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Cryopreservation of Orchid Seeds, Protocorms, and Pollen

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The popularity of orchids has a worldwide reach, placing them as the top cut flower and potted plant in the international market. Last year, the United States Department of Agriculture upgraded orchids ranking first as flowering potted plant in the United States with wholesale value estimates of about $200 million. Orchids are indicator plants to the health of a habitat. Over collection and habitat destruction have promoted the reduction in wild orchid natural populations. Therefore, conservation of orchids needs immediate attention. Cryopreservation is an efficient technique for long-term storage of orchid plant material and can assist in the preservation of endangered orchids. Furthermore, it offers an alternative for long-term storage of orchid genetic material for breeding and genetic improvement programs. In this paper we summarize several studies with cryopreservation of orchid material, which have been developed in our laboratory over the past seven years at the University of Florida's Tropical Research Center. We have developed an efficient protocol for cryopreservation of hybrid mature seeds, protocorms, and pollen of *Dendrobium* Sw. species and hybrids. Improved cryopreservation protocols were developed using phloroglucinol as a cryoprotectant. Seedlings from cryopreserved *Oncidium flexuosum* Lodd. seeds were also assessed for genetic stability after cryopreservation.

Orchids represent one of the largest families of flowering plants and have gained popularity and interest over the past decade from both the production and conservation points of view. With about 30,000 known species, orchids have great ornamental appeal because of their flower colors, shapes, and patterns, added to the over 100,000 hybrids, which bring additional variety in flower characteristics of commercial interest. Orchids rank top among flowering plants, providing high returns as either cut flowers or potted plants, and represent 8% of the world floriculture trade (Chugh et al., 2009). The total wholesale value of orchids was estimated at over $200 million in 2011, placing them as the second highest potted flowering plant in the United States (USDA, 2011). However, in 2013 orchids became the top potted flowering plant in the country (USDA, 2014). The increase demand for orchids has been promoting breeding programs for the development of new plant material with enhanced characteristics (Johari et al., 2009). Besides their ornamental appeal, orchids have also medicinal and ethnobotanical value.

However, most orchids are listed under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), as either threatened or endangered. Habitat destruction has been the major factor contributing for the reduction of genetic variability in orchids (Nikishina, 2001). Therefore conservation programs are important to preserve the existing genetic diversity for breeding programs and also for long-term conservation of germplasm.

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Cryopreservation is a technique used for the long-term storage of germplasm in ultra low temperatures (–196 °C) in liquid nitrogen, being simple, safe and cost-effective (Benson, 2008). Cryopreservation allows the storage of diverse orchid tissues and organs, such as seeds, protocorms and pollen, allowing preservation and distribution of material at reduced costs, as well as representing an important tool for orchid breeders. Vitrification has been widely used in as the cryopreservation method for orchids (Pritchard et al., 1999; Thammasiri, 2000; Popova et al., 2003; Hirano et al., 2005). More recently we have developed a number of studies to verify the effectiveness of the vitrification method for the cryopreservation of seeds (Vendrame et al., 2007), pollen (Vendrame et al., 2008), and protocorms (Vendrame and Faria, 2011). We have also evaluated alternative cryoprotectants with low toxicity for the cryopreservation of protocorms (Vendrame and Faria, 2011) and seeds (Galdiano et al, 2012; 2013) and the genetic stability of seedlings resulting from the cryopreservation of seeds (Galdiano et al., 2013).

The objective of this review is to report the different studies performed with orchid cryopreservation at the Tropical Research and Education Center (TREC), Institute of Food and Agricultural Sciences, University of Florida.

Cryopreservation of Orchid Seeds

The long-term storage of orchid seeds plays an important role for both conservation and breeding programs. Studies with orchid cryopreservation at TREC have focused on vitrification protocols due to their efficiency, low cost, little space required, facility of germplasm material distribution, and reliability.

Mature seeds of several *Dendrobium* hybrids (Fig. 1A) were submitted to different vitrification treatments aiming at improved

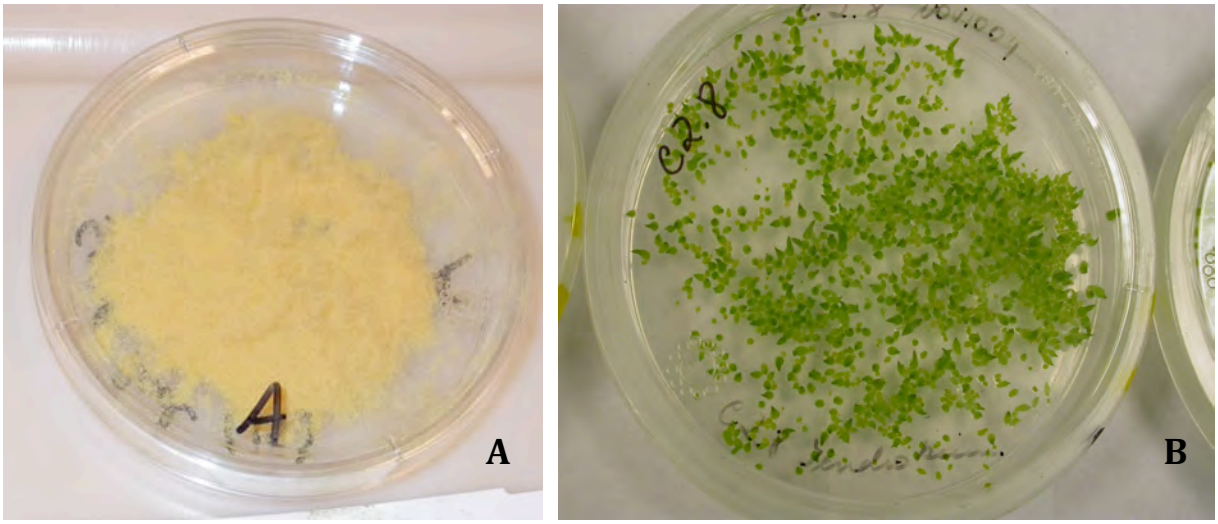

Fig. 1. (**A**) Seeds of *Dendrobium* hybrid. (**B**) Seed germination of *Dendrobium* hybrid after cryopreservation (LN at –196 °C). Seeds were pre-cooled in ice (0 °C) for 1–3 h prior to immersion in liquid nitrogen.

germination of cryopreserved seeds (Vendrame et al., 2007). The plant vitrification solution (PVS2, Sakai et al., 1990) used for cryopreservation consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS medium (Murashige and Skoog, 1962) with 0.4 M sucrose (pH 5.7). The PVS2 solution is traditionally used in cryopreservation protocols and results showing a germination percentage equal or above 50% of the initial germination rate of seeds is considered successful.

In this study, pre-cooling treatments were introduced to evaluate their effectiveness in improving germination rates post cryopreservation. Pre-cooling treatments include placement of seeds in PVS2 in ice (0 °C) for 1, 2, 3, 4, and 5 h prior to immersion in liquid nitrogen (LN). The controls (direct seed germination, no LN) showed germination percentages between 63.7% and 96.1%. Additional controls (no pre-cooling prior to LN) had germination percentages between 0% and 26.9%.

The best treatments included pre-cooling of seeds for a period of 1 to 3 hours prior to immersion in LN, with post cryopreservation germination percentages varying from 47.2% to 63.8%. Pre-cooling of seeds for 1–3 h prior to LN provided germination percentages of at least 66% of the initial germination percentage, therefore confirming the success of the study. The success in seed germination (Fig. 1B) post cryopreservation was also determined by subsequent plant development and survival.

In a second study, the effectiveness of phloroglucinol and Supercool X-1000® as cryoprotectants was evaluated for the cryopreservation of *Dendrobium* hybrid seeds (Galdiano et al., 2012). Phloroglucinol (1,3,5-trihydroxybenzene) is a benzenetriol extracted from brown algae known for its antioxidant properties and for enhancing in vitro shoot production of plants (Kang et al., 2006; Kim and Kim, 2010). Supercool X-1000® is a copolymer of polyvinyl alcohol and vinyl acetate and a low cost alternative to antifreeze proteins.

Mature seeds were placed in PVS2 solution for 60 min, after which they were submitted to treatments containing either phloroglucinol (1%), Supercool X-1000® (1%), or a combination of both. Controls consisted of seeds in PVS2 solution for 60 min with no cryoprotectants. All treatments and control were immersed in LN for 60 min.

Although PVS2 alone returned germination percentages (51.1%), the addition of 1% phloroglucinol enhanced germination significantly (79.3%). The addition of 1% Supercool X-1000® did not enhance germination (52.5%) as compared to PVS2 alone (51.1%). The combination of PVS2 with 1% phloroglucinol and 1% Supercool X-1000® improved germination (76.2%), but to levels similar to PVS2 + 1% phloroglucinol (79.3%). Although the specific mechanism of action of phloroglucinol was not investigated, it appears that a potential synergistic effect occurred between PVS2 and phloroglucinol, warranting additional studies.

Cryopreservation of Orchid Protocorms

Cryopreservation of orchid protocorms using vitrification protocols has been reported (Wang et al., 1998; Popova et al., 2003; Nikishina et al., 2007; Yin, 2009). However, vitrification solutions contain cryoprotectants such as DMSO (dimethyl sulfoxide) that can be toxic to plant tissues, particularly the tender tissues of orchid protocorms, and therefore require careful handling and determination of non-lethal concentrations. Phloroglucinol protects cells against oxidative stress and has cryoprotective properties (Benson and Bremmer, 2004).

Seeds of *Dendrobium nobile* were germinated in vitro and 60-day-old protocorms (Fig. 2A) were selected for cryopreservation studies (Vendrame and Faria, 2011). Protocorms were immersed in PVS2 solution and treatments consisted of different cryoprotectants either alone or in combination, including glycerol (2 M), sucrose (0.4 M), and phloroglucinol (1%). Controls contained neither cryoprotectants, nor PVS2 (control 1), or contained PVS2 alone (control 2). Controls and treatments were immersed in LN for 3 months.

Treatments with sucrose (0.4 M) resulted in very low protocorm survival percentages (6.2% to 8.9%). The combination of glycerol (2M) with PVS2 increased survival to 31%. However, among all treatments, the combination of PVS2 with 2M glycerol and 1% phloroglucinol returned the highest protocorm survival (68%) with about 62% of protocorms forming seedlings (Fig. 2B). The successful survival and subsequent development of

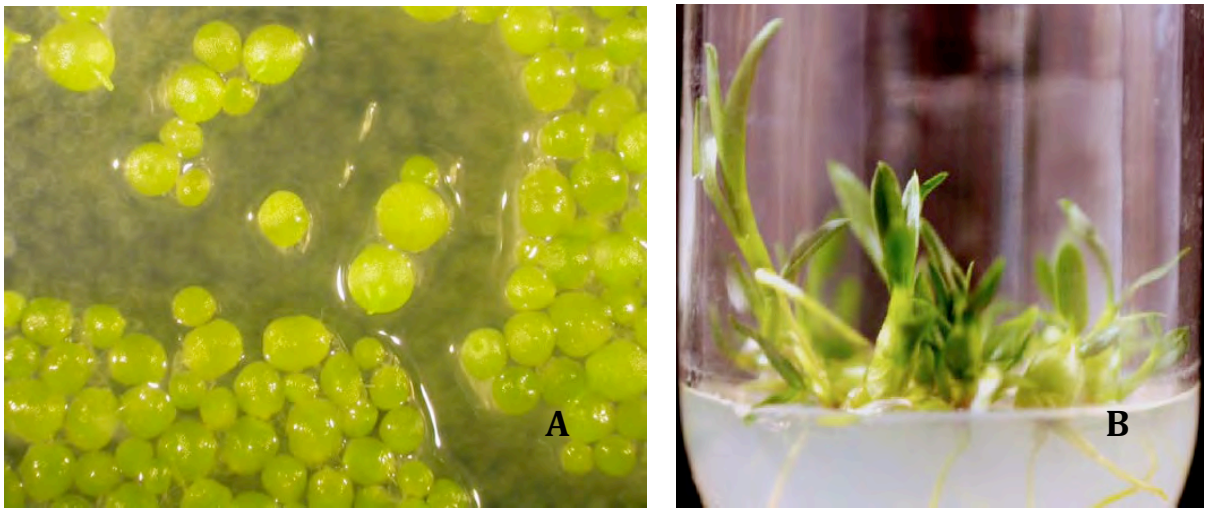

Fig. 2. (**A**) Protocorms of *Dendrobium nobile* prior to cryopreservation (liquid nitrogen at –196 °C). (**B**) Seedling formation from protocorms cryopreserved with PVS2, 2 M glycerol and 1% phloroglucinol (Vendrame and Faria, 2011).

Fig. 3. (**A**) Pollinia of *Dendrobium* hybrid selected for cryopreservation. (**B**) Capsules of *Dendrobium* hybrid formed as a result of successful pollination using cryopreserved pollinia. (Vendrame et al., 2008).

protocorms into seedlings was essential to validate the effectiveness of phloroglucinol as a feasible cryoprotectant.

Cryopreservation of Orchid Pollen

Orchid pollen storage is important for orchid breeding and conservation, allowing crosses between plants with temporal and spatial separation between periods of sexual reproduction, and for long-term conservation of germplasm. Pollen grains in orchids are fused together into a structure called the pollinium and many orchids have groups of 2 or more pollinia.

A protocol for cryopreservation of orchid pollinia was developed by Vendrame et al. (2008). Pollinia from *Dendrobium* hybrids (Fig. 3A) were collected and submitted to vitrification procedures and additional different treatments, followed by immersion in LN for 48 h. Treatments consisted of pollinia in PVS2 submitted to either room temperature (27 °C ± 2 °C) or ice pre-cooling (0 °C) for 1, 2, 3, and 4 h prior to LN. Four controls were established; fresh pollinia used to pollinate a flower with no LN, pollinia in PVS2 for 2 h with no LN, pollinia with PVS2 in LN, and pollinia with no PVS2 in LN.

As a method of evaluation of success for the cryopreservation of pollen, usually pollen is germinated in an in vitro medium after cryopreservation. In this study, the success of pollinia germination was verified by pollinating flowers of *Dendrobium* hybrids with pollinia that were previously cryopreserved in LN. Subsequent capsule development (Fig. 3B), seed formation and seed germination were also evaluated.

Interestingly, in this study pollinia germination as evaluated by successful pollination of flowers and subsequent seed formation was high for all treatments. Among all controls, pollination percentages were between 60% to 80%. For controls in room temperature, pollination percentages were between 70% to 80% and for pre-cooling treatments, between 60% to 80%. Therefore, no significant differences were observed, leading to the conclusion that *Dendrobium* hybrid pollinia can be placed

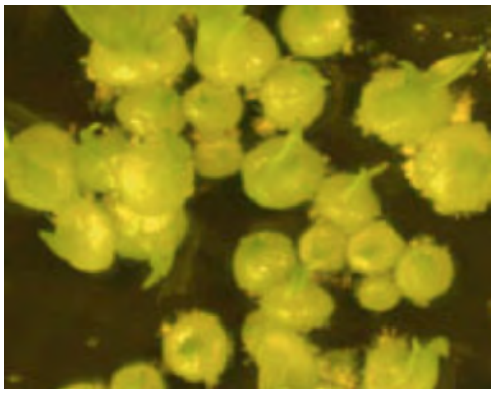

Fig.4. Germinating cryopreserved seeds of *Oncidium flexuosum* (Galdiano et al., 2013).

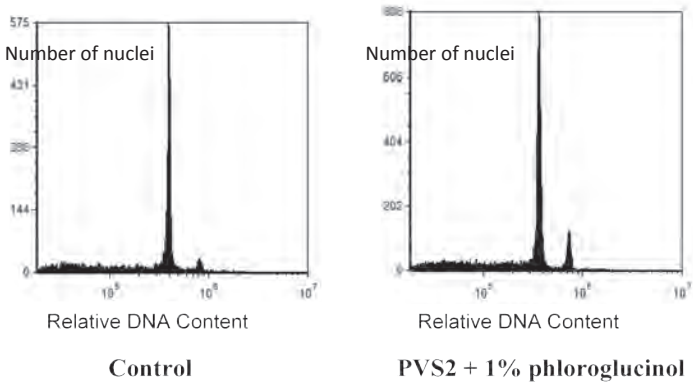

Fig. 5. Flow cytometry analysis of seedlings germinated from cryopreserved seeds (Galdiano et al., 2013). The relative DNA content was the same when comparing the control with the treatment (PVS2 + 1% phloroglucinol).

directly into LN with no previous treatment and without the need for a vitrification solution. This is breakthrough in orchid pollinia cryopreservation, as it makes the procedure simpler and cost-effective.

Genetic Stability of Seedlings from Cryopreserved Seeds

In any cryopreservation procedure, it is important to maintain the genetic integrity of the plant material being cryopreserved. Among different techniques, flow cytometry is a common technique used for fast and large-scale analysis of DNA content in plants, as well as for detecting genetic differences in plants. The genetic stability of seedlings from cryopreserved seeds of *Oncidium flexuosum* was evaluated using flow cytometry (Galdiano et al., 2013).

Mature seeds of *O. flexuosum* were cryopreserved using the vitrification method with PVS2. Seeds exposed to PVS2 for either 60 or 120 min were compared, as well as the addition of 1% phloroglucinol or 1% Supercool X-1000®.

All cryopreserved seeds showed germination (Fig. 4), although results were variable among treatments. Seeds cryopreserved in PVS2 with phloroglucinol had significantly higher germination percentages as compared to Supercool X-1000®. Controls (direct seed germination) had 91.4% germination, while the control with PVS2 alone had only 41.2% germination. Supercool X-1000® returned germination percentages of 47.4% (PVS2 60 min) to 58.9% (PVS2 120 min). However, seeds in PVS2 for 60 min with 1% phloroglucinol had 69.6% germination, while seeds in PVS2 for 120 min with 1% phloroglucinol had 78.4% germination.

Flow cytometry analysis revealed no changes in ploidy levels in seedlings resulting from cryopreserved seeds (Fig. 5), therefore conforming the seedlings were true-to-type.

Conclusions

In this review we outlined several studies that clearly demonstrate the importance of cryopreservation for orchid seeds, protocorms and pollen. Cryopreservation protocols were shown to be efficient, fast and reliable with significantly positive results. We have also demonstrated that the effectiveness of cryopreservation protocols depends on the proper selection of cryoprotectants. The evaluation of new and safer alternatives for cryoprotectants, such as phloroglucinol is essential for the improvement of cryopreservation protocols. Furthermore, the protocols here described serve as a basis for most orchid species and hybrids, but refinements and adjustments might be needed when considering different species and/or hybrids due the large diversity in the family and with new intergeneric hybrids being created. Cryopreservation can definitely be considered a valuable tool for the conservation of orchid germplasm, being used for the preservation of rare and/or endangered species. The technique can also have commercial value for breeders by allowing the preservation of genetic material for use in breeding and genetic improvement programs, as well as for distribution and exchange of material. Additional efforts for continuous studies and the development of cryopreservation protocols for other species and hybrids are warranted and recommended.

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