RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2007.33.223

ScienceAsia 33 (2007): **223-227**

Cryopreservation of *Vanda coerulea* Griff. ex Lindl. Seeds by Vitrification

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Received 23 Jun 2006
Accepted 11 Oct 2006

ABSTRACT: An effective protocol for cryopreservation of seeds of *Vanda coerulea* Griff. ex Lindl., an endangered Thai epiphytic orchid, in liquid nitrogen (LN) was developed by vitrification. Seeds from 7-month-old pods were sufficiently dehydrated in 2 ml cryotubes filled with highly concentrated plant vitrification solution (PVS2), consisting of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide, prepared in modified Vacin and Went (1949) (VW) liquid medium at 25 ± 2°C for 70 min. The seeds were then rapidly plunged into LN. After recovering from LN and rapid warming in a waterbath at 40°C for 2 min, the PVS2 solution was replaced with 0.5 ml of 1.2 M sucrose in VW solution and kept at 25 ± 2°C for 20 min prior to transfer on VW agar medium. About 67% of cryopreserved seeds treated with PVS2 solution for 70 min were able to develop into normal seedlings *in vitro*, while without PVS2 treatment, there was no survival after LN storage.

KEYWORDS: *Vanda coerulea* Griff. ex Lindl., Thai orchid, orchid seeds, vitrification, cryopreservation.

INTRODUCTION

Vanda coerulea Griff. ex Lindl. is a medium-sized epiphytic orchid with beautiful pale to fairly deep blue flowers. It is found in the Himalayan mountains and ranges across an enormous stretch of territory, from India (Assam), through to Thailand and even into southern China; between 800 and 1700 m altitude, growing on stunted trees in exposed situations. [1,2]

Due to its outstanding horticultural characteristics of flower color, size, shape and spray, it is used worldwide for breeding blue vandaceous hybrids for commercial cut-flowers and potted orchids.[3,4] *Vanda coerulea* was listed as one of the few 'truly' threatened orchid species by the Convention on International Trade in Endangered Species (CITES) in the 1970s. The conservation of threatened *Vanda coerulea* is urgently needed. Orchids can be preserved in their natural habitat (*in situ* conservation) and outside their natural habitat (*ex situ* conservation), e.g. as living plants in greenhouses, seed storage, living plants and tissues in aseptic culture (*in vitro* culture). Orchid seeds are dry when they reach maturity, so they can be stored with high viability in cool and dry places for a short-term (a few years).[5]

Cryopreservation is potentially of great value for the conservation of orchid seeds, because small storage containers could be used and the system has low cost and maintenance needs. Furthermore, tropical, subtropical and temperate orchid seeds can be conserved and stored together in liquid nitrogen (LN) tanks without the problems of unsuitable climatic conditions.[5] In addition, one pod has abundant seeds, possibly up to thousands of seeds. Terrestrial and epiphytic orchid seeds with moisture content lower than 14% can be conserved in liquid nitrogen (LN) (-196°C).[5,6] Seeds of *Encyclia vitellinum* survived storage at -40°C for 35 d and those of *Cattleya* hybrids and *Dendrobium nobile* tolerated 465 d at -79°C.[7] Seeds of *Dendrobium candidum* with less than 12% moisture content gave 95% survival when stored in LN.[8]

Vitrification (glass formation) is a simple, fast and effective method for cryopreservation. It eliminates the need for controlled slow freezing and permits cells and meristems to be cryopreserved by direct transfer into LN.[9,10,11] This method was first presented by Fahy et al. in 1984 on rabbit kidney and later has been used successfully with various storage forms of plant groups.[12] There are some reports published on cryopreservation of orchids, such as zygotic embryos and immature seeds of *Bletilla striata*,[13,14] protocorms and protocorm-like bodies of *Dendrobium candidum*,[15,16] shoot tips of *Dendrobium* Walter Oumae,[17] seeds of *Doritis pulcherrima*,[18] suspension culture cells of *Doritaenopsis*,[19] immature seeds of *Ponerorchis graminifolia*, [20] *in vitro*-grown shoot apices of *Arachnis* species, [21] and seeds of *Bratonia* hybrid.[22] There is no report on cryopreservation of rare and endangered Thai orchid species; therefore, the purpose of this study was to develop protocols for cryopreservation of *Vanda coerulea* by vitrification.

Materials and Methods

Plant Materials and Experimental Design

Vanda coerulea plants were self-pollinated in the saranhouse of the Institute of Science and Technology for Research and Development, Mahidol University, Salaya Campus, Nakhon Pathom province. Mature pods were harvested 7 months after pollination.

The moisture content of the seeds from freshly harvested pods was determined using the hot air oven method (103°C for 17 h).[6] A completely randomized design (CRD) with four replications was used in all experiments at the Plant Science Department Laboratory, Faculty of Science, Salaya Campus.

Scanning Electron Microscopy

Vanda coerulea seeds from control and those treated with and without exposure to PVS2 solution for 70 min prior to rapid cooling in LN, were prefixed in a solution of 4% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4, at 4°C for 2 h. The seeds were washed 3 times with 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at 4°C for 1 h. They were then washed 3 times with water at 4°C, followed by dehydration in a graded series of ethanol (50-90%) at 4°C for 15 min each, 95% at 4°C for 30 min and 100% at room temperature for 20 min. The seeds were dried in a critical point dryer (HITACHI HCP-2), mounted on aluminum stubs and coated with an 18 nm platinum and paladium coat using a HITACHI E102 Ion Sputterer. Micrographs of seeds were taken using a scanning electron microscope (HITACHI S-2500).

Cryopreservation Using Vitrification

Mature pods, 7-month-old, were harvested, cleaned and washed with running tap water for a few minutes. Subsequently, the pods were brought inside a laminar air-flow cabinet, soaked in 95% ethyl alcohol and flamed with a lamp until the flame stopped. The pods were then cut on a sterile Petri dish and the seeds were taken out. For each treatment, approximately 8 mg of seeds were put in a 2 ml cryotube. Into the cryotubes was added 0.5 ml fresh plant vitrification solution (PVS2), which was developed by Sakai et al. in 1990. The solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide, prepared in modified Vacin and Went (VW) liquid medium.[8] Vitrification was carried out at 25 ± 2°C for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min, before plunging the cryotubes into LN where they were kept for 1 d.

The cryotubes were taken out of LN and warmed rapidly in a waterbath at 40°C for 2 min. The PVS2 solution was replaced with 0.5 ml of 1.2 M sucrose in VW liquid medium in which the seeds were kept at 25 ± 2°C for 20 min. Each cryotube (one replication), which contained approximately 150-200 seeds, was then transferred and cultured on VW agar medium to check for survival. The cultures were incubated at 25 ± 2°C under illumination of about 37 $\mu molm^{-2}s^{-1}$ provided by F30/GRO (GE) fluorescent tubes for 16 h per d. Survival, assessed by counting the seeds that had turned green, was measured weekly for 3 months after culturing on VW agar medium.

After seedlings were fully developed into roots and shoots, they were transferred from flasks and grown in community pots, which were filled with charcoal (at the bottom) and osmunda fibres at the top, under 50% shade in the saranhouse (greenhouse).

Results

Fig 1 shows the flowers and mature pods of *Vanda coerulea*. The characteristics of seeds of *Vanda coerulea* at 7 months after pollination are shown in Table 1. The pod is quite large and heavy due to the flower size of 7-10 cm across. It took about 7 months for the pod to mature when the tip of the pod turned yellow. Seed

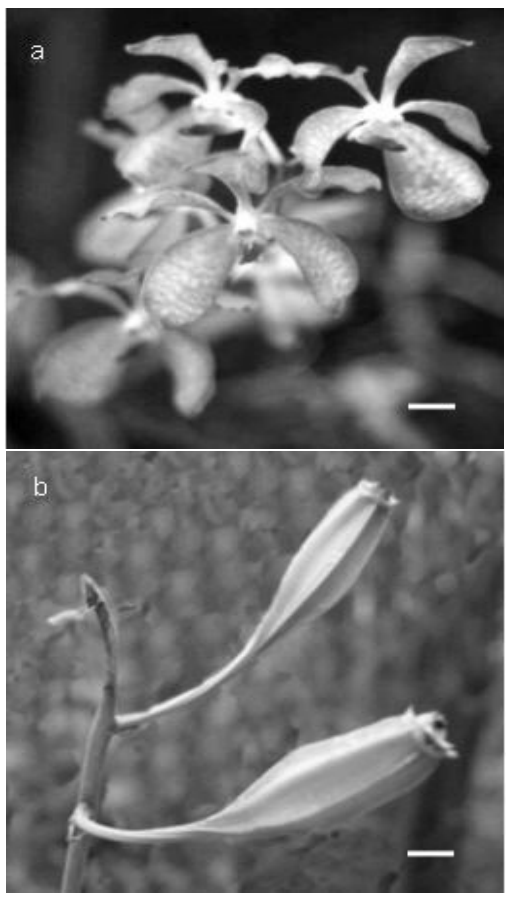

Fig 1. Flowers (a) and mature pods (b) of *Vanda coerulea*. Bar = 1 cm.

Table 1. Characteristics of seeds of *Vanda coerulea* at 7 months after pollination.

Character	
Pod size[a](l x w cm)	7.0 x 2.5
Seed size[b](l x w mm)	0.18 x 0.08
Pod weight[a](g)	5.73
Seed weight of 100 seeds[c](mg)	1.20
Seed moisture content[c](% f.wt.)	33

[a]average from 10 pods.
[b]average from 50 seeds.
[c]average from 5 pods.

moisture content was 33% of fresh weight. The effect of exposure time to PVS2 solution at 25 ± 2°C on seed survival with and without (treated control) cooling to -196°C (LN) is shown in Fig 2 . For treated control, the survival percentage was decreased a little from about 74 to 53 when exposure times were increased from 0 to 100 min. For exposure times to PVS2 solution followed by cooling to -196°C, the statistical analysis shows significantly different values among treatments (p=0.01). There was no survival when exposed for 0-20 min, but the survival percentage increased continuously up to the exposure time of 70 min and the survival decreased sharply upon longer exposures to about 11% when exposed for 100 min.

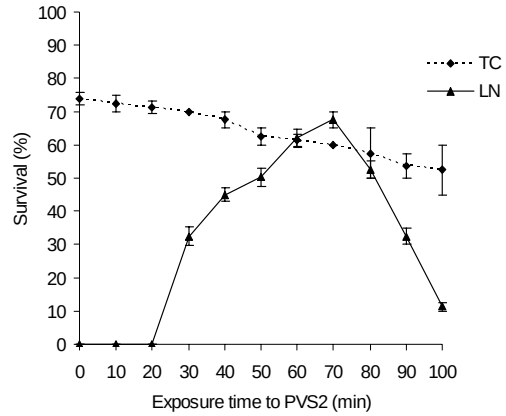

Fig 2. Effect of exposure time to PVS2 solution at 25±2°C on survival of *Vanda coerulea* seeds (11 weeks after culture) and cooled to -196°C in liquid nitrogen (LN). Treated controls (TC) were exposed to PVS2 but not subjected to -196°C. Approximately 100-200 seeds were used in each of the 4 replicates. Bar represents standard deviation.

The development of *Vanda coerulea* seeds from germination to 11 wks after culture for both control and cryopreserved seeds is shown in the scanning electron micrograph in Fig 3. Fig 4 shows the comparison of the normal development of non-

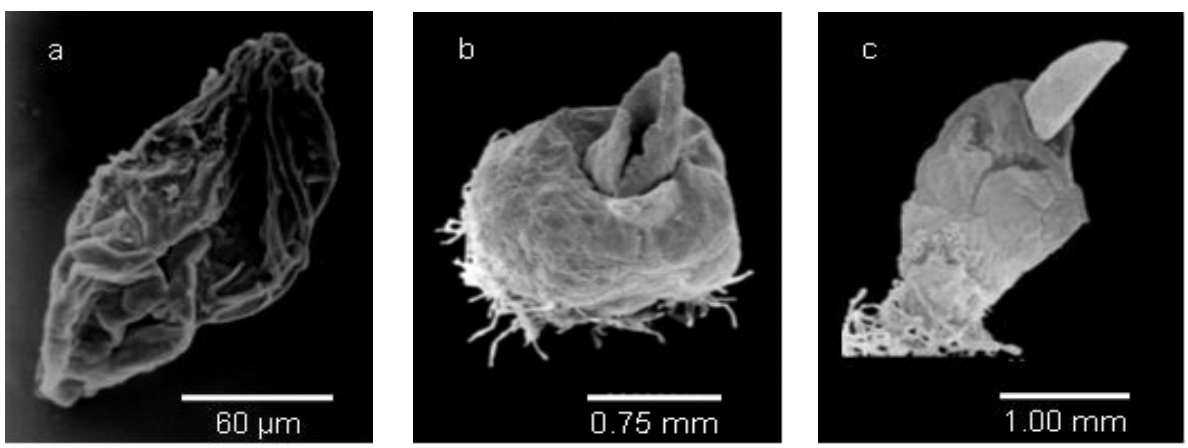

Fig 3. Scanning electron micrograph of *Vanda coerulea* seeds at the beginning of the experiment (a), a germinating seed (control) at 11 wks after culture (b), a germinating seed with exposure to PVS2 solution for 70 min + LN at 11 wks after culture (c).

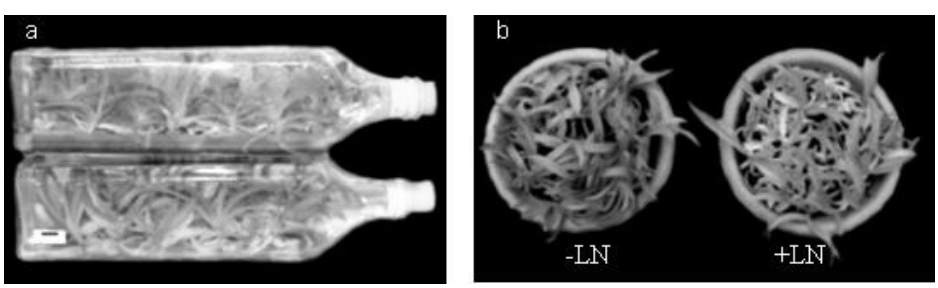

Fig 4. Development of *Vanda coerulea* seedlings from 7-month-old seeds. a: one-year-old seedlings developed from cryopreserved seeds dehydrated with PVS2 solution for 70 min (lower) and control (upper). Bar = 1 cm. b: 1.5-year-old seedlings (control) (left) and 1.5-year-old seedlings developed from cryopreserved seeds dehydrated with PVS2 solution for 70 min (right). They were grown in 7 inch pots.

cryopreserved and cryopreserved seeds in the bottles at one year old, and in pots, grown in the saranhouse at 1.5 year old.

Discussion

The desiccation characteristics of seeds differ among species, being broadly grouped as desiccation-tolerant (orthodox), intermediate and desiccation sensitive (recalcitrant) behaviors.[12] The seed longevity of the first group can be increased by lowering seed moisture and storage temperatures.[23] Orchid seeds are orthodox, very tiny, and without cotyledon and endosperm.[5] The embryos are composed of homogeneous small parenchyma cells with mostly lipid reserves.[24,5] Due to their minute size and homogeneous cells, the seeds can be sufficiently dehydrated which leads to successful cryopreservation.[25]

Although orchid seed desiccation methods were used successfully in many genera as mentioned before, vitrification could be an appropriate and practical method for conservation of many accessions of orchid seeds.[13,18] At the beginning of this experiment, seed moisture content (Fig 5) was 33% of fresh weight which was too high for preserving in LN.[5,8] There was no survival without exposure to PVS2 solution (Fig 2). Therefore, the seeds had to be dehydrated with PVS2 solution before plunging into LN. The highest survival of about 67% was obtained with exposure to PVS2 solution for 70 min. Exposure less than 20 min was not enough for *Vanda coerulea* seed dehydration, while over 70 min decreased the survival because of excessive dehydration and the inability to tolerate that dehydration level. The growth and development of cryopreserved and non-cryopreserved mature seeds were not obviously different at ages of one year (Fig 4a) and 1.5 year (Fig 4b).

Since *Vanda coerulea* seeds can germinate well (about 74%) at mature stage, their seeds can be cryopreserved successfully at this stage. This result is the same as *Doritis pulcherrima,* a Thai terrestrial and monopodial orchid.[18] In contrast, *Ponerorchis graminifolia* var. *Suzukiana*, an endangered Japanese terrestrial orchid, germinates more readily when the seeds are immature.[20] Therefore, the appropriate stage of the seed should be considered during cryopreservation. The survival rate of cryopreserved *Vanda coerulea* seeds using this vitrification protocol was about 67% without the difficulties of preculture and loading treatments. The results are quite satisfactory because there is an enormous number of seeds in one pod.

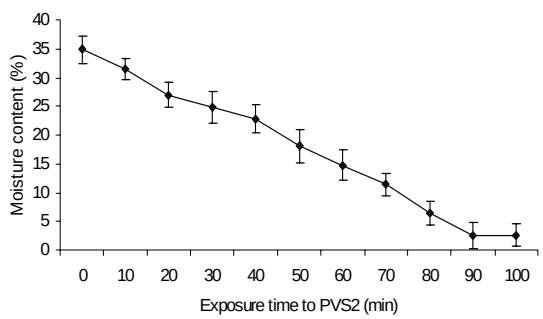

Fig 5. Effect of exposure time to PVS2 solution at 25±2°C on *Vanda coerulea* seed moisture content. Seeds were obtained from pods at 7 months after self pollination. Bar represents standard deviation.

Acknowledgements

The authors would like to thank Dr. Akira Sakai, retired Professor from Hokkaido University, for valuable suggestions. This work was partially supported by the Thailand National Research Council; the Royal Chitralada Projects, Chitralada Palace; the Faculty of Science and the Institute of Science and Technology for Research and Development, Mahidol University; and the Horticultural Research Institute, Department of Agriculture.

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