrose for 15 min. The cryopreserved and non-cryopreserved pollinia were used to pollinate flowers of the same species. The results showed that cryopreserved pollinia retained fertilizing ability, and had similar fruit formation as those pollinated with non-cryopreserved pollinia. The fruit formation from cryopreserved pollinia in V cryo-plate and D cryo-plate methods was 55.6% and 50%, respectively. Seeds were successfully produced using non-cryopreserved and cryopreserved pollinia and these germinated and developed into seedlings with well-formed leaves and roots. Multiple stems (4.8 stems/plant) were formed when seedlings were cultured on modified VW agar medium (Vacin and Went, 1949) supplemented with 100 gl^{-1} banana, 150 ml coconut water, 50 gl^{-1} potato, and 20 gl^{-1} sucrose for 120 days at 25±2°C (Fig. 5).

Table 1. Effect of different dehydration time of encapsulation-dehydration method on *Arundina graminifolia* seed germination.

Time (min)	-LN	+LN
0	76.15±4.10^{ab}	0^{d}
30	61.68±4.72^{c}	0^{d}
60	82.65±3.84^{a}	0.92±0.91^{d}
90	71.03±4.41^{abc}	12.00±3.78^{c}
120	82.57±3.65^{a}	54.84±5.18^{b}
180	68.00±4.68^{bc}	69.91±4.33^{a}
240	77.11±4.64^{ab}	74.07±4.24^{a}
300	80.43±4.16^{ab}	52.83±4.87^{b}

Table 2. Effect of different dehydration time of D cryo-plate method on *Arundina graminifolia* seed germination.

Time (min)	-LN	+LN
0	95.00±2.19^{a}	0^{c}
30	92.25±2.25^{a}	42.21±3.99^{b}
60	67.31±4.62^{c}	75.00±4.04^{a}
90	93.38±2.14^{a}	82.24±3.71^{a}
120	79.41±3.48^{b}	82.00±3.15^{a}

Fig. 6. A-L. Cryopreservation of *Rhynchostylis gigantea* pollinia using V cryo-plate method for hand-pollination with *R. gigantea* var. *harrisoniana* flowers: A, *R. gigantea* flowers; B, Pollinia; C, Pollinia were placed on aluminium cryo-plates embedded in 3% sodium alginate solution, then soaked in 100 mM $CaCl_2$ and PVS2, respectively; D, Cryo-plates with pollinia transferred to uncapped 2 ml cryotubes held on cryo-canes and then directly plunged into liquid nitrogen; E, Non-cryopreserved and cryopreserved pollinia stained red were considered viable while the unstained pollinia were considered non-viable; F, *R. gigantea* var. *harrisoniana* flowers; G-H, Fruit set after hand-pollination of cryopreserved pollinia of *R. gigantea* with *R. gigantea* var. *harrisoniana* flowers; I, Fruit and seeds of *R. gigantea* var. *harrisoniana* × *R. gigantea*; J-K, Seeds of *R. gigantea* var. *harrisoniana* × *R.* gigantea (L.) Ridl. germinated and developed into protocorms and seedlings on modified VW agar medium; L, Hybrid plantlets.

Table 3. Effect of exposure time to PVS2 on viability of pollinia and fruit set after 60 days of hand-pollination with non-cryopreserved and cryopreserved pollinia of ten orchid taxa (by V cryo-plate method).

Orchid taxa	Exposure time	Viability (%)		Fruit set (%)	
	to PVS2 (min)	-LN	+LN	-LN	+LN
Dendrobium chrysotoxum L.	20	60.0±8.7	40.0±2.3	60.0±3.8	20.0±0.6
Spathoglottis plicata Blume.	20	100	100	100	100
Coelogyne trinervis L.	20	100	100	100	100
Encyclia bractescens L.	20	100	100	100	66.7±33.3
Seidenfadenia mitrata (Rchb.f.) Garay.	40	100	100	50.0±1.0	50.0±2.9
Pomatocalpa spicatum Breda.	40	100	100	100	100
Vanda bensonii Batem.	40	100	100	100	100
V. brunnea Rchb.f.	60	100	100	100	100
Aerides houlettiana Rchb.f.	60	100	100	66.7±1.0	66.7±1.0
Rhynchostylis gigantea var. *harrisoniana* × *R. gigantea* (L.) Ridl.	60	100	100	100[a]	66.7±33.3

Values represent means±SD.

Preserving pollinia viability and fertility for pollination is very important in orchid breeding. Cryopreservation of orchid pollinia using aluminium cryo-plate is the new tool for long term storage of plant genetic resources. Presently, an attempt was made to determine the procedure of V cryo-plate method for cryopreservation of orchid pollinia and apply to some orchid species for breeding.

Material and Methods

Pollinia of *Rhynchostylis gigantea* (L.) Ridl. were collected from completely open flowers in the morning and then these were placed on aluminium cryo-plates embedded in 3% sodium alginate solution. Aluminium cryo-plates with pollinia were immersed in loading solution containing 2M glycerol and 0.4M sucrose

Table 4. Effect of exposure time to PVS2 on fruit size after hand-pollination for 60 days with non-cryopreserved and cryopreserved pollinia of ten orchid species by V cryo-plate method.

Orchid taxa	Exposure time	Fruit size (cm) (width x length)	
	to PVS2 (min)	-LN	+LN
Dendrobium chrysotoxum L.	20	3.5±0.2 × 1.1±0.1	3.5±0.2 × 1.1±0.1
Spathoglottis plicata Blume	20	0.4±0.0 × 2.3±0.0	0.4±0.0 × 2.3±0.1
Coelogyne trinervis L.	20	0.9±0.0 × 3.1±0.0	0.9±0.0 × 3.2±0.0
Encyclia bractescens L.	20	0.4±0.2 × 3.9±2.0	0.4±0.0 × 5.0±0.7
Seidenfadenia mitrata (Rchb.f.) Garay.	40	0.3±0.0 × 3.1±1.0	0.3±0.0 × 2.5±0.0
Pomatocalpa spicatum Breda.	40	0.3±0.0 × 1.7±0.0	0.3±0.0 × 1.3±0.0
Vanda bensonii Batem.	40	0.9±0.0 × 4.4±0.2	0.9±0.0 × 5.8±0.3
V. brunnea Rchb.f.	60	1.0±0.1 × 4.0±0.2	0.9±0.0 × 3.7±0.2
Aerides houlettiana Rchb.f.	60	0.8±0.0 × 1.7±0.0	0.7±0.0 × 1.6±0.1
Rhynchostylis gigantea var.*harrisoniana* × *R. gigantea* (L.) Ridl.	60	1.2±0.2 × 2.9±0.3	0.6±0.4 × 1.8±1.0

Values represent means±SD.

for 15 min at room temperature (29±2°C), and then dehydrated with PVS2 solution for 0-60 min at 29±2°C. The cryo-plates with pollinia were directly plunged into liquid nitrogen for 40 min, and rapidly warmed in 1 M sucrose for 15 min. The cryopreserved and non-cryopreserved pollinia were used to hand-pollinate flowers of the same species and flowers of *R. gigantea* var. *harrisoniana* for producing hybrids. Subsequently, this procedure was successfully applied for cryopreservation of other eight orchid species (Table 3).

Results

The viability of non-cryopreserved and cryopreserved pollinia dehydrated with PVS2 was 100%. The pollinia stained red were considered viable while the unstained pollinia were considered non-viable. The highest fruit set (100%) was obtained after pollinating flowers with cryopreserved pollinia after dehydration with PVS2 for 40 min. The procedure for cryopreservation of *R. gigantea* pollinia using V cryo-plate method was successfully applied for cryopreservation of other eight orchid species.

The exposure time to PVS2 has affected the viability of pollinia and fruit set in all species; viability of pollinia ranged from 40% to 100% with an average of 93.33% and per cent fruit set ranged from 20% to 100% with an average of 78.16% (Table 3). Fruit set was successful after pollination of cryopreserved pollinia and seed production was observed. The exposure time to PVS2 has also affected the size of the fruit in all species; thus the fruit size ranged from 0.3-3.5 cm (Table 4).

Seeds germinated into protocorms and these developed into seedlings when cultured on modified Vacin and Went (1949; VW) agar medium. Hybrid plantlets were grown in the greenhouse at Rajamangala University of Technology Isan Surin campus, Surin province, Thailand for 1 year.

Perusal of literature indicates that Prasongsom *et al.* (2019) made studies in an extinct epiphytic orchid, *Dendrobium cruentum* (originated from SouthWestern of Thailand). Though the cultivation of this species was successful, there was a problem for long-term storage. Encapsulation-vitrification and V cryo-plate methods were used by using 2% sodium alginate with various exposure times (0, 30, 60, 90, and 120 min) to determine the different water content. The maximum germination percentage (68.1%) was observed in V cryo-plate method with 60 min exposure time of PVS2 solution; while, the encapsulation-vitrification method showed lower germination percentage (21.6%) under the same conditions. Non-precultured protocorms of *Acampe rigida* were used to find suitable duration time for dehydration on V cryo-plate and D cryo-plate methods (Imsomboon *et al.,* 2019). These were dehydrated with PVS2 solution for 0, 20, 40, 60, and 120 min. The results showed that protocorms dehydrated with PVS2 solution for 40 min gave the highest survival score at 0.08 and gave the highest percentage of survival at 16.67% higher than control (+LN; 0 min), but most of protocorms showed dark green colour and developed slower than control (-LN; 0 min). The suitable treatment for V cryo-plate was exposing these to PVS2 solution for 40 min. For D cryo-plate method, non-precultured protocorms were dehydrated with silica gel for 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 hrs. The results showed that protocorms dehydrated with silica gel for 1 hr gave the highest survival score at 0.56 and gave the highest percentage of survival up to 74%. Most protocorms were with dark green colour and these developed slower than control. The suitable treatment for D cryo-plate method was dehydrating with silica gel for 1 hr. An unpaired t-test was used to compare the data of survival score between suitable treatments from V cryo-plate and D cryo-plate methods. The suitable treatment for cryopreservation of *A. rigida* protocorms is with D cryo-plate method dehydrated with silica gel for 1hr. Imsomboon and Thammasiri (2020) cryopreserved *Paphiopedilum exul* seeds using encapsulation-vitrification method with exposure time to PVS2 solution for 40 min and encapsulation-dehydration with air drying in a laminar air-flow cabinet for 2 hrs gave 30% and 14% germination, respectively.

Conclusion

Cryopreserved seeds and protocorms of some Thai orchid species were able to develop into normal seedlings and cryopreserved pollens were also successfully used for pollination, though with different per cent fruit sets. The vitrification, encapsulation-dehydration, encapsulation-vitrification, droplet-vitrification, and cryo-plate methods appear to be promising techniques for cryopreservation of some Thai orchid species.

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