БИОТЕХНОЛОГИЯ РАСТЕНИЙ

UDK 582.794.2:58.085.(593)

CONSERVATION OF THAI ORCHID SPECIES USING CRYOBIOTECHNOLOGY

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Thailand is the origin of about 1,300 tropical orchid species and 180-190 genera. Deforestation and over-collection of wild Thai orchids for trade has placed orchid species at a risk of extinction. Therefore, the conservation, as well as sustainable use is urgently needed to conserve orchids by various means. The genus *Paphiopedilum and Dendrobium cruentum* are listed in Appendix I of CITES. At the Department of Plant Science, Faculty of Science, Mahidol University, various methods of cryopreservation of Thai orchid species are 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 aiuminium plate) dehydrated with silica gel and drying beads. Application of these methods in seeds was successful in *Dendrobium chrysotoxum* (99%, vitrification). *Dendrobium cruentum* (32%, virification), *Dendrobium draconis* (95%, vitrification), *Dendrobium hercoglossum* (80%, encapsulation-vitrification). *Doritis pulcherrima* (62%, vitrification). *Rhynchostylis coelestis* (85%, vitrification), *Vanda coerulea* (67%, vitrification), as well as in protocorms of *Dendrobium cruentum* (33%, vitrification; 27%, encapsulation-dehydration), *Dendrobium cariniferum* (15%, encapsulation-vitrification), *Grammaytophyllum speciosum* (14%, encapsulation-vitrification), *Rhynchostylis gigantea* (19%, vitrification), *Vanda coerulea* (40%, encapsulation-dehydration) and *Seidenfadenia mitrata* (67%, vitrification) and *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). Cryopreserved seeds and protocorms were able to develop into normal seedlings. These methods appear to be promising techniques for cryopreservation of some Thai orchid germplasm.

Key words: *vitrification; encapsulation-dehydration; encapsulation-vitrification; droplet-vitrification; cryo-plate; drying beads; silica gel*

Introduction

Thailand is the origin of about 1,300 tropical orchid species and 178 genera. Many Thai orchid species have good horticultural characteristics and are used as parents for breeding, making Thailand the No.1 orchid exporter. Climate change, deforestation (habitat destruction) and over-collection of wild Thai orchids for trade has 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 [13]. At the Department of Plant Science, Faculty of Science, Mahidol University, various methods of ex situ conservation of Thai orchid species are implemented, namely cryopreservation, seed stores under Orchid seed stores for sustainable use (OSSSU) project and micropropagation [4, 8].

After meeting and discussing with Professor Akira Sakai at the International Workshop on *In Vitro* Conservation of Plant Genetic Resources, July 4-6, 1995 in Kuala Lumpur, Malaysia, he came to demonstrate vitrification-based methods for plant cryopreservation at the Department of Plant Science. A little while later, research on cryopreservation of jackfruit embryonic axes has started and the results were very successful

and novel [11]. Dr. K. Thammasiri then shifted his interest in cryopreservation research into orchids and discovered a new scientific direction. In 2000, the first paper on *Doritis pulcherrima*, a wild Thai orchid, on seed cryopreservation by vitrification with 62% was published [11]. It was also the first paper on seed cryopreservation by vitrification (dehydration in PVS2 solution, consisting of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide, prepared in modified Vacin and Went liquid medium).

Later M.Sc. and Ph.D. students of Dr. K. Thammasiri studied recent methods, namely vitrification (dehydration in PVS2 solution), 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) and droplet-vitrification (fast freezing from small drops of PVS2 solution with plant materials inside on 7 x 20 mm sterile aluminium foil strip). Application of these methods on seeds was successful for many Thai orchid species. Cryopreserved seeds and protocorms were able to develop into normal seedlings. These methods appeared to be promising techniques for cryopreservation of many Thai orchid species.

Dr. Thammasiri [12] presented "Preservation of Seeds of Some Thai Orchid Species by Vitrification" at the 16th World Orchid Conference. *Dendrobium chrysotoxum, Dendrobium draconis, Doritis pulcherrima and Rhynchostylis coelestis* had 99%, 95%, 62% and 85% germination, respectively after seed cryopreservation by vitrification. Other Thai orchid seeds were later successfully cryopreserved, such as in *Dendrobium cruentum* (32% by vitrification) [3], *Vanda coerulea* (67% by vitrification) [14], *Dendrobium hercoglossum* (80% by encapsulation-vitrification) [7], as well as in protocorms of *Dendrobium cruentum* (33% by vitrification and 27% by encapsulation-dehydration) [3], *Dendrobium cariniferum* (15% by encapsulation-vitrification) [6], *Vanda coerulea* (40% by encapsulation-dehydration) [2] and *Seidenfadenia mitrata* (67% by vitrification) [5].

The aim of this study was to develop efficient cryopreservation methods for endangered Thai orchid species.

Objects and Investigation methods

Plant materials

Protocorms of *Grammatophyllum speciosum* and *Arundina graminifolia* were used in this study.

Mature fruits, 8-month-old, derived from self-pollination of *G. speciosum* were used. Fruits were cleaned and wiped with 70% ethanol, then placed inside a laminar air-flow cabinet and soaked in 95% ethanol for 1 min, then flamed with an alcohol burner. After cooling, fruits were cut and seeds were removed and placed on sterile Petri dish. Seeds were sown on half strength Murashige and Skoog agar medium (½MS) containing 2% (w/v) sucrose. The medium was adjusted to pH 5.7 before adding 0.2% (w/v) gelrite. Cultures were maintained at standard conditions; 25± 2°C, under white fluorescent light ("Philips", Thailand) at the intensity of 37 μmol m^{-2} s^{-1} for 16 h d^{-1}. Protocorms, 0.1 cm in diameter, developed from 2 month-old germinating seeds were used in this study [8].

Mature seeds of *Arundina graminifolia* were germinated on half strength Murashige and Skoog (½ MS) agar medium supplemented with 0.1 mg L^{-1} α-naphthaleneacetic acid, 0.1 mg L^{-1} kinetin and 20 g L^{-1} sucrose at the Orchid Research Laboratory, Mahidol University, Salaya Campus. The pH was adjusted to 5.8 prior to autoclaving. Cultures were incubated at 25±3°C under white fluorescent light ("Philips", Thailand) at the intensity of 37 μmol m^{-2} s^{-1} for 16 h d^{-1}. This medium stimulated the development of protocorms after 70 days. Protocorms, 1-2 mm diameter were used in the experiments.

Desiccation materials used were 50 g silica gel (2.8-3.0 mm diameter) per Petri

dish and 30 g drying beads (6.5 mm diameter) per Petri dish to dehydrate encapsulated protocorms and encapsulated protocorms adhered to cryo-plates. Silica gel, considered to be a low cost and effective product, is largely used to effectively remove moisture from plant materials. Drying beads, developed by the combination of aluminosilicate with clay, allow for easy handling and the ability to be used virtually indefinitely. After these beads have absorbed their maximum amount of moisture, they can be dried in an oven and used again.

Droplet-vitrification

For droplet-vitrification method [9] (Figure 1), selected protocorms were precultured on filter paper soaked with ½MS medium containing 0.4 M sucrose at 25 ± 2°C for 0, 1, 2 and 3 days. The precultured protocorms were treated with loading solution (2 M glycerol and 0.4 M sucrose in ½MS liquid medium) for 20 min at 25 ± 2°C. Following preculture, the protocorms were treated with loading solution (2 M glycerol and 0.4 M sucrose in ½MS liquid medium) for 20 min at 25 ± 2°C, then dehydrated with PVS2 solution (30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide in ½MS liquid medium with 0.4 M sucrose at pH 5.7) for 0, 15, 30, 60, 90, and 120 min. Ten small drops of PVS2 solution were dropped on 7x20 mm sterile aluminium foil strip, and then treated protocorms were placed one by one in a prepared drop of PVS2 solution. After that aluminium foil strip containing beads was rapidly transferred into a 1.8 ml cryotube filled with liquid nitrogen (LN) and then the cryotube was incubated in LN for at least 1 h. For rapid warming, aluminium foil strips were removed from LN and transferred into a new cryotube filled with 1.5 ml of ½MS liquid medium containing 1.2 M sucrose for 20 min at 25 ± 2°C at the intensity of 37 μmol m^{-2} s^{-1} for 16 h d^{-1}.

Encapsulation-dehydration

For encapsulation-dehydration method [9] (Figure 2), encapsulated protocorms were precultured in ½MS liquid medium containing 0.4 M sucrose on a shaker (110 rpm) at 25 ± 2°C for 0, 1, 2 and 3 days. The precultured beads were exposed to sterile air-flow at 0.5 inches/water column (0.12 kPa) inside a laminar air-flow cabinet at c. 28°C and 34% RH for 0-12 h. After dehydration, ten dehydrated beads were transferred into 1.8 ml cryotube and then directly plunged into LN and stored for at least 1 h. For rapid warming, cryotubes were removed from LN and rapidly warmed in a waterbath at 40 ± 2°C for 2 min under white fluorescent light ("Philips", Thailand) at the intensity of 37 μmol m^{-2} s^{-1} for 16 h d^{-1}.

Encapsulation-vitrification

For encapsulation-vitrification method [9] (Figure 3), encapsulated protocorms were precultured in ½MS liquid medium containing 0.4 M sucrose on a shaker (110 rpm) at 25 ± 2°C for 0, 1, 2 and 3 days Then, precultured beads were treated with loading solution for 20 min 25 ± 2°C. The precultured beads were dehydrated with PVS2 solution for 0, 15, 30, 60, 90, and 120 min. 10 beads were then transferred into 1.8 ml cryotube containing 1.5 ml PVS2 solution. The cryotubes containing treated beads were directly plunged into LN and stored for 1 h. For rapid warming, the cryotubes were rapidly warmed in a waterbath at 40 ± 2°C for 2 min, then PVS2 solution were removed from cryotubes and replaced by adding 1.5 ml of ½MS liquid medium containing 1.2 M sucrose for 20 min at 25 ± 2°C under white fluorescent light ("Philips", Thailand) at the intensity of 37 μmol m^{-2} s^{-1} for 16 h d^{-1}.

Cryo-plate method

For cryo-plate method [1] (Figure 4), *Arundina graminifolia* protocorms, 1-2 mm, were placed into 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 of cryo-plates pre-filled with the alginate solution containing 2% (w/v) sodium alginate in calcium-free ½ MS liquid medium with 0.4 M sucrose. The cryo-plates were hardened for 20 min by

slowly dispensing the calcium chloride solution containing 0.1 M calcium chloride in ½MS liquid medium with 0.4 M sucrose. Then, the cryo-plates 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 until the moisture content was 25% and then placed into 2 mL cryotubes (one cryo-plate/cryotube) and plunged directly into liquid nitrogen for 1 day. The cryo-plates were removed from cryotubes and warmed in unloading solution (1.2 M sucrose solution) for 20 min. Protocorms were then removed from the the cryo-plate and placed and cultured on ½ MS agar medium at 25±3°C under white fluorescent light ("Philips", Thailand) at the intensity of 37 µmol m^{-2} s^{-1} for 16 h d^{-1} and determined regrowth after 90 days

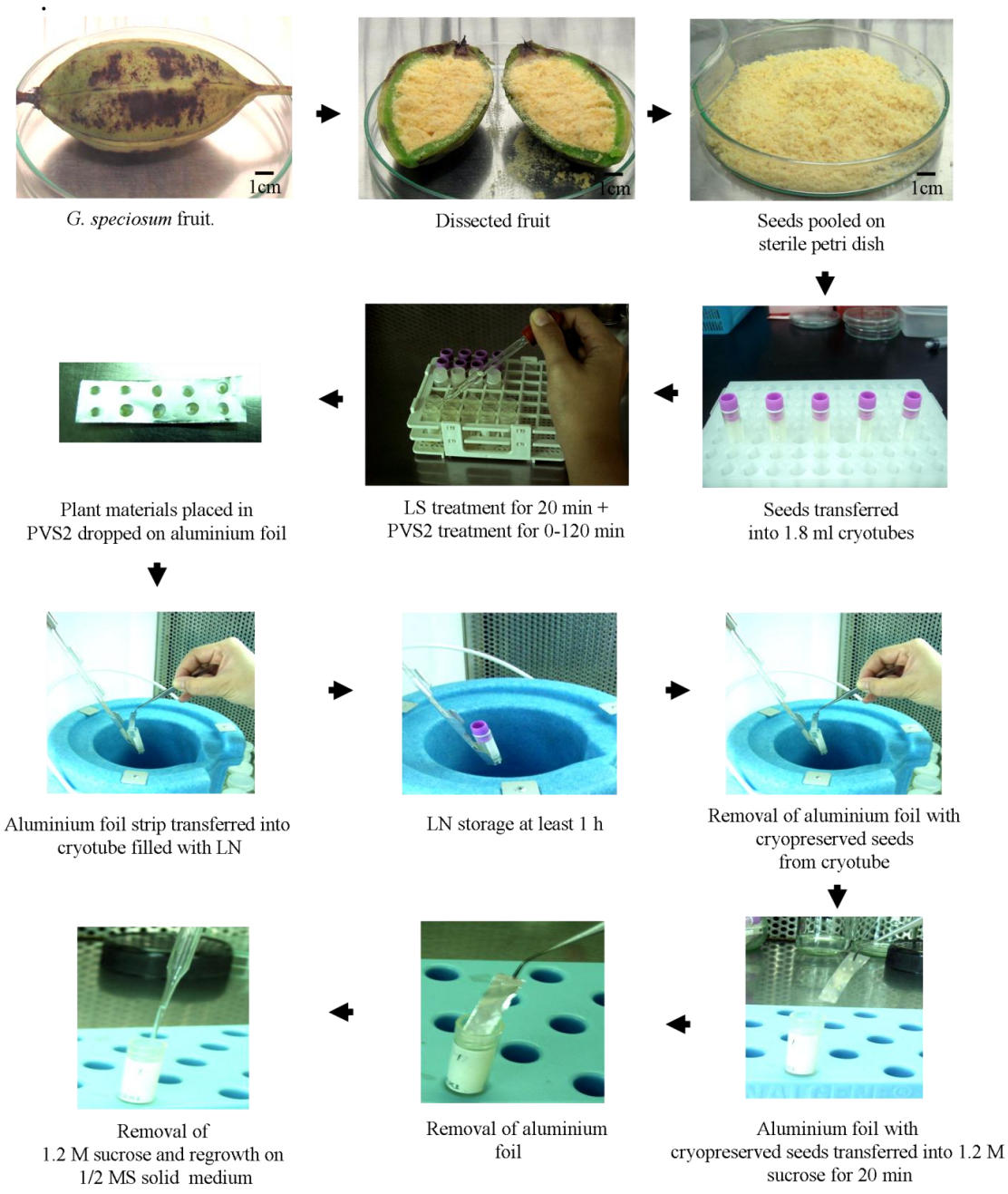

Figure 1 Established protocol for cryopreservation of *G. speciosum* seeds by droplet-vitrification

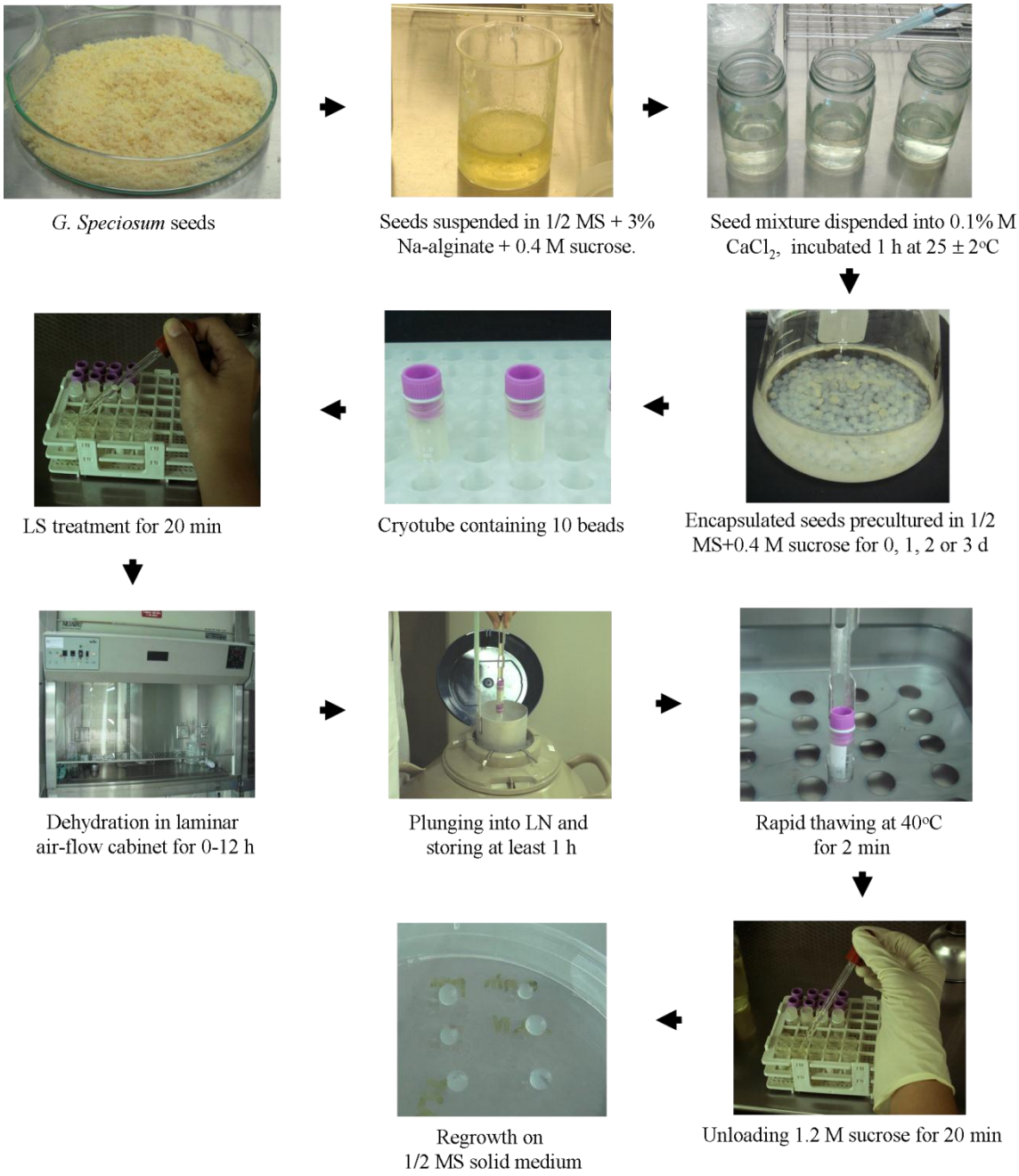

Figure 2 Established protocol for cryopreservation of *G. speciosum* seeds by encapsulation-dehydration

Statistical analysis

All experiments were arranged in a completely randomized design (CRD). Data from the experiments were subjected to analysis of variance (ANOVA) and the means were compared using the least significant difference (LSD) test based on four replications.

Results and Discussion

Droplet-vitrification method

Preculture and treatment with PVS2 solution affected the water content of the protocorms. Water content of protocorms precultured with 0.4 M sucrose for 0, 1, 2 and 3 days and without exposure to PVS2 solution was 83%, 80%, 78% and 75%, respectively. After exposure to PVS2 solution, protocorms without preculture showed significantly higher water content ($P < 0.05$) than protocorms precultured with 0.4 M sucrose for 1, 2 and 3

days at any PVS2 solution exposure time. The preculture of protocorms on 0.4 M sucrose before dehydration with PVS2 solution helped to further reduce the water content of protocorms. Longer preculture times resulted in more desiccation. The water content of precultured protocorms after exposure to PVS2 solution decreased rapidly for the first 30 min, followed by a gradual decline.

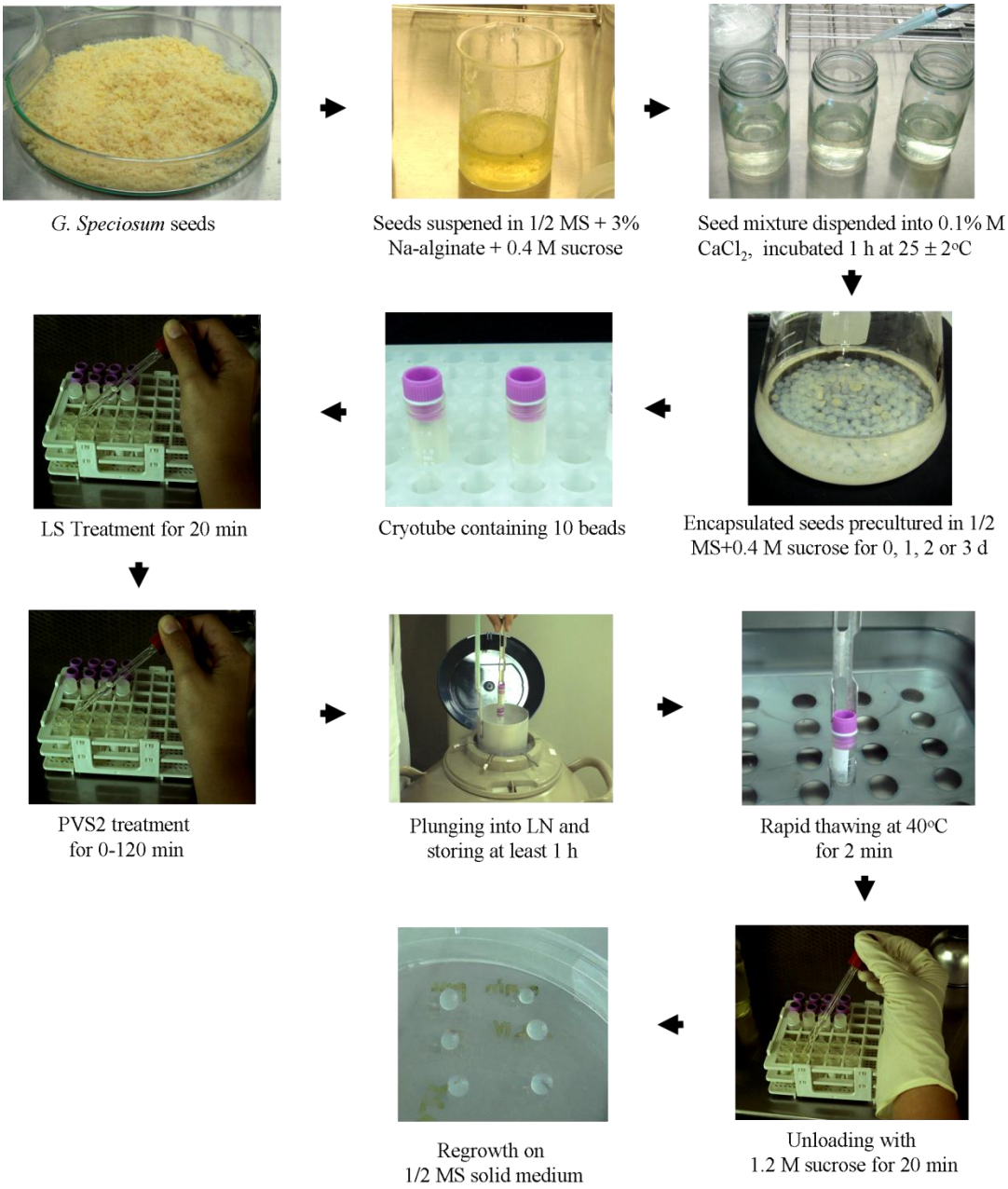

Figure 3 Established protocol for cryopreservation of *G. speciosum* seeds by encapsulation-vitrification

The exposure time to PVS2 solution affected the regrowth rate of protocorms. Increasing exposure time to PVS2 solution led to reduced regrowth in the control treatment (non- cryopreserved). The regrowth rate of non-cryopreserved protocorms decreased rapidly after 30 min of exposure time to PVS2 solution, whether the protocorms were precultured with 0.4 M sucrose or not. The optimum exposure time to PVS2 solution was 30 min. Longer exposure times to PVS2 solution also reduced the growth rate of cryopreserved protocorms. The highest regrowth rate of 38% after cryopreservation was

achieved when protocorms were precultured on 0.4 M sucrose for 2 days, followed by dehydration with PVS2 solution for 30 min.

Encapsulation-dehydration method

In contradiction to the previous experiment, the sucrose preculture did not affect the water content of the encapsulated beads when precultured for 0, 1, 2 or 3 days. When the beads were dehydrated under a laminar air-flow cabinet, the water content of encapsulated protocorms linearly decreased. The water content of encapsulated beads was about 20% after 12 h of dehydration. The regrowth rate of non-cryopreserved beads decreased rapidly after 8 h dehydration and was less than 40% when dehydrated for 12 h. After cryopreservation, the highest regrowth rate of 24% was achieved when encapsulated protocorms were precultured with 0.4 M sucrose for 2 days and then dehydrated for 8 h. The survival of encapsulated protocorms after cryopreservation by encapsulation-dehydration was successful when precultured for 0, 1, 2, or 3 days and dehydrated for 6 to 10 h. There was no survival of encapsulated protocorms after cryopreservation when dehydrated for 12 h.

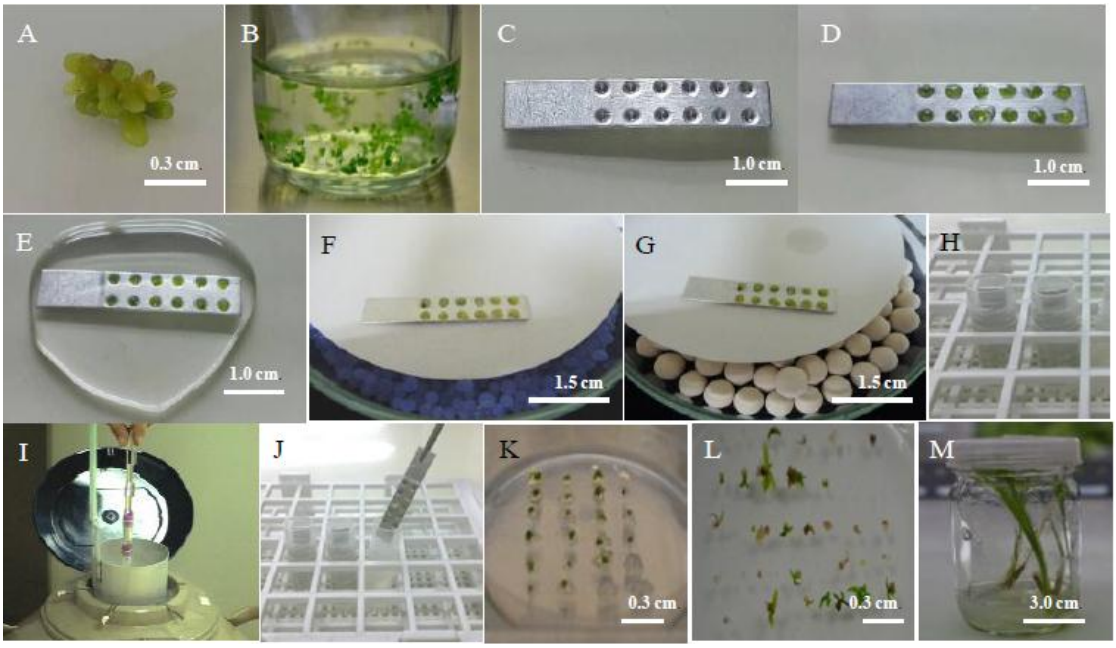

Figure 4 Cryo-plate method dehydrated with silica gel or drying beads:
A – protocorm development; B – preculture of protocorms in 1/2 MS liquid medium with 0.7 M sucrose for 1 day; C – pour the alginate solution containing 2% (w/v) sodium alginate in calcium-free 1/2 MS basal medium with 0.4 M sucrose in the wells; D - place the precultured protocorms in the wells one by one; E - pour the calcium chloride solution containing 0.1 M calcium chloride in 1/2 MS basal medium with 0.4 M sucrose; F – dehydration with 50 g silica gel; G – dehydration with 30 g drying beads; H – put each cryo-plate in a 2 ml cryotube; I - plunge 2 ml cryotubes into liquid nitrogen for 1 day; J - warming in 1.2 M sucrose solution for 20 min; K – plate on 1/2 MS agar medium; L – regrowth; M - regrowth after 60 days

Encapsulation-vitrification method

This experiment showed that sucrose preculture time did not have much effect on water content of encapsulated beads. At 0 min exposure time to PVS2 solution, the water content was 85%, 85%, 85% and 84% when precultured with 0.4 M sucrose for 0, 1, 2 and 3 days, respectively. The water content of encapsulated protocorms after exposure to PVS2 solution decreased rapidly in the first 60 min of the treatment.

Exposure time to PVS2 solution affected the regrowth rate of non-cryopreserved beads, increasing exposure time to PVS2 solution significantly reduced the regrowth rate. After cryopreservation by encapsulation-vitrification, the highest regrowth rate of about 14%

was achieved when encapsulated protocorms were precultured with 0.4 M sucrose for 1 and 2 days, and then dehydrated with PVS2 solution for 60 min.

Cryo-plate method

Preculture, encapsulation adhered to cryo-plates and dehydration using 50 g silica gel or 30 g drying beads per Petri dish for 5 h had a little effect on survival (92% for silica gel; 95% for drying beads) and regrowth (90% for silica gel; 92% for drying beads) of non-cryopreserved encapsulated protocorms adhered to cryo-plates. Survival and regrowth of cryopreserved encapsulated protocorms adhered to cryo-plates were hardly different (74-77%) and close to values observed for non-cryopreserved materials.

Regrowth was observed at the 2nd week after culture on ½MS agar medium. Dehydration using silica gel or drying beads did not obviously affect regrowth rate. Subsequently, protocorms developed into normal plantlets.

In this study, *G. speciosum* protocorms cryopreserved by droplet-vitrification gave the highest regrowth rate (38%) among these three methods. This regrowth rate seems to be low but it is sufficient for *Grammatophyllum* plants because the cost to maintain these large plants in the greenhouses is high. Droplet-vitrification combines fast freezing and the application of concentrated intracellular solution which is important for successful vitrification-based cryopreservation. Placing plant materials on aluminium foil strips and then directly plunging into LN has a cooling rate of around 130°C s^{-1}.

Pretreatment which prepares the plant materials to the freezing process is an important process for successful cryopreservation. However, the suitable condition depends on species. In this study, preculture of protocorms and encapsulated protocorms with 0.4 M sucrose induced freezing tolerance. Protocorms precultured with 0.4 M sucrose for 2 days before exposure to PVS2 solution and LN showed higher survival compared to protocorms without preculture. Encapsulated protocorms precultured with 0.4 M sucrose for 1 and 2 days before exposure to PVS2 solution and LN showed higher regrowth rates than encapsulated protocorms without preculture. Osmosis occurs in the presence of high concentrations of sucrose and results in a slow reduction of moisture content. Sucrose is able to enter the cells which leads to soluble sugar accumulation and acts as the protectant of proteins and membranes from dehydration and freezing damage. The preculture of mature seed, 3-day germinating seed and protocorms of *Bletilla striata* with high concentrations of sucrose and sorbitol enhanced their tolerance of PVS2 treatment and improved the regrowth rate of cryopreserved materials [2].

PVS2 solution is the most commonly used cryoprotectant for plant cryopreservation; however, it could be highly toxic. Optimization of the exposure time to PVS2 treatment is known to reduce the injurious effects that might occur during the dehydration process. Our results showed that optimum exposure time to PVS2 solution depended on plant materials, 30 min for protocorms and 60 min for encapsulated protocorms. PVS2 solution is highly toxic to *G. speciosum* protocorms. Regrowth rate of non-cryopreserved was significantly decreased when exposure times to PVS2 solution was increased. The regrowth rate of non-cryopreserved protocorms was <60% when protocorms or encapsulated protocorms were exposed to PVS2 solution for 90 min, and the regrowth rate was less than 40% when plant materials were exposed to PVS2 solution for 12 h.

Encapsulation of protocorms with 3% Na-alginate affected the regrowth rate of non-cryopreserved protocorms. The regrowth rate of non-cryopreserved protocorms without exposure to PVS2 was 93%, while the regrowth rate of encapsulated protocorms without exposure to PVS2 or before being subjected to dehydration by air-flow was about 83%.

It was noted that droplet-vitrification is the most suitable method for the cryopreservation of *G. speciosum* protocorms among the three methods tested. However, the regrowth rate obtained from droplet-vitrification was quite low. To obtain higher survival and

regrowth rate, protocorms of different developmental stages, sucrose or other sugar concentrations for preculture, preculture conditions, exposure times to loading solution or recovery method after cryopreservation will need to be further investigated for droplet-vitrification.

Cryopreserved protocorms of *Arundina graminifolia* using the cryo-plate method had the highest regrowth (77%) using drying beads after 90 days. This could be attributed to the removal of a reasonable amount of water from the cells that allowed for regeneration without ice crystal formation. It can also be suggested that good cold / heat conduction of the cryo-plate at 4,500°C min^{-1}, and the reduction in harmful chemicals had an effect on the regrowth of the protocorms.

Using the cryo-plate method permits for the encapsulation of protocorms directly on the plate itself. Doing so may increase survival and decrease cell destruction due to dehydration. Utilizing the cryo-plate method does not require the use of toxic cryoprotectants, such as DMSO and ethylene glycol, which has been applied in standard vitrification techniques. This work allows for the development of new research techniques, such as the testing of genetic loss over time in orchid species that have been cryopreserved. The technique might also be suitable for application to other species of rare and endangered plants. In conclusion, this is the first report of using silica gel and drying beads successfully to dehydrate orchid materials cryopreserved by the cryo-plate method.

Acknowledgements

This research project was supported by the Faculty of Science, and the Faculty of Graduate Studies, Mahidol University, The Royal Golden Jubilee Ph.D. Program, The National Research Council and the Biodiversity-Based Economic Development Office (Public Organization).

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Статья поступила в редакцию 20.07.2016 г.

Канчит Таммасири Сохранение тайских видов орхидей с помощью криобиотехнологии // Бюл. Никит. ботан. сада – 2016. – № 120. – С. 7 – 16.

Таиланд считается родиной более 1300 видов орхидей из 180-190 родов. Вырубка леса и чрезмерный уровень экспорта дикорастущих орхидей приводит к их исчезновению. Следовательно, проблема рационального их использования и сохранения видов любыми способами стоит достаточно остро. Роды *Paphiopedilum* и *Dendrobium cruentum* включены в список Конвенции СИТЕС. На кафедре ботаники (Факультет естественных наук, Махидол Университет), применяются различные методы криоконсервации с целью сохранения видов орхидей, произрастающих на территории Таиланда. В последнее время для криоконсервации используются методы витрификации (дегидротация в растворе PVS2, состоящем из 30% (вес/объём) этилена гликоля и 15% (вес/объём) диметилсульфоксида, приготовленном в модифицированной VW жидкой среде), инкапсуляции и дегидротации (инкапсуляция в шариках из альгината кальция с последующим высушиванием в камере с приграничным потоком воздуха), инкапсуляции-витрификации (инкапсуляция в шариках из альгината кальция с последующей дегидротацией в растворе PVS2), капельной витрификации (сверхбыстрый способ замораживания маленьких капель раствора PVS2 на алюминиевой пластине) и применение крио-плато (комбинирование метода инкапсуляции и капельной витрификации на алюминиевых пластинах сверхбыстрого замораживания) путем дегидротации силикагелем и высушивающими шариками. Использование этих методов было довольно успешным при работе с семенами растений следующих родов: *Dendrobium chryosotoxum* (99%, витрификация), *Dendrobium cruentum* (32%, витрификация), *Dendrobium draconis* (95%, витрификация), *Dendrobium hercoglossum* (80%, инкапсуляция-витрификация), *Doritis pulcherimma* (62%, витрификация), *Rhynchostylis coelestis* (85%, витрификация), *Vanda coerulea* (67%, витрификация), так же как и в протокормах *Dendrobium cruentum* (33%, витрификация; 27%, инкапсуляция-дегидротация), *Dendrobium cariniferum* (15%, инкапсуляция-витрификация), *Grammaytophyllum speciosum* (14%, инкапсуляция-витрификация), *Rhynchostylis gigantean* (19%, витрификация), *Vanda coerulea* (40%, инкапсуляция-дегидротация) и *Seidenfadenia mitrata* (67%, витрификация) и *Arundina graminifolia* (76% и 74%, испльзование крио-плато, дегидротация высушивающими шариками и силикагелем, соответственно; 64%, инкапсуляция-витрификация с использованием высушивающих шариков или силикагеля). Семена и протокормы после криосохранения способны образовать стандартные саженцы. Данные методы являются перспективными для криосохранения зародышевой плазмы Тайских видов орхидей.

Ключевые слова: *витрификация; инкапсуляция-дегидротация; инкапсуляция-витрификация; капельная витрификация; крио-плата; высушивающие шарики; силикагель*