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REVIEW

In vitro conservation of *Dendrobium* germplasm

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Abstract *Dendrobium* is a large genus in the family Orchidaceae that exhibits vast diversity in floral characteristics, which is of considerable importance to orchid breeders, biotechnologists and collectors. Native species have high value as a result of their medicinal properties, while their hybrids are important as ornamental commodities, either as cut flowers or potted plants and are thus veritable industrial crops. Thus, preservation of *Dendrobium* germplasm is valuable for species conservation, breeding programs and the floriculture industry. Cryopreservation represents the only safe, efficient and cost-effective long-term storage option to facilitate the conservation of genetic resources of plant species. This review highlights 16 years of literature related to the preservation of *Dendrobium* germplasm and comprises the most comprehensive assessment of thorough studies performed to date, which shows reliable and reproducible results. Air-drying, encapsulation–dehydration, encapsulation–vitrification, vitrification and droplet-vitrification are the current cryopreservation methodologies that have been used to cryopreserve *Dendrobium* germplasm. Mature seeds, pollen, protoplasts, shoot primordia, protocorms and somatic embryos or protocorm-like bodies (PLBs) have been cryopreserved with different levels of success. Encapsulation–vitrification and encapsulation–dehydration are the most used protocol, while PLBs represent the main explant explored.

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Keywords Cryopreservation · Genetic breeding · Micropropagation · Orchidaceae · Plant biotechnology · Protocorm-like body

Abbreviations

DMSO Dimethyl sulfoxide
FDA Fluorescein diacetate
LN Liquid nitrogen
PLB Protocorm-like body
TTC Triphenyl tetrazolium chloride

Introduction

Orchid cultivation is one of the most economically significant global nursery industries, constituting a multi-billion dollar market (Teixeira da Silva 2013a). *Dendrobium* is a large orchid genus represented by over 1,100 species distributed from India and Sri Lanka then eastward to Japan and southward through the Philippines, Malaysia, Indonesia, and New Guinea to Australia and through the Pacific Islands to New Zealand (Pridgeon and Morrison 2006; Xu et al. 2006). The name is derived from 'Dendron' which means tree and 'bios' means 'life' that is an epiphytic plant that exists by clinging to the branches and trunks of host trees (Pradhan et al. 2013). This genus represents one of the most important orchids either as pot plants or as cut flowers. Several *Dendrobium* hybrids are very important to the orchid cut-flower industry in a number of countries. As a result, the production of *Dendrobium* hybrids plants has spiked, with large-scale production occurring in many countries like the Netherlands, Germany, China, Taiwan, Thailand, Philippines, Unites States, and Japan (Puchooa 2004). In general, orchids rank second in potted flowering plants in the United States, with a wholesale value of US$ 200 million for the year 2011 (US Department of Agriculture 2012). However, the USDA Floriculture report of 2013 indicated an 11 % increase in the value of potted orchids in 2012 from the previous year, while all other crops in this category had their values reduced (US Department of Agriculture 2013). Among the main orchid genera produced in the US, *Dendrobium* ranks second as potted orchids, behind *Phalaenopsis*. The State of Hawaii is the main producer of *Dendrobium* in the US with a total sales value of US$ 5.25 million for 2011 (U.S. Department of Agriculture 2011, 2012).

In addition to their ornamental value, some native *Dendrobium* species have medicinal (Hou et al. 2012; Ng et al. 2012) and ethnopharmacological importance in many Asian countries. Flower extracts of the epiphytic species *D. nobile* possessed antimicrobial and antitumor properties in addition to antiperoxidative activity (Devi et al. 2009), and extracted polysaccharide fractions from its stem could be considered as effective natural antitumor agents (Wang et al. 2010). Several members of the *Dendrobium* genus have alkaloids, amino acids, trace elements, while others contain active ingredients that can enhance immunity, promote the secretion of digestive juice, inhibit platelet aggregation, lower blood pressure or hypoglycemia, or display a range of pharmacological effects such as antioxidant, anti-aging, antipyretic and analgesic (Ng et al. 2012).

There are several in vitro preservation methods available for the preservation of *Dendrobium* germplasm. The choice of method, however, usually depends on the storage duration required. For short-term and medium-term storage, the aim is to reduce growth and to increase the intervals between subcultures. For long-term storage, cryopreservation is the only current method available to achieve this goal (Engelmann 2011). In vitro slow growth storage techniques are routinely used for medium-term conservation of numerous species and growth reduction is generally achieved by modifying the environmental conditions and the culture medium (Kulus and Zalewska 2014). Changes in environment conditions include a reduction in temperature, while modifications of the culture medium can include dilution of mineral elements, reduction of sugar concentration, changes in the nature and/or concentration of plant growth regulators (for example, the use of sub-lethal levels of growth retardants) and addition of osmotically active compounds (Negash et al. 2001).

Conservation of *Dendrobium* germplasm

The reduction in wild orchid stocks by orchid collectors, over-exploitation for medicinal purposes, deforestation and destruction of habitats for urbanization, and unauthorized trade have led to a reduction in natural populations of many orchids (Swarts and Dixon 2009) leading many orchid species to become extinct and a large number of species to become rare or endangered (Yam et al. 2010). The entire Orchidaceae is included in the CITES (Convention on International Trade in Endangered Species) Appendix II (Shefferson et al. 2005). There are a number of techniques devised to conserve orchids: ex vitro, such as botanic garden collections or ex situ germplasm banks (Hou et al. 2012; Merritt et al. 2014), as well as in vitro, including seed bank development, slow growth conservation and cryopreservation of seed, meristem, tissue-cultured shoot primordia, somatic embryos, and pollen (Table 1). In *Dendrobium*, immature seeds have not yet been used as an explant for cryopreservation, although they have been used for *Bletilla striata* (Hirano et al. 2005a), *Ponerorchis graminifolia* (Hirano et al. 2005b), *Dactylorhiza fuchsii*

Table 1 In vitro conservation applied to *Dendrobium* germplasm

Species and/or cultivar	Explant used	Method applied	Method and key findings	Reference
D. candidum	Seeds	Air-drying method and low temperature	Seeds were desiccated with silica gel for 3, 4, 5, 6, 24, 48, 72, 96 h, then placed into cryo-tubes and plunged into LN. After 24 h or 30 days, seeds were recovered from LN and rapidly thawed in a 40 °C water bath for 1 min or at RT (20 °C) for 30 min, then cultured in ½ MS medium. Seed water content was a key factor for cryopreservation in LN. Seeds were dried to 8–19 % water content before added into LN for a 48–96 h, and then thawed in a 40 °C water bath for 1 min. Seed GP reached 92–95 %. Seeds stored in LN could form normal plantlets by morphological observations	Zhang et al. (1997)
D. candidum	4-month-old seeds, protocorm, PLBs	ED and EV	Freshly harvested 4-month-old seeds contained about 43 % water (fresh weight basis) and did not survive exposure to LN. Seeds were desiccated with silica for 0–72 h, then placed into cryo-tubes and plunged into LN. After recovering from LN and rapid thawing in a water bath 40 °C. PLBs were cryopreserved after pretreatment with 25 % PVS2 for 10 min at 22 °C followed by 100 % PVS2 for 0, 7, 15, 30 min at 0 °C, then plunged into LN. After recovering from LN and rapid thawing in a water bath 40 °C. High survival (about 95 %) and rapid recovery growth could be achieved by drying 4-month-old seeds with water contents ranging from 12 to 19 %. 88 % survival possible when PLBs cultured for 3 weeks on ½ MS media + 0.5 mg ABA, were incubated for 15 min in PVS2 and then exposed to LN. Dark-cultured PLBs with vigorous growth were desiccated to a water content of 30 ± 2 % for 6–7 days. After cryopreservation, survival was 48–80 %	Wang et al. (1998, 1999)
D. officinale	Seedlings	In vitro	Plantlets from seed germination could be conserved for 12 months without subculture on ½ MS + 0.5 mg/l NAA + 20 g/l sucrose + 7 g/l agar at 25 ± 2 °C under 2,000 lux irradiance. Survival could reach 100 %. Seedlings could also be conserved in vitro for 12 months without subculture at 4 °C in the dark. Survival on ½ MS (100 %) and ¼ MS media (91.67 %) was higher than that on MS medium (41.67 %). The suitable concentrations of sucrose were 20–40 g/l. Supplement with 0.5–1.0 g/l AC could improve the growth of stored plantlets	Shi et al. (1999, 2000)
D. candidum	Protoplasts PLBs	EV	Protoplasts could be cryopreserved after pretreatment with 25 % PVS2 for 5 min at 0 °C followed by 100 % PVS2 for 3 min at 0 °C, then plunged into LN. After recovering from LN and thawing rapidly in a 37 °C water bath, then washed with 1.2 M sorbitol and MS solution, survival was 48 %, as assessed under a fluorescent microscope. PLBs could be cryopreserved after pretreatment with 25 % PVS2 for 10 min at 22 °C followed by 100 % PVS2 for 15 min, then plunged into LN. After recovering from LN and rapid thawing in a 40 °C water bath, then covering with 1.2 M sorbitol and ½ MS solution, survival was 88 % after 6 weeks.	Chen (2000), Chen et al. (2001)
D. candidum	PLBs	EV and ED	Using clay:vermiculite:water (2:1:2) as the encapsulating media, germination reached 56.8 %. When 1.0 % AC alone or 1.0 % AC + 0.5 % starch were added, the corresponding average germination of artificial seeds increased to 76.7 and 80.3 %, respectively	Zhang et al. (2001)
D. candidum	PLBs	Air-drying method and low temperature	PLBs were desiccated to 20–30 % moisture content. Half the PLBs was transferred to 2-ml polypropylene cryo-tubes and immersed into LN. The other half of PLBS was cultured, under aseptic conditions, and not submitted to LN, thus allowing the evaluation of desiccation effects. The optimal moisture content before freezing ranged from 0.1 g H_2O/g DW (11 % on FW basis) to 0.5 g H_2O/g DW (33 % on FW basis). Exogenous 0.1 mg/l ABA for 3 d at 25 ± 2 °C on a rotary shaker at 80 rpm in the light. Treatment of PLBs before desiccation also induced the accumulation of soluble sugars, heat-stable proteins and dehydrins	Bian et al. (2002)
D. candidum	PLBs	Air-drying method and low temperature	PLBs were dehydrated by the air-drying method then plunged into LN. The optimal water content for cryopreservation was from 0.1 to 0.5 g/g [(FW–DW)/DW]. The amount of heat-stable proteins increased significantly when water content decreased to 1.0 g/g [(FW–DW)/DW]. Two bands of heat-stable proteins with respective molecular masses of 28.7 and 34.3 kD were dehydrins from immunological detection	Lin et al. (2004)
D. 'Walter Oumae'	Shoot tips	ED	Shoot tips were encapsulated in calcium-alginate. Encapsulated shoot tips were precultured on modified VW medium solidified with agar + 0.3, 0.5 or 0.7 M sucrose for 2 days. Culture conditions were 25 ± 2 °C, 37 μmol m^{-2} s^{-1}, 16-h PP. Encapsulated shoot tips were dehydrated on a laminar air-flow cabinet for 0–10 h, and added immediately to LN. Beads were removed from LN and thawed rapidly in a 40 ± 2 °C water bath. The TTC assay was used to assess survival. Regrowth was assessed by culturing the beads on regeneration medium. The ideal protocol was preculture of encapsulated shoot tips in 0.3 M sucrose (2 days) and 6–6 h dehydration prior to cryopreservation in LN. Highest survival was 16.18 and 13.33 % of encapsulated explants regrew	Lurswijidjarus and Thammasiri (2004)

Table 1 continued

Species and/or cultivar	Explant used	Method applied	Method and key findings	Reference
D. hybrid 'Sena Red', 'Mini WRL', 'Jaquelyn Thomas', 'BFC Pink'	Mature seeds	V	Before immersion in LN, mature seeds were exposed to PVS2 pre-vitrification solution either at RT (27 ± 2 °C) or in ice (0 °C) for periods of 1, 2, 3, 4, or 5 h. Germination was higher for seeds in ice-cold PVS2 for 1–3 h before cryopreservation than seeds in PVS2 at RT for the same time periods. Percentage of germination was not different when comparing pre-vitrification in PVS2 at 1, 2 or 3 h for 'Sena Red', 'Mini WRL', and 'BFC Pink'. However, higher germination was observed for 'Jaquelyn Thomas' seeds in PVS2 for 1 h before cryopreservation. The precooling treatment in ice combined with a dehydration treatment in PVS2 for a period of 1–3 h was essential to allow proper germination of cryopreserved seeds. Plant growth and development were not adversely affected by cryopreservation	Vendrame et al. (2007)
D. lituiflorum	PLBs	EV	PLBs encapsulated in sodium alginate solution were dropped into 100 mM of $Ca(NO_3)_2 \cdot 4H_2O$ for 30 min on a gyratory shaker (75 rpm). Encapsulated PLBs stored at different temperatures (RT, 4, 8 °C) and periods of time (30, 60, 90, 120 days) had the potential to germinate and regenerate on MS medium. Only 3.3 % of encapsulated PLBs germinated at RT and 80 % encapsulated PLBs germinated after 90 days of storage	Das et al. (2008)
D. hybrid 'Sena Red', 'Mini WRL'	Pollen	V	Pollinia were exposed to PVS2 pre-vitrification solution in ice (0 °C) or at RT (27 ± 2 °C) for a period of 1–4 h prior to immersion in LN. After cryopreservation, pollinia were removed from LN and pollination of flowers of the same hybrids was performed to assess pollinia viability and germination. Subsequent to the crosses, the percentage of pollinia germination was high (>80 %). However, *D.* 'Sena Red' pollinia exposed to 3 h of precooling at 0 °C in PVS2 with subsequent immersion into LN and pollinia exposed to 3 h of RT in PVS2 without precooling had lower germination percentages (60 and 70 %, respectively). Yet, capsules with seeds were obtained for all treatments and hybrids. A seed viability test (TTC and FDA) revealed high viability (90–95 %) for all treatments and for both hybrids	Vendrame et al. (2008)
D. 'Sonia-28'	PLBs	EV	PLBs of two parameters of length (0.01–0.20 and 0.21–0.40 cm) were precultured under five different sucrose concentrations (0.06, 0.10, 0.25, 0.50, 0.75 M) for two different periods (24 and 48 h). Dehydration treatment for six different periods (5, 10, 15, 20, 25, 30 min) was the main parameter tested to assess PLB survival after storage in LN. Cryopreserved PLBs treated with 20 min PVS2 at 0 °C possessed highest viability (OD = 0.0085 at 530 nm) in the TTC assay	Tiau et al. (2009)
D. candidum Wall ex. Lindl.	PLBs	EV	PLBs (0.2–0.3 cm in diameter) were excised and used for cryopreservation. PLBs were first precultured in liquid MS medium + 0.2 mg/l NAA + 0.5 mg/l BA + 0.75 M sucrose under continuous light (36 µmol m^{-2} s^{-1}) at 25 ± 1 °C for 5 days. PLBs were osmoprotected with 2 M glycerol + 1 M sucrose for 80 min at 25 °C. PLBs were encapsulated within 15 min in solution (0.5 M $CaCl_2$ + 0.5 M sucrose) at 25 ± 1 °C, then dehydrated for 150 min at 0 °C in PVS2 + 30 % (w/v) glycerol + 15 % (w/v) ethylene glycol + 15 % (w/v) DMSO in 0.5 M sucrose. PLBs were added directly to LN for 1 h, thawed at 40 °C for 3 min, then washed 3× every 10 min with MS medium + 1.2 M sucrose. 85 % of cryopreserved PLBs survived and developed morphologically normal plantlets	Yin and Hong (2009)
D. chrysanthum	Wild mature seeds	V	Seed germination decreased with increasing treatment period in PVS2. Cryo-tolerance (evaluated by germination percentage after treatments) of seeds increased obviously following PVS2 pretreatment. When pretreatment period was <45 min, seed cryo-tolerance increased (highest germination 80 %) with increasing pretreatment time of PVS2, but decreased after 60 min, only approximately 10 % of seeds pre-treated for 120 min germinated. All seeds that survived could germinate and develop into normal seedlings after storage in LN	He et al. (2010)
D. 'Bobby Messina'	PLBs with a size range of 1–2 and 3–4 mm	ED	PLBs were pre-treated with 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 M sucrose and/or sorbitol supplemented in semi-solid ½ MS medium at 24 °C for 0–5 days under a 16-h PP. Pre-treated PLBs were encapsulated in 3 % sodium alginate + 0.1 M $CaCl_2$, both based on a liquid ½ MS medium base and supplemented with 0.4 M sucrose. The resulting Ca-alginate beads were osmoprotected in liquid ½ MS medium containing 0.75 M sucrose on an orbital shaker (110 rpm) at 24 °C for 1 day under a 16-h PP. The osmoprotected beads were dehydrated for 0–10 h in a Parafilm®-sealed culture jar containing 50 g of oven-sterilized silica gel followed by rapid freezing in LN. After thawing in a 40 °C water bath for 90–120 s, the cryopreserved beads were cultured on growth recovery media containing semi-solid ½ MS media supplemented with 2 % sucrose. Survival of the cryopreserved PLBs was assessed by the TTC test. Highest survival (OD = 0.14 at 490 nm) was observed in 3–4 mm cryopreserved PLBs	Antony et al. (2010, 2011a, b)
D. 'Sonia-17′	PLBs	ED	Using ED, the cryopreservation of PLBs was assessed based on the effects of four dehydration periods (0, 1, 3 and 5 h) and four different concentrations of 24-h sucrose pretreatment (0, 0.3, 0.5 and 0.7 M). All cryopreserved PLBs resulted in very low survival (OD from 0.103 to 10.32 at 530 nm), irrespective of the dehydration period. Best survival was obtained when 0.5 M sucrose pretreatment and 3 h dehydration were applied	Subramaniam et al. (2011)

Table 1 continued

Species and/or cultivar	Explant used	Method applied	Method and key findings	Reference
D. nobile	Protocorms	V	Protocorms (60-day-old, 1–2-mm) exposed to 2 M glycerol osmoprotective solution for 20 min followed by 10 min in PVS2 vitrification solution with 1 % PG resulted 68 % of protocorm recovery and survival. Pretreatment with sucrose reduced protocorm survival by 90 %	Vendrame and Faria (2011)
D. wardianum Warner	PLBs	ED and EV	Optimal cryopreservation of PLBs was possible when subcultured twice from germination for 60 days on proliferation medium, then precultured on ½ MS solid medium containing 0.8 mol/l (273.6 g/l) sucrose in darkness at 4 °C for 6 day. Subsequently, transfer to osmotically protected solution composed of ½ MS, 2.0 mol/l (184 g/l) glycerol and 0.4 mol/l (137 g/l) sucrose at RT for 40 min then dehydrated with PVS2 at 0 °C for 40 min. Thereafter, samples placed into cryo-tubes, fresh PVS2 added, plunged into LN for l h. Finally, samples thawed in a water bath at 40 °C for 1 min, and washed three times with ½ MS liquid medium containing 1.2 mol/l (410 g/l) sucrose for 10-min intervals. After 30 days, regrowth on recovery medium ½ MS containing 0.2 mg/l BA, 0.5 mg/l NAA and 20 g/l sucrose resulted in 20 % survival. Physiological changes occurred in pretreatment: PLB water content reduced significantly and relative survival reached a maximum of 46.6 %, when the water content was 22.72 g/g for untreated PLBs, 1.06 g/g [(FW–DW)/DW] before freezing. Pretreatment and dehydration improved the relative conductivity (4.2–4.5-fold) with an increase in MDA content during dehydration and thawing (6-fold). A single peak was observed by HPLC for soluble sugar and soluble protein content, the trend increased (42.5 % higher), and then decreased (55 % lower). Plasma membranes were markedly damaged during pretreatment and dehydration through TEM observation. Starch grains and protein mostly accumulated in the intact apical meristem and protein content varied, for example a 18.20 % increase then a decrease, corresponding to the physiological and biochemical characteristics of the PLBs	Wu and Shen (2011), Liu et al. (2013)
D. 'Bobby Messina'	PLBs	ED	1–2 and 3–4 mm PLBs were precultured in semi-solid ½ MS medium supplemented with various sucrose concentrations (0.0, 0.2, 0.4, 0.6, 0.8, 1.0 M). PLBs were then encapsulated to form beads in liquid ½ MS media supplemented with different concentrations of sodium alginate (2.5, 3.0, 3.5 %). The beads were placed in PVS2 in 2 ml cryovials and plunged into LN for 24 h. Beads were then thawed in a 40 °C water bath for 90 s and were placed in recovery medium (semi-solid ½ MS medium + 2 % sucrose) for 4 days in the dark. Highest viability was possible from PLBs (1–2 mm wide) precultured in semi-solid ½ MS medium + 1.0 M sucrose for 24 h and encapsulated in 2.5 % sodium alginate. Viability of cryopreserved PLBs was assessed by Evans blue dye assay. Antony et al. (2012) then verified, using 6 RAPD primers, that the genetic stability of cryopreserved PLBs was maintained following cryopreservation	Zainuddin et al. (2011), Antony et al. (2012)
D. 'Sonia-28′	PLBs	EV	1–2 and 3–4 mm PLBs were precultured in semi-solid ½ MS medium supplemented with various sucrose concentrations (0, 0.25, 0.5, 0.75 and 1.0 M) at different periods (0, 3, 6 and 9 days). Precultured PLBs were encapsulated and osmoprotected for 24 h then dehydrated in PVS2 at 0 °C at different periods (0, 30, 60, 90, 120, 150, 180 and 210 min) prior to storage in LN for at least 24 h. After rapid thawing (40 ± 2 °C) for 2 min, the beads were unloaded with 1.2 M sucrose and then cultured on semi-solid ½ MS medium devoid of PGRs. The TTC assay was used to determine the viability of the treated PLBs 2 weeks after recovery. The optimized EV parameters were: preculture of 3–4 mm PLBs for 6 days in 0.5 M sucrose followed by dehydration in PVS2 at 0 °C for 150 min. Histological observations of non-cryopreserved and cryopreserved PLBs indicated that the majority of cells were injured either during freezing or thawing or during the osmotic dehydration step with PVS2. TTC assay was used to determine the viability of the treated PLBs after 2 weeks of recovery	Ching et al. (2012)
D. hybrid 'Dong Yai'	Mature seeds (vitrification) Protocorms (droplet-vitrification)	V	Seed was cryopreserved in PVS2 for 60 min at 0 °C containing 1 % PG, resulting in 79 % seed germination. Protocorms could be cryopreserved after pretreatment with 0.3 M sucrose for 24 h followed by exposure to PVS2 solution containing 1 % PG for 15 min at 0 °C in the DV method with 14 % protocorm survival after 75 days. In controls and other treatments, only 0–6 % survival resulted. 1 % Supercool X1000® was not an effective cryoprotectant, unlike PG. In Galdiano et al. (2014) paper, mature seeds submitted to PVS2 (0–6 h) returned germination of 51–58 % (at 1–3 h of exposure). A moderate germination rate for the 6-h treatment in PVS2 was observed (39 %), leading to the hypothesis that this hybrid could have an intrinsic trait of high dehydration tolerance. FCM analysis revealed no chromosomal changes on seedlings from vitrified seeds, as well as seedlings germinated from the control treatment (seeds not cryopreserved)	Galdiano et al. (2012, 2014)

Table 1 continued

Species and/or cultivar	Explant used	Method applied	Method and key findings	Reference
D. nobile	PLBs	ED and EV	Cryopreservation of PLBs based on ED and EV was established. In both cryogenic procedures, PLBs were initially osmoprotected with a mixture of 0.4 M sucrose and 2 M glycerol, incorporated in an encapsulation matrix comprising 3 % (w/v) sodium alginate and 0.1 M $CaCl_2$. EV resulted in higher survival (78.1 %) and regrowth (75.9 %) than ED (53.3 and 50.2 %, respectively). Incorporation of 0.4 M sucrose and 2 M glycerol followed by treatment with the same concentration of loading solution for about 60 min (the authors did not indicate the concentrations) in the encapsulation matrix resulted in higher survival percentage after cryopreservation. EV was the most appropriate method to cryopreserve PLBs. Regenerated plantlets showed normal morphology, similar to control plants	Mohanty et al. (2012, 2013b)
D. 'Bobby Messina'	PLBs	V	Optimization of preculture period, adjusting incubation conditions in PVS2 solution as well as conditions for regrowth after cryopreservation using PLBs 3–4 mm in size resulted in 40 % growth in a recovery period for 2 months after replating. Optimal preculture period for PLBs in 0.2 M sucrose: 1 day. Thereafter, PLBs were treated with a mixture of ½ MS containing 2 M glycerol + 0.4 M sucrose at 25 °C for 20 min, dehydrated with PVS2 solution at 0 °C for 20 min, then plunged into LN. PLBs were recovered from LN in a 40 °C water bath for 90 s, washed with ½ MS containing 1.2 M sucrose, then cultured on semi-solid ½ MS medium containing 2 % (w/v) sucrose. Using AA (0.6 mM) in all steps of the protocol (preculture, loading, unloading, regrowth), recovery decreased from 100 to 30 % in non-cryopreserved PLBs and from 40 to 10 % in cryopreserved PLBs	Antony et al. (2013)
D. chrysanthum	PLBs	EV	Exact same procedure as Mohanty et al. (2012) protocol for *D. nobile*. Optimal growth, i.e., 63 % survival and 60 % regrowth, were observed when encapsulated beads containing PLBs were treated with loading solution for 80 min followed by PVS2 for 100 min. Plants derived from cryopreservation were similar to control plants	Mohanty et al. (2013a)
D. 'Sonia-28′	PLBs	EV	1–2 mm PLBs were precultured for 24 h on ½ MS semi-solid medium supplemented with 0.5 M sucrose, placed in a loading solution for 10 min and dehydrated in PVS2 at 0 °C for 20 min prior to thawing. Thawed PLBs were unloaded into 1.2 M sucrose and recovered on semi-solid recovery medium composed of ½ MS + 2 % sucrose for 3 weeks. There was a general decrease in the total protein content (from 3.31 to 0.73 U mg^{-1}) of cryopreserved PLBs as vitrification progressed with a general increase in SOD activity. The SOD activity of cryopreserved PLBs increased slightly to 64.95 U mg^{-1} during thawing, and reduced to a third of the previous value at the unloading stage, at 27.76 U mg^{-1}. The SOD activity of cryopreserved PLBs increased at both recovery stages, peaking at 148.03 U mg^{-1} in the second growth recovery stage. Cryopreserved PLBs also underwent excessive post-thawing oxidative stress due to decreased levels of catalase (from 114 to 52.93 U g^{-1}) at the post-thawing recovery stage, which contributed to the poor survival rates of the cryopreserved PLBs	Poobathy et al. (2013a, b)
D. 'Sonia-28′	PLBs	V	PLBs which were precultured on semi-solid ½ MS + 0.4 M sucrose for 48 h were placed in a loading solution containing 0.4 M sucrose + 2.0 M glycerol for 20 min, then dehydrated in PVS2 solution at 0 °C for 50 min, and finally stored in LN for 24 h. Cryopreserved PLBs were thawed in a 40 ± 2 °C water bath for 90 s then added to unloading solution (1.2 M sucrose) for 20 min. The survival of cryopreserved PLBs 3–4 mm in size in the recovery stage improved (16 %) by adding AA (0.6 mM) in all steps of the procedure (preculture, loading, PVS2, unloading, regeneration medium) and by adding 0.2 % (w/v) AC to regeneration medium (semi-solid ½ MS medium + 0.6 mM AA)	Poobathy et al. (2013c)
D. 'Bobby Messina'	PLBs	ED	Histological and SEM analyses proved that plasmolysis (depending on its degree) resulted in different changes in the cellular processes of cryopreserved PLBs as compared to non-cryopreserved PLBs. As the duration of dehydration increased, cryopreserved PLBs accumulated a mass of homogenous cells whose cytoplasm became denser since dehydration induced plasmodesmata breaking and plasmolysis. Lack of regrowth may have been caused by severe damage to the surface of PLBs caused by cryopreservation, as observed by SEM	Antony et al. (2014)

AA ascorbic acid, *ABA* abscisic acid, *AC* activated charcoal, *BA* N^6-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva (2012); *$CaCl_2$* calcium chloride, *d* day(s), *DMSO* dimethyl sulfoxide, *DW* dry weight, *DV* droplet-vitrification, *ED* encapsulation–dehydration, *EV* encapsulation–vitrification, *FDA* fluorescein diacetate, *FCM* flow cytometry, *FW* fresh weight, *GP* germination percentage, *h* hour(s), *LN* liquid nitrogen, *MDA* malondialdehyde, *min* minute(s), *MS* Murashige and Skoog (1962), *NAA* α-naphthaleneacetic acid, *OD* optical density, *PG* phloroglucinol, *PGR* plant growth regulator, *PLB* protocorm-like body, *PP* photoperiod, *PVS2* plant vitrification solution (Sakai et al. 1990), *RAPD* randomly amplified polymorphic DNA, *RH* relative humidity, *RT* room temperature, *s* second(s), *SEM* scanning electron microscopy, SOD superoxide dismutase, *TEM* transmission electron microscopy, *TTC* 2,3,5-triphenyl tetrazolium chloride, *V* vitrification, *VW* Vacin and Went medium (Vacin and Went 1949), *w* week(s)

(Nikishina et al. 2007), and *Cyrtopodium hatschbachii* (Surenciski et al. 2007). Cryopreservation, which can ensure the safe and cost-efficient long-term conservation of *Dendrobium* germplasm, has a wide applicability for orchids both from temperate and tropical origin in which seeds, shoot meristems, protocorms, somatic embryos (i.e., protocorm-like bodies, or PLBs) and cultured cells can be preserved in liquid nitrogen (LN; −196 °C) to create cryobanks for conserving rare and endangered species (Hirano et al. 2006; Hossain et al. 2013). Until 2006, three cryopreservation techniques had been applied to orchid species, namely desiccation (air-drying), vitrification and encapsulation–dehydration (Hirano et al. 2006). However, in dehydration, since naked PLBs that are very sensitive to dehydration are first dehydrated, and since vitrification uses chemicals that can be toxic (Teixeira da Silva 2013b), this list was broadened to include five methods: air-drying, encapsulation–dehydration, encapsulation–vitrification, vitrification and droplet-vitrification. Encapsulation–dehydration method may be the most suitable as it results in a high survival frequency after cryogenic storage. Table 1 indicates that from a total of 37 documented studies in the literature, 12 used encapsulation–vitrification while 9 used encapsulation–dehydration, 7 used vitrification alone, 3 reported air-drying methods and low temperature, and a single study used in vitro methods. The encapsulation of explants in alginate beads for cryopreservation has some benefits as compared to the use of non-encapsulated samples. The alginate beads provide enhanced protection of dried materials from mechanical and oxidative stress during storage and ease of handling of small samples during pre- and post-cryopreservation.

Many studies examined the cryopreservation of *Dendrobium* PLBs including *D.* 'Walter Oumae' (Lurswijidjarus and Thammasiri 2004) and *D. candidum* (Chen 2000; Chen et al. 2001; Bian et al. 2002; Lin et al. 2004; Yin and Hong 2009). Seeds of *D. candidum* (Wang et al. 1998, 1999), *D. crystallinum* and *D. virgineum* (Huehne and Bhinja 2012) were also successfully cryopreserved. Vendrame and Faria (2011) and Galdiano et al. (2012) showed that the cryopreservation of mature seeds was possible with vitrification, while droplet-vitrification was effective for protocorms, with phloroglucinol being an important aspect of the vitrification solution. Phloroglucinol has many biological effects in vitro and has shown to enhance a wide range of organogenic processes in plants (Teixeira da Silva et al. 2013). Orchid seeds are morphophysiologically different from other plants, to some extent limiting the application of cryopreservation and thus modification and proper adjustment of the techniques are required. Cryopreservation of vegetative tissue involves several stages, specifically the establishment of in vitro cultures, conditioning of these tissues, addition of an appropriate cryoprotectant, exposure of cultures to ultra-low temperature, re-warming and regeneration of plant cells and tissues (Kulus and Zalewska 2014). Each stage, which needs to be optimized, plays an important role in determining the survival of tissue upon re-warming.

Some orchid cryopreservation papers applied deep-freezing to seeds with a moisture content of 20 % or less (for vitrification protocols), while other studies dealt with direct freezing of orchid seeds. Seed moisture is a key factor because the presence of unbound water in seeds considerably reduces their germinability causing the embryo tissues to perish because of the formation of ice crystals in their cells during freezing in LN (Sakai et al. 1991; Sakai and Engelmann 2007). In the cryopreservation of terrestrial and epiphytic orchids with a moisture level below 11 %, seed germinability did not change after cryoconservation for most species examined (Pritchard 1984; Pritchard et al. 1999). In seeds of *D. candidum*, a high survival rate of 95 % was also obtained when desiccated seeds with 8–19 % water content with silica gel were directly plunged into LN (Wang et al. 1998). However, mature seeds of *Dendrobium* commercial hybrids with comparable seed moisture (9–18 %) did not survive after direct freezing (Vendrame et al. 2007; Galdiano et al. 2012, 2014), and the desirable dehydration with plant vitrification solution #2, PVS2 [30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose; Sakai et al. (1990)] for a period of time between 1–3 h prior to cryopreservation was essential to allow proper germination of cryopreserved seeds.

Conservation through storage should be supported by an appropriate explant viability test, since the success of any cryopreservant-based protocol is determined by the recovery of viable propagules. Viability tests such as the triphenyl tetrazolium chloride (TTC) reduction assay (Singh 1981) and the fluorescein diacetate (FDA) staining technique (Pritchard 1985) have been largely explored for seeds, protocorms and PLBs. The TTC assay, qualitative for large tissues and organs, is often used for orchid seeds and embryos because TTC reduction assay and regrowth observations used in the assessment of seedlings growth or plantlets survival were correlated (Lurswijidjarus and Thammasiri 2004; Vendrame et al. 2008; Tiau et al. 2009; Antony et al. 2010, 2011b; Subramaniam et al. 2011; Ching et al. 2012; Galdiano et al. 2012, 2014; Poobathy et al. 2013a). Dehydrogenases, through respiration in mitochondria, reduce colorless TTC to red triphenylformazan or reduced TTC (Verleysen et al. 2004). Figure 1 shows the assessment of survival using the TTC test in mature seeds and 45-day-old protocorms of *Dendrobium* hybrid 'Uniwai Royale' after cryopreservation by vitrification (unpublished data).

Cryopreservation of seeds, pollen, protocorms and PLBs of many *Dendrobium* orchids has been successfully

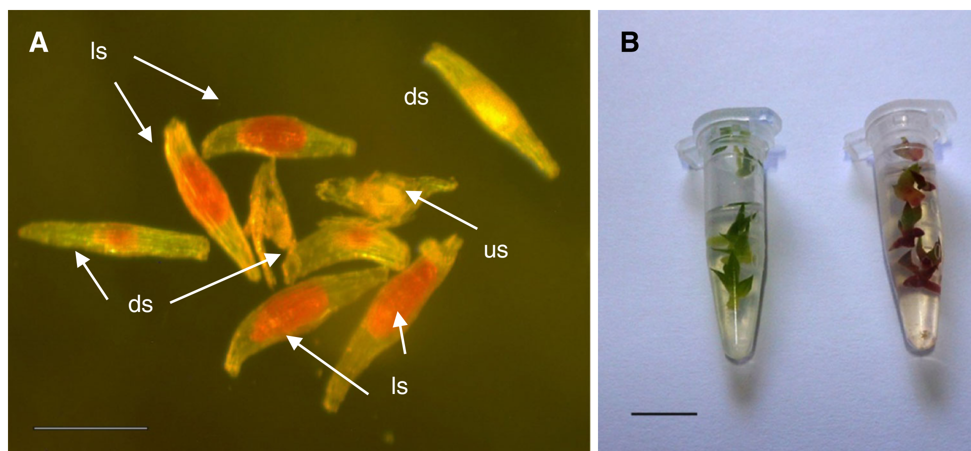

Fig. 1 Stained seeds and protocorms of *Dendrobium* hybrid ‘Uniwai Royale’ with the 2,3,5-triphenyl tetrazolium chloride (TTC) reduction assay. Viable explants show insoluble scarlet formazan because the dehydrogenase enzyme in living tissues reduces soluble colorless TTC to insoluble scarlet formazan. **a** Mature seeds after cryopreservation by vitrification. **b** 45-days-old protocorms directly cryopreserved in liquid nitrogen (*left Eppendorf tube*) or PVS2-treated protocorms (*right Eppendorf tube*). *ds* dead seeds, *ls* living seeds, *us* unviable seed. *Bars* **a**: 1 mm, **b**: 1 cm. (Unpublished photos: Renato Fernandes Galdiano Jr.)

attempted for long-term conservation. Vitrification and air-drying methods have resulted in both low and slow rates of regrowth of plantlets of *D. candidum* (Bian et al. 2002). Therefore, the cryopreservation protocol widely used in the literature is encapsulation–vitrification, followed by encapsulation–dehydration (Table 1). The encapsulation–vitrification method has been developed for apices of numerous species from tropical origin and involves the incubation of explants in a Na-alginate solution (2–5 %) and their subsequent release (immersed in a drop of alginate) into a complexation agent (50–100 mM $CaCl_2$ solution), where bead hardening occurs within 20–30 min. Encapsulated explants are then precultured in liquid medium with a high sucrose concentration and partially desiccated before exposure to LN (Sakai et al. 2008). In encapsulation–dehydration, encapsulation protects explants against extreme treatments such as high sucrose concentrations, which allow the removal of most freezable water from the cells and thereby allowing internal solutes to enter a vitrified state when plunged into LN and subsequently preventing the formation of lethal intracellular ice (Engelmann et al. 2008).

PLBs have been the main *Dendrobium* explant explored for the (cryo)preservation of *Dendrobium* germplasm (Table 1). They are clonal in nature (i.e., they are genetically identical), they have the potential to regenerate plants with an independent root and shoot system (hence their consideration as somatic embryos in the Orchidaceae), and represent thus a suitable and reliable source of material for germplasm conservation. PLBs behave as embryogenic cultures (similar to what was found in hybrid *Cymbidium*; Teixeira da Silva and Tanaka 2006) and are extremely susceptible to cryoinjuries due to the high water content within the cells (Renato Fernandes Galdiano Jr., personal observation). Consequently, additional steps such as extra preculture treatment (Antony et al. 2011b) or dehydration are required prior to preserving in LN (Yin and Hong 2009; Mohanty et al. 2012). During dehydration and freezing in LN, proteins and membranes are protected when soluble sugars such as sucrose accumulate in the cytoplasm (Sakai and Engelmann 2007; Yin and Hong 2009). Using protocorms as explants, vitrification and droplet-vitrification were explored by Vendrame and Faria (2011) and Galdiano et al. (2012) for the cryopreservation of *Dendrobium* germplasm. Droplet-vitrification provided limited effectiveness recovery and was influenced by sucrose pretreatment. For seeds, only vitrification has been used for an endangered species *D. chrysanthum* (He et al. 2010), as well as commercial hybrids *D.* ‘Sena Red’, *D.* ‘Mini WRL’, *D.* ‘Jaquelyn Thomas’, *D.* ‘BFC Pink’ and *D.* ‘Dong Yai’ (Vendrame et al. 2007; Galdiano et al. 2012, 2014).

In short-term conservation and cryopreservation, there may be some alterations to the genome, thus the determination of genetic integrity is necessary after (cryo)storage. Those processes can cause dehydration-, osmotic pressure- or freezing-related stresses that may result in genetic instability (Hazubska-Przbyl et al. 2010). Genetic instability and somaclonal variations may cause some differences in genotype and phenotype profiles of cryopreserved plants (Harding 2004). Some papers reported the use of randomly amplified polymorphic DNA (RAPD) for evaluating the genetic stability of *Dendrobium* PLBs after storage (Antony et al. 2012). Mohanty and Das (2013) had also reported one such protocol, but that study was subsequently retracted due to figure manipulation, casting

doubts on the veracity, reliability or reproducibility of the results. RAPD results from selected primers indicated that the genetic stability of PLBs following storage (short-term and long-term) was maintained. However, molecular markers sometimes failed to detect somaclonal variations in ornamental plants, including in orchids (Park et al. 2009). Phenotypic characteristics of plantlets regenerated from *D. candidum* PLBs (Yin and Hong 2009) and of greenhouse-acclimatized seedlings associated with flow cytometry (FCM) for cryopreserved *Dendrobium* hybrid 'Dong Yai' seeds (Galdiano et al. 2014) were also used to assess genetic stability. In both studies, regenerated plantlets derived from cryopreserved PLBs or seeds were similar to controls (explants not cryopreserved). The discovery and development of new molecular markers to assess diversity are also fundamental for the study of genetic stability and to establish conservation programs for *Dendrobium*. Ding et al. (2008) analyzed *D. officinale* with sequence-related amplified polymorphism (SRAP) and obtained 96 polymorphic bands from a total of 109. Hou et al. (2012) developed 15 new and highly polymorphic microsatellite loci for *D. officinale* to evaluate the genetic diversity of wild and *ex situ* conserved populations.

There are no studies on the re-introduction of *Dendrobium* species back into the wild for in situ conservation, nor are there any studies that examine the storage of genetically modified material. These aspects need to be the focus of future projects.

Conclusions and future perspectives

Cryopreservation is a suitable means for preservation of *Dendrobium* germplasm. It requires minimal storage space and maintenance, while ensuring the stability of phenotypic or genotypic characteristics. Of the total of three cryopreservation techniques that have been applied to *Dendrobium* hybrids and species conservation and/or preservation, vitrification is the simplest technique because it does not require expensive equipment or laborious steps, and is thus particularly useful for *Dendrobium* seed and pollen. However, encapsulation–vitrification and encapsulation–dehydration are most likely the most suitable methods explored for this orchid genus because they resulted in a high survival frequency after cryogenic storage of PLBs, the major explant explored. Such techniques have been explored to a great extent and future perspectives may include optimization of existing cryopreservation protocols, as well as innovative approaches to cryopreservation.

Conflict of interest The authors declare no conflicts of interest, financial, or other.

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