
Research Article

Cryopreservation of the North Borneo *Phalaenopsis gigantea* J.J.Sm. Using a Vitrification Approach

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Received 08 April 2023 | Reviewed 24 May 2023 | Accepted 16 June 2023 | Published 15 October 2023

Doi: <https://10.51200/jtbc.v20i.4651>

ABSTRACT

Phalaenopsis gigantea is an endangered orchid found in Borneo and Kalimantan. A cryopreservation protocol using vitrification was developed using seeds as explants. Seeds from green unburst capsules were precultured on New Dogashima Medium supplemented with 0–0.5 M sucrose prior to treatment with loading solution and vitrification with PVS2. Loading durations from 0–60 min and PVS2 dehydration time from 0–7 hours were tested. Following vitrification, seeds were stored in liquid nitrogen for one week before being recovered and subsequently cultured on a germination medium. The viability of the seeds post cryopreservation was evaluated based on 2,3,5-triphenyltetrazolium chloride reduction assay (TTC) by the seeds and germination. Seeds survived the cryopreservation treatments and germinated. Seeds precultured on the medium containing 0.3 M sucrose exhibited the highest germination ($8.3\% \pm 2.3$), while a 10-min loading time yielded the maximum germination ($13.4\% \pm 2.9$). Seeds dehydrated in PVS2 for 7 hours had the highest germination percentage ($13.9\% \pm 2$) after liquid nitrogen storage. The TTC and the germination test did not give similar results for the viability of seeds. Cryopreserved seeds developed into seedlings and showed normal morphology. Given that the seeds of *Phalaenopsis* lost viability at room temperature very fast, this protocol can potentially be used for its long-term storage which can assist in the conservation programme of the species.

Keywords: *Phalaenopsis gigantea*; cryopreservation; vitrification; sucrose preculture; loading time; PVS2 dehydration

Introduction

Phalaenopsis gigantea J.J.Sm., commonly known as the Elephant Ear Orchid, is endemic to Borneo (Malaysia - Sabah and Sarawak; Brunei and Indonesia -

Kalimantan). Previously, the species could be found in abundance in the lowland forest on the east coast of North Borneo. However, due to illegal collection activity and clearing of forests for agriculture and development, it is now challenging to find them in the wild habitat. Although its population in the wild is dwindling, it is still currently listed in Appendix II of the Convention on International Trade in Endangered Species (CITES). Under this category, the trading of a species must be controlled to avoid utilization incompatible with their survival (CITES, 2013). Hence, it is essential to preserve the species before it disappears completely. Current conservation programmes for the species include growing it in gardens and nurseries managed by government agencies as well as maintaining plant materials *in vitro*. These methods have limitations - in gardens and greenhouses, the species is exposed to climatic and disease threats, whereas the *in vitro* materials are exposed to contamination and genetic alteration. Storing the seeds of *P. gigantea* is a potential approach to the preservation of the species. But, using the conventional seed banking method, the longevity of orchid seeds is poor (Schofeld et al., 2018). A storage method that can maintain seed viability would be an advantage for the species conservation programme.

Cryopreservation is a method of storing cells, tissues, and organs at a super-low temperature (-196 °C) which causes very minimal loss of viability when the conditions involved are optimal. The method has been used to conserve plant materials of endangered species; compared to conventional practices of conservation, cryopreservation offers more benefits which include requiring little space, being free from climatic and disease threats, and effective for long-term storage (Niino and Valle Arizaga, 2015; Streczynski et al., 2019). The method has been used successfully to preserve the seeds of several orchids such as *Dendrobium* hybrid (Galdiano et al., 2014), *Cattleya tigrine* (Vettorazzi et al., 2019), *Paphiopedilum dayanum* (Petrus et al., 2019) and *Paphiopedilum bellatulum* (Rchb.f.) Stein (Rodpradit et al., 2022).

Cryopreservation of orchid seeds can be done in several ways, one of which is vitrification. Vitrification is a process where cryoprotectant solution solidifies into a metastable amorphous solid known as glass (Roque-Borda et al., 2021). The method uses concentrated cryoprotectants that can penetrate and dehydrates the cell, hence preventing the formation of intra- and extracellular ice crystals in cells and tissues that can cause damage to the cells (Londe et al., 2018). The procedure does not require the use of an expensive programmable freezer (Jiroutová & Sedlák, 2020) making it cheaper than the other methods of cryopreservation of plant materials. Examples of orchids that had been

cryopreserved using vitrification are *Rhynchostylis gigantea* (Lindl.) Ridl (Jariyajirawattana et al., 2020), *Aranda Broga* Blue orchid (Khor et al., 2020), *Dendrobium cruentum* Rchb. f. (Prasongsom et al., 2019), *Caladenia latifolia* (Bustam et al., 2016) and *Coelogyne dayanum* (Hakim et al., 2015). These previous works reported that the conditions for vitrification of orchids vary depending on the species and the types of explants used (Seol, 2020). The success of cryopreservation by vitrification requires the optimization of critical variables such as the conditions of preculture, the time of exposure to loading solution, and the time of dehydration in cryoprotectant solutions (Zamecnik et al., 2021). One of the goals in optimizing these conditions is to reduce the water content in the explants.

At present, there is no report on the cryopreservation of *P. gigantea*; therefore, this study aimed to develop an effective vitrification protocol for the cryopreservation of *P. gigantea* seeds by optimizing the conditions involved in the method.

Materials and Methods

Plant Materials

Phalaenopsis gigantea growing in the Orchid Garden at Tawau Hill National Park were self-pollinated. Green, unburst capsules were harvested five months after pollination and sterilized by immersion in 25% (v/v) Chlorox containing one drop of Tween 20 for 20 mins, followed by three rinses with sterilized distilled water. Each capsule was longitudinally dissected, and the seeds were cultured on a germination medium (Murdad et al., 2006) for 10 days. This culture medium was New Dogashima Medium (NDM) (Tokuhara & Mii, 1993) supplemented with 20 gL⁻¹ sucrose and 1 gL⁻¹ activated charcoal. After this, the seeds were transferred to preculture media, which were NDM, with different concentrations of sucrose.

Vitrification

i) Preculture in sucrose

The seeds were precultured on semi-solid NDM media containing 1 gL⁻¹ activated charcoal supplemented with different concentrations of sucrose (0.1 M, 0.2 M, 0.3 M, 0.4 M, or 0.5 M) for five days followed by exposure to loading solution. The loading solution consisted of 2 M glycerol and 0.4 M sucrose (Matsumoto et al., 1995) in an NDM medium at 25 °C for 20 mins. After the loading stage, the seeds were treated with PVS2 solution (Sakai et al., 1990) at 0 °C for 5 hours and subsequently stored in liquid nitrogen (LN) for 1–7 days. The PVS2 solution

was made of 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethyl sulfoxide (DMSO), and 0.4 M sucrose in NDM medium (pH 5.7). The control consisted of seeds that were precultured on sucrose-free media.

ii) Loading time

The seeds were precultured on the medium with 0.3 M sucrose for five days followed by incubation in loading solution for 10, 20, 30, 40, 50, or 60 mins at 25 °C followed by treatment in PVS2 as above and stored in liquid nitrogen (LN) for 1–7 days. For the control, the seeds were treated with loading solution but the solution was immediately removed and replaced with PVS2. Because the loading solution was not allowed time to take effect on the seeds, this was referred to as 0 min loading time.

iii) PVS2 dehydration time

The seeds were precultured on 0.3 M sucrose for five days and treated with loading solution for 20 mins at 25 °C before being exposed to PVS2 solution for 1, 2, 3, 4, 5, 6, or 7 hours at 0 °C. The vitrified seeds were then stored in LN for 1–7 days. The control consisted of seeds that were immediately transferred into the LN tank after the PVS2 solution was added into the cryotubes. Because the PVS2 was not allowed time to take effect, this was referred to as PVS2 for 0 h. The seeds that were cultured on the germination medium with no vitrification treatment and no exposure to LN were used as the negative control (Non-Cryo). Each treatment was replicated 3–4 times, and 100–300 seeds were tested in each replicate.

Thawing

After storage in LN, cryopreserved seeds were rapidly re-warmed in a water bath at 37 °C for 1 min, after which the PVS2 solution was removed and replaced with 1 ml of unloading solution which contained 1.2 M sucrose (Matsumoto et al., 1995) in NDM medium for 15 mins at room temperature. Finally, the seeds were rinsed five times with a washing solution (NDM medium containing 2% w/v sucrose) (Hakim et al., 2015).

Viability test

Viability was accessed by 2, 3, 5-triphenyltetrazolium chloride (TTC) reduction assay (Hu et al., 2013) and germination of seeds. For the TTC reduction, seeds were incubated in a 1% (w/v) TTC solution for 24 hours at 30 °C in the dark. The number of seeds with TTC-stained embryos were counted, and the viability was defined as the percentage of seeds showing staining. At least 100 seeds were counted.

Seed germination and regrowth

Thawed seeds were cultured on the germination medium which consisted of NDM containing 20 gL⁻¹ sucrose and 1 gL⁻¹ charcoal (Murdad et al., 2006) in Petri dishes and incubated at 25 °C ± 2 in the dark. Seeds were considered to have germinated when the seed coats cracked, and the protocorms emerged. Germination percentages were determined every 10 days for 210 days. The results were interpreted as germination percentage after 210 days. After 210 days, the germinated seeds were transferred to light conditions so that the protocorms turned green.

Seedling growth and acclimatisation

Green protocorms were transferred to a development medium, which consisted of Experimental Ernst Robert medium (XER) (Ernst, 1994) containing fructose. Protocorms were monitored for shoot and root development.

Data collection and analysis

This study was carried out in a Completely Randomized Design (CRD). Three replicates were prepared for each type of treatment. The germination rates of the seeds were analyzed using One-WayANOVA, means were compared using Turkey Range Test at $p < 0.05$. While data for seed viability based on TTC and seed germination were subjected to Correlation Coefficient Analysis to measure the strength of the relationship between two variables using IBM SPSS Version 21.0 (IBM Corp., Chicago, IL, USA). Pearson Correlation Test was employed to determine a significant difference at $p < 0.05$.

Results and Discussion

This study evaluates the viability of the seeds of *P. gigantea* post cryopreservation based on TTC and germination; hence results for both tests are presented. The embryos of the seeds that were taken out of liquid nitrogen storage slowly swelled up (**Figure 1B**) and broke out of the testea, forming protocorm (**Figure 1C**) before developing into roots and leaves (**Figure 1D**). Generally, cryopreserved seeds of *P. gigantea* exhibited lower germination as compared to the seeds that did not undergo cryopreservation treatment and were not stored in LN (Non-Cryo). These results suggest that damage had occurred in the seeds. This reduction in germination could have been caused by injury due to the formation of intracellular ice crystals during freezing (Chang & Zhao, 2021). Similarly, a reduction in the germination of the seeds of *Vanda tricolor* Lindl. post-cryopreservation was also reported by Jitsopakul et al. (2012).

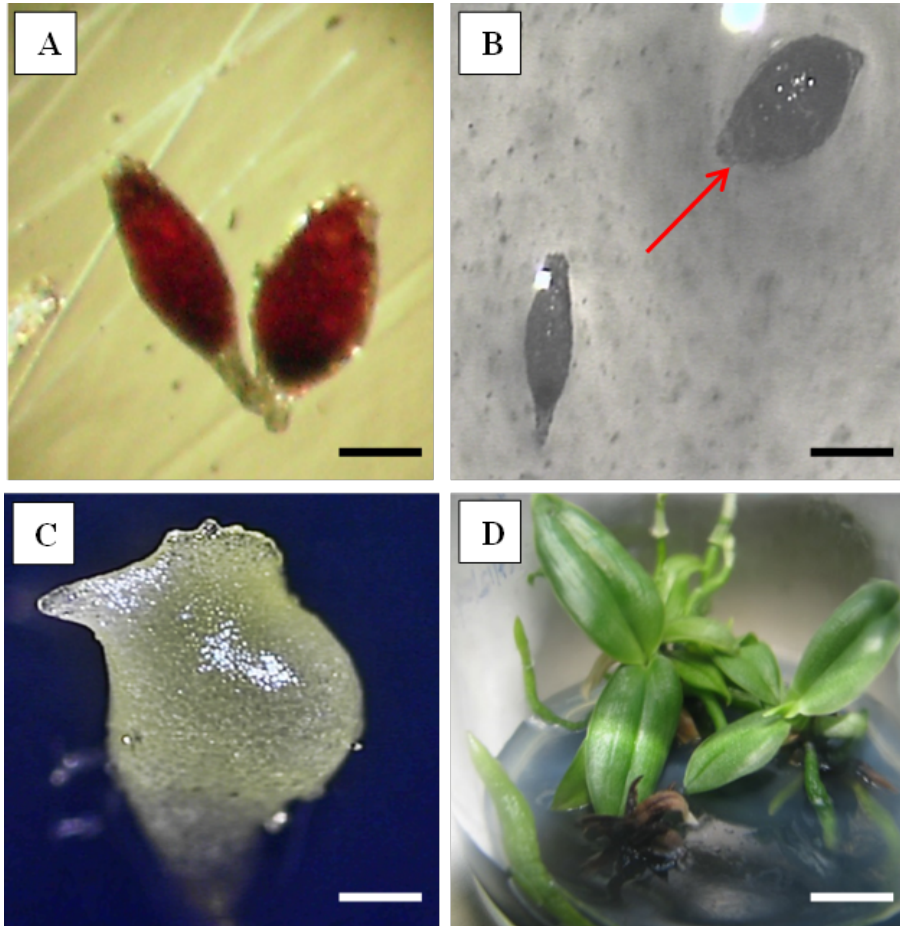


Figure 1. Development of cryopreserved seeds of *Phalaenopsis gigantea* after cryopreservation. (A) Seeds-stained red following TTC test, magnification x 100; (B) A swollen seed (arrow) on germination medium, following recovery from liquid nitrogen, Bar = 1.1×10^{-4} mm; (C) Germinated seed showing leaf primordium, Bar = 1.3 mm; (D) Seedlings from cryopreserved seeds, Bar = 8.3 mm.

Effect of preculture in sucrose on TTC test and the germination

Following recovery from liquid nitrogen, the viability of the seeds based on TTC test showed that precultured on 0.5 M sucrose gave the highest viability (41.8%), followed by those precultured on 0.2 M sucrose (34.3%) (**Figure 2**). However, the viability based on the germination test revealed that the germination of cryopreserved seeds was the highest ($8.3\% \pm 2.3$) when they were precultured on the medium containing 0.3 M sucrose, this is only 1.5-fold increment in germination as compared to the seeds that were pre-treated on the medium

without sucrose $5.7\% \pm 1.5$) (Control). The seeds precultured on the medium containing 0.25M sucrose showed the lowest germination ($1.3\% \pm 0.6$), this was a 97% reduction in germination as compared to the Non-Cryo seeds ($42.0\% \pm 12.5$) (Figure 2). Observation over 210 days revealed that germination declined when the sucrose concentration was further increased to 0.4 or 0.5 M (Figure 2). Our results indicated that preculturing *P. gigantea* seeds in sucrose improved their germination after storage in liquid nitrogen, however, there was no significant ($p < 0.05$) difference in the germination of seeds precultured in different sucrose concentrations.

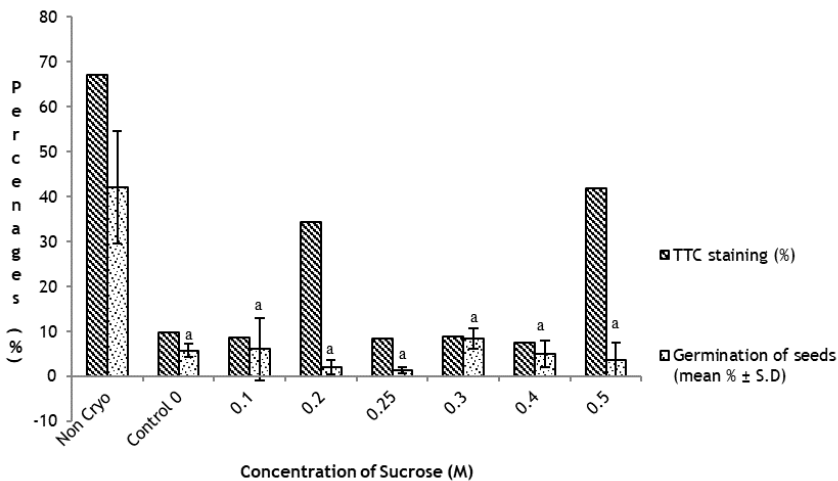


Figure 2. Survival of *Phalaenopsis gigantea* seeds precultured on sucrose after cryopreservation (LN), based on TTC and germination tests.

The beneficial effect of sucrose preculture is due to sucrose enhancing the physiological tolerance of plant materials that are to be cryopreserved by vitrification (Zhang et al., 2023). Enhancement of freeze tolerance because of preculture with sucrose is attributed to the sugar's ability to stabilize membranes by forming hydrogen bonds with membrane phospholipids (Crowe et al., 1987). In our work, 0.3 M sucrose is the best concentration for the preculture of *P. gigantea* seeds. But for other species such Jewel Orchid, *Ludisia discolor* sucrose at 0.2 M for 24 h was the best sucrose preculture treatment that enhanced germination (Burkhan et al., 2022). While *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer required 0.5 M sucrose preculture for the best recovery post-cryopreservation (Diengdoh et al., 2019). On the other hand,

there are other orchid species that did not require the sucrose preculture step for successful cryopreservation, this was reported for *Bletilla formosana* (Hayata) Schltr. by Hu et al. (2013).

Effect of loading time on TTC test and germination

The viability of cryopreserved seeds based on TTC analysis was the highest (14.8%) when the seeds were incubated with loading solution for 30 mins (**Figure 3**). But evaluation of growth based on seed germination showed that the highest germination (13.4% \pm 2.9) occurred when seeds were incubated in the loading solution for only 10 min (**Figure 3**). Germination percentages decreased when the loading period exceeded 10 mins, and the seeds which were treated with the loading solution for 0 min (Positive) had the lowest germination (4.3% \pm 1.5). A significant ($P < 0.05$) increase in germination was observed when the seeds were treated for 10 mins in loading solution as compared with 0 min of loading time.

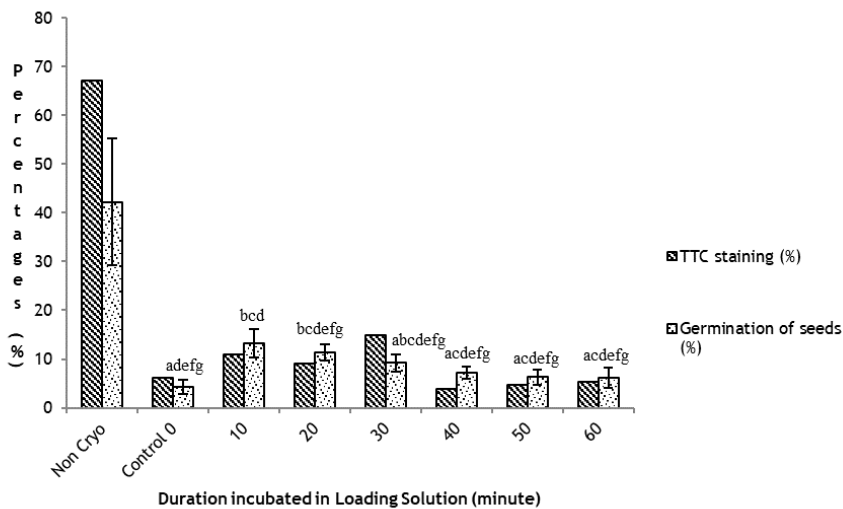


Figure 3. Survival of *Phalaenopsis gigantea* seeds incubated in loading solution for different times after cryopreservation (LN), based on TTC and germination tests.

Our studies showed that the loading treatment is an important step in enhancing the germination of *P. gigantea* seeds post-cryopreservation. When cells are incubated in concentrated PVS2 prior to storage in liquid nitrogen, the cells experience severe osmotic differences which can cause injury due to excessive dehydration, and chemical toxicity (Kaviani & Kulus, 2022). To reduce this

osmotic shock when samples are exposed to the concentrated PVS2, treatment of samples with a loading solution having less concentrated osmoticum prior to dehydration is necessary (Matsumoto, 2017). According to Kim et al. (2009), loading treatment acts as an osmotic neutralizer and/or induces a physiological adaptation of tissues, cells, and membranes before the dehydration and freezing steps. In our present study, the protective effect of loading treatment for 10 mins improved germination post-storage by three-fold in LN. The benefit of loading treatment was also reported in the cryopreservation of *B. formosana*, a Taiwanese terrestrial orchid; it was postulated that treating *B. formosana* at different times of loading solution reduces the injuries on the membrane due to severe dehydration in PVS2 (Hu et al., 2013).

Effect of PVS2 dehydration time on the TTC test and germination

The germination test revealed that germination increased as the dehydration time in PVS2 increased; germination was maximal ($13.9\% \pm 2$) with 7 h incubation and minimal ($3.4\% \pm 0.6$) with 0 h (Control) incubation in PVS2 (Figure 4). As much as a 4-fold increase in germination was observed when the seeds were dehydrated in PVS2 for 7 h as compared with 0 h of incubation. A significant ($p < 0.05$) improvement in germination was observed when the dehydration time in PVS2 was longer than 3 h. In the vitrification procedure, explants must be dehydrated sufficiently to avoid cell damage due to the formation of ice crystals during storage (Rohmah et al., 2022). It is also known that PVS2 solution not only facilitates the survival of cells that are to be cryopreserved but is also toxic to cells when in contact with them (Zamecnik et al., 2021). Hence, it is vital to identify the optimal time of exposure to the vitrification solution so that cells are not damaged, but sufficiently dehydrated (Nakkanong & Nualsri, 2018). This present study indicated that *P. gigantea* seeds showed high tolerance towards desiccation, a desiccation time of 7 h resulted in the highest germination. The longer dehydration time required by *P. gigantea* seeds might be due to the high-water content of the seeds as the seeds were taken from green unburst capsules (immature). According to Wu et al. (2018), the drying time directly affects the water content of orchid seeds, hence affects the formation of intracellular ice nuclei. Desiccation time is species dependent, orchids such as *B. formosana* required 24 h for optimum germination (Wu et al., 2018), on the other hand, seeds of *Coelogyne dayanum* 3 h in PVS2 to yield 80.9% germination (Hakim et al., 2015), while *Coelogyne nitida* (Wall. ex D. Don) Lindl. seeds needed only 40-min incubation in PVS2 to enhance regrowth (Chaudhury et al., 2021). In contrast to the germination test, the viability test using TTC analysis showed that 2 h of incubation in PVS2 gave the highest viability (Figure 4).

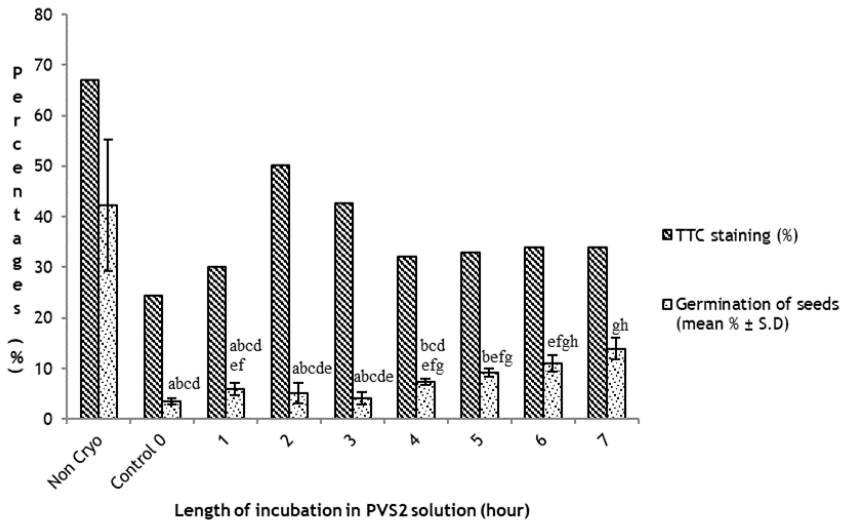


Figure 4. Survival of *P. gigantea* seeds incubated in PVS2 solution for different times after cryopreservation (LN), based on TTC and germination tests.

Throughout the study, the TTC test and the germination test did not give similar results for the viability of seeds, and correlation coefficient analysis revealed that there is no significant ($p < 0.05$) correlation between TTC and germination tests. The TTC test depends on the dehydrogenase enzymes, the enzymes present in living tissues reduce the colorless TTC salt into red-colored triphenylformazan (França-Neto & Krzyzanowski, 2019), hence viable seeds will be stained red (Figure 1A). The contradictory results between the TTC and germination tests may be due to the TTC test giving false positives when the enzymes present in the cell even after the cell death resulted in a viable score (Pradhan et al., 2022). A similar result was reported by Kolomeitseva et al. (2022) in *Dendrobium nobile* Lindl. and in *Rhynchosyilis gigantea* (Lindl.) Ridl. (Jariyajirawattana et al., 2020). This study showed that TTC is not an accurate indicator of viability for the species, hence, for *P. gigantea* seeds, it is essential to confirm the survival of cryopreserved seeds through germination tests.

Seedling growth and acclimatization

Plantlets with developed roots were acclimatized and showed normal morphology like that of the parents (Figure 1D). When using cryopreservation technique in any conservation programme, it is very important that the cryopreserved materials retain their genetic stability (Engelmann, 2011). The genetic integrity of plants surviving cryopreservation can be assessed using

several methods, one of which is through the phenotypic level (Harding, 2004). Previous studies that compared the vegetative growth and morphology of seedlings from cryopreserved seeds to that of non-cryopreserved seeds and the parents reported that there were no significant differences in vegetative characteristics. The works included that of *Vanilla planifolia* (González-Arno et al., 2022), *B. formosana* (Hu et al., 2013), and *Vanda coerulea* Griff. ex Lindl. (Thammasiri & Soamkul, 2007). This present study had shown that no morphological variation was observed as a result of using our method of cryopreservation by vitrification.

Conclusions and Recommendations

This study showed that *P. gigantea* seeds survived cryopreservation by vitrification and germinated successfully. Optimizing the vitrification condition, namely, preculture, loading time, and incubation time in PVS2 improved the survival of cryopreserved *P. gigantea* seeds. Because *Phalaenopsis* seeds lose their viability when stored at ambient temperatures, cryopreservation using this procedure can potentially be applied to conserve *P. gigantea* seeds for long-term storage.

Acknowledgments

This work was financially supported through a grant from the Malaysian Ministry of Science and Innovation (MOSTI) and in collaboration with Sabah Parks. The authors would like to thank Sabah Parks for providing the orchid capsules.

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