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PRODUCTIVITY AND CYTOGENETIC STABILITY OF PROTOCORM-LIKE BODIES OF HYBRID *CYMBIDIUM* CRYOPRESERVED BY ENCAPSULATION-DEHYDRATION AND VITRIFICATION

Jaime A. TEIXEIRA DA SILVA[1,2]

Abstract: Transformed and untransformed protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light' were cryopreserved using two methods (encapsulation-dehydration and vitrification) to form new PLBs (*neo*-PLBs) on Teixeira *Cymbidium* (TC) medium. The organogenic response was quantified and genetic stability was assessed by flow cytometry. Intact PLBs produced significantly more *neo*-PLBs and fresh weight of *neo*-PLBs than half-PLBs or PLB longitudinal thin cell layers (lTCLs), for two vitrification protocols and for an encapsulation-dehydration method. The addition of 2% sucrose significantly improved the number of *neo*-PLBs than half-PLBs or PLB lTCLs but any other concentration of sucrose, and all other osmotic agents (mannose, PEG-6000, DMSO) at any concentration resulted in significantly worse *neo*-PLB formation. However, the length of exposure to sucrose did not significantly affect the number or fresh weight of *neo*-PLBs. The desiccation (air current or silica gel) method applied negatively affected the number and fresh weight of *neo*-PLBs. The length of the cryopreservation period negatively impacted the number of *neo*-PLBs and fresh weight of transformed or untransformed *neo*-PLBs. Most severe negative effects were registered after one year of cryopreservation. Cryopreserved *neo*-PLBs showed high levels of endopolyploidy (8C-64C) relative to non-cryopreserved PLBs.

Keywords: alginate bead, orchids, PLB, thin cell layers, Teixeira *Cymbidium* (TC) medium, transgenic

Introduction

Orchids are an important group of flowering plants due to their ornamental value. Cryopreservation, or preservation in liquid nitrogen (LN; –196 °C), provides a useful way of preserving rare or endangered orchid tissue over a long period of time and can be used to create cryobanks [BENSON, 2008]. For example, many members of the orchid family (Orchidaceae) are included in the Convention on International Trade in Endangered Species (CITES) Appendix II (2014), making the search for suitable regeneration and preservation protocols all the more important. To date, several orchid explants (seed, meristems, tissue-cultured shoot primordia, immature seeds, somatic embryos or protocorm-like bodies (PLBs), callus) have been cryopreserved with different levels of success [WANG & PERL, 2006; HOSSAIN & al. 2013]. The number of studies reporting on the cryopreservation of orchid PLBs or protocorms remains limited, for example *Geodorum densiflorum* [DATTA & al. 1999], *Doritaenopsis* [TSUKAZAKI & al. 2000], *Dendrobium* Walter Oumae [LURSWIJIDJARUS & THAMMASIRI, 2004], *Dendrobium candidum* [YIN & HONG, 2009], *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], *Cymbidium eburneum*

[1] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa, 761-0795 – Japan, e-mail: jaimetex@yahoo.com
[2] Current address: P. O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken, 761-0799, Japan

[GOGOI & al. 2012], hybrid *Cymbidium* Twilight Moon 'Day Light' [TEIXEIRA DA SILVA, 2013c], and *Aerides odorata* [HONGTHONGKHAM & BUNNAG, 2014] while more work has been achieved with orchid seeds. The cryopreservation of *Dendrobium* germplasm was recently reviewed [TEIXEIRA DA SILVA & al. 2014]. SAIPRASAD & POLISETTY (2003) encapsulated *Dendrobium*, *Oncidium* and *Cattleya* PLBs at the leaf primordium stage, 13-15 days after culture. Half-PLB explants that had developed for two weeks prior to encapsulation resulted in higher survival of alginate beads [TEIXEIRA DA SILVA, 2012a]. NAGANANDA & al. (2011) encapsulated the PLBs of *Flickingeria nodosa* and achieved 95% conversion to plants after 3 months' storage at 4 °C. Cold storage, however, was not successful for *Cymbidium* hybrid PLBs [TEIXEIRA DA SILVA, 2012a].

In all cases, however, once the cryopreserved tissue emerges from cryostorage, it must have the ability to resume growth and regenerate. In that sense, tissue culture remains an essential complement to cryostorage, at least for orchids. This study employs one form of cryopreservation, encapsulation-dehydration [FABRE & DEREUDDRE, 1990] which generally involves three steps: 1) encapsulation in alginate beads, the synseeds (the former term will be used throughout this manuscript); 2) treatment with a high concentration of sucrose to reduce moisture content to 20-30%; 3) rapid freezing in LN. In another popular cryopreservation method, vitrification, explants are treated with a concentrated vitrification solution for variable periods of time (15 min to 2 h), followed by a direct plunge into LN, which results in both intra- and extra-cellular vitrification, which refers to the physical process of transition of an aqueous solution into an amorphous and glassy (non-crystalline) state [LAMBARDI & al. 2006]. The vitrification solution consists of a concentrated mixture of penetrating and non-penetrating cryoprotectant substances. The most commonly applied solution, named PVS2 (Plant Vitrification Solution n° 2), consists of 30% glycerol, 15% ethylene glycol, 15% DMSO (all v/v) and 0.4 M sucrose [SAKAI & al. 1990]. For example, PVS2 was used for the cryopreservation of *Vanda coerulea* [JITSOPAKUL & al. 2007]. Due to its popularity, vitrification was also tested in this study.

Generally, 4 °C has been found to be most suitable for storage of orchid alginate beads or synseed [SAIPRASAD & POLISETTY, 2003; IKHLAQ & al. 2010] although there is a wealth of literature on the choice of optimum temperature and light conditions (reviewed by SHARMA & al. 2013). Encapsulation-dehydration is likely to be the most suitable form of cryopreservation because it results in a high survival frequency. One reason is that the encapsulation of PLBs within calcium alginate beads protects them from direct damage when subjected to desiccation [KHODDAMZADEH & al. 2011] while avoiding toxic cryoprotectants through the use of more "natural" osmotica such as sucrose. The sucrose molarity in a bead (synseed) is further increased by desiccation which results in a glass transition during cooling in LN, preventing ice crystal formation (a cause of lethal damage to living cells) during exposure to ultra-low temperature [ENGELMANN, 2011].

A synthetic seed is an encapsulated clonal product that can eventually grow into a plant, either *in vitro* or *ex vitro*, through "conversion" or "germination", somewhat like a conventional zygotic seed [SHARMA & al. 2013]. Since PLBs are considered to be somatic embryos in orchids with a high degree of cytogenetic stability [TEIXEIRA DA SILVA & TANAKA, 2006], they have been used as explants in this study and, when encapsulated for cryopreservation, are termed alginate beads. TEIXEIRA DA SILVA (2012a) preserved hybrid *Cymbidium* PLBs as alginate beads in the short- and mid-term (1-12 months). In that study, in one treatment that involved cryopreservation, PLBs were

cryostored in LN in the dark for 1, 6 and 12 months without any pre-treatment, i.e., a crude or simple form of cryostorage. The result was understandably poor, and only 6% of cryostored PLBs formed *neo*-PLBs (i.e. new PLBs) after plating on optimized *Cymbidium* PLB regeneration medium, Teixeira *Cymbidium* (TC) medium [TEIXEIRA DA SILVA, 2012b]. Direct cryopreservation would not surprisingly result in cellular damage, in the form of ice crystal formation, due to the expansion of cells without a suitable osmotic treatment to prepare them for the cold shock treatment by LN. Based on a previously established protocol for the cryopreservation of a popular *Cymbidium* hybrid cultivar, Twilight Moon 'Day Light' [TEIXEIRA DA SILVA, 2013c], *neo*-PLB formation from PLB explants was further quantified in this study. Furthermore, the viability of explants post-cryopreservation and pre-re-plating was also assessed by flow cytometry.

Materials and methods

All protocols strictly follow TEIXEIRA DA SILVA (2012a, 2013c), almost *verbatim* in parts, except for flow cytometry.

Chemicals and reagents

All chemicals and reagents were of analytical grade and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan) originally developed from shoot-tip culture on VACIN & WENT (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium) every 2 months on VW medium supplemented with 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin (Kin), 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA), following TEIXEIRA DA SILVA & al. (2005) and TEIXEIRA DA SILVA & TANAKA (2006). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100-ml Erlenmeyer flasks, double-capped with aluminium foil, at 25 °C, under a 16-h photoperiod with a photosynthetic photon flux density of 45 µmol/m^2/s provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally dissected as two pieces of PLB (3-4 mm in diameter) segments, 10/flask, were used as explants for PLB induction and proliferation. Culture conditions and media followed the recommendations previously established for medium formulation [TEIXEIRA DA SILVA & al. 2005], biotic [TEIXEIRA DA SILVA & al. 2006b] and abiotic factors [TEIXEIRA DA SILVA & al. 2006a] for PLB induction, formation and proliferation.

Explant preparation

Based on a protocol for the encapsulation of PLBs [TEIXEIRA DA SILVA, 2012a], three explant types were tested: intact PLBs, half-PLBs, and PLB longitudinal thin cell layers (lTCLs). lTCLs are useful explants in orchid regeneration due to the ability to control organogenesis at a fine scale relative to larger explants [TEIXEIRA DA SILVA & TANAKA 2006; TEIXEIRA DA SILVA, 2013a], although the risk is that they can also lose viability due to increased chances of damage during explant preparation. Intact PLBs were prepared by removing the terminal shoot apical meristem and white base of callus-like non-regenerative tissue, half-PLBs were prepared by cutting prepared intact PLBs along a

longitudinal axis while PLB lTCLs were prepared by 1-mm deep 1 mm^2 sections (5 or 6 per PLB) made from stock PLBs (see detailed preparation of three explant types in TEIXEIRA DA SILVA, 2013a).

Explant encapsulation and bead (synseed) preparation, and cryopreservation

Explant preparation and handling is described broadly in Fig. 1. Explants were encapsulated by immersing each explant with sterilized forceps in a 3.5% (w/v) sodium alginate solution made up in TC medium supplemented with 10% (v/v) coconut water [TEIXEIRA DA SILVA, 2012a] (Fig. 2A). Using wide-mouth glass pipettes (i.e., 5- or 10-ml pipettes with terminal end sawn off), this solution was fed into a complexing solution made of 100 mM $CaCl_2$ for 45 min, drop by drop, each drop containing a single explant. While half-PLBs and PLB lTCLs could pass through the neck of the 5-ml pipettes, 10-ml pipettes were required for intact PLBs. The complexing solution was stirred gently with a sterilized metal spoon once every 10 min to prevent the alginate beads from adhering to each other and fusing. After hardening, the alginate beads were rinsed gently for 10 min in sterile distilled water under sterile conditions on the clean bench to wash away any remaining $CaCl_2$ residue. Since the cryopreservation of raw (i.e., non-encapsulated) PLBs was poor in previous trials [TEIXEIRA DA SILVA, 2012a], in this study, encapsulated explants (intact PLBs, half-PLBs, PLB lTCLs) were removed from the alginate beads and transferred onto TC basal medium under the same conditions as in PLB initiation and proliferation (indicated above), following cryopreservation, to assess the ability to form *neo*-PLBs (Fig. 2B). Simultaneously, a set of explants was left in the alginate case and plated onto TC basal medium.

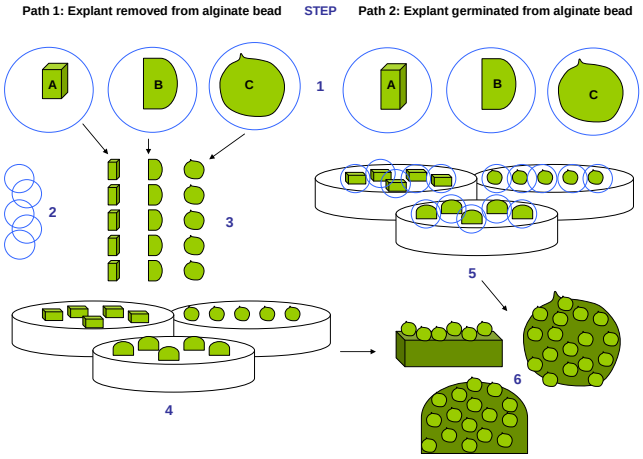

Fig. 1. General schematic of explant preparation and processing for different objectives of this experiment. For all studies, alginate beads (syn. synseeds) were created using 3.5% (w/v) sodium alginate-based TC medium [TEIXEIRA DA SILVA, 2012b] supplemented with 10% (v/v) coconut water [TEIXEIRA DA SILVA, 2012a] from three explants, in step 1: A = PLB lTCLs, B = half-PLBs, C = intact PLBs (see detailed preparation of all three explant types in TEIXEIRA DA SILVA, 2013a, 2013c). In path 1, any explant type was excised from the alginate bead, alginate bead sodium alginate cases were discarded (step 2) and explants were used in a "naked" form (step 3), plated separately on Petri dishes (step 4). In path 2, any explant type was retained within its alginate bead and alginate beads were separately plated on and germinated in Petri dishes (step 5). Finally, the development of *neo*-PLBs (i.e., new PLBs) from explants A, B and C was assessed from paths 1 and 2 (step 6). Explant sizes, shapes, distributions and densities are not proportional or to scale, and depending on the sampling time, can reveal completely different quantitative organogenic outcomes [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013].

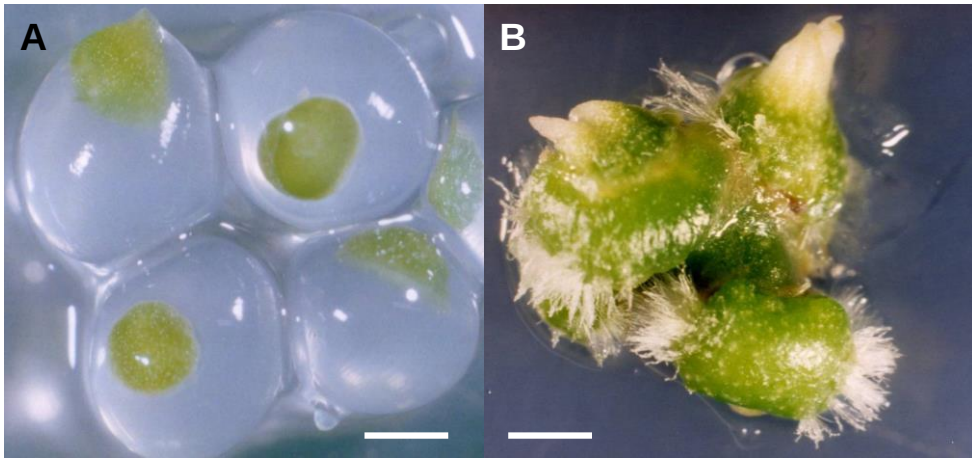

Fig. 2. (A) Encapsulated half-PLBs in sodium alginate synseeds. (B) Young (30 days old), actively dividing *neo*-PLBs from cryopreserved half-PLB growing on TC medium. Scale bars: A = 1 cm; B = 5 mm.

Preculture and priming of PLB-alginate beads

Two parameters were assessed: osmotic hydration (i.e., osmoticum loading) and desiccation.

1. Osmotic hydration

Intact PLBs were confirmed to be the best explant for encapsulation due to higher survival and percentage of explants forming PLBs (explants removed from the encapsulated alginate bead) or percentage re-growth (encapsulated explants) (see Tab. 1 of TEIXEIRA DA SILVA, 2013c). Encapsulated PLBs (25/dish) (i.e., alginate beads) were placed in 25-cm diameter, 1-cm deep Petri dishes (As-One, Osaka, Japan) and precultured in stationary liquid sucrose-free TC medium (50 ml/dish) (control), 1, 2, 3, or 4% (w/v or v/v) sucrose, mannose, polyethylene glycol (PEG-6000) or DMSO for 24 h. After it had been established that 2% sucrose was the best osmoticum, the same explants were plated under the same conditions for 0, 12, 24, 36 or 48 h. Precultured, encapsulated PLBs were plunged into LN (10 alginate beads/treatment, each within 5-cm wide stainless steel cages (maker unknown) or within 2-ml polypropylene tubes (2.0 ml Conical Screw Cap Microtube; Quality Scientific Plastics Inc., Kansas, USA), 5/tube ensuring that all vials and tubes were appropriately labeled). Pre-trials showed that there was no difference between the use of polypropylene tubes or stainless steel cages. After storage in LN for 1 h, the vials were rapidly re-warmed (3 min in a 35 °C water bath) and PLBs were retained in the alginate beads or removed from the alginate beads and replated on TC basal medium under the same conditions as PLB initiation and proliferation indicated above to assess survival and PLB-formation ability. Once it had been established that pre-culture of intact PLBs in 2% sucrose (Tab. 2 of TEIXEIRA DA SILVA, 2013c) for 24 h (Tab. 3 of TEIXEIRA DA SILVA, 2013c) was the best treatment and explant type combination, in terms of explant survival and ability to regenerate PLBs (i.e., germinate), intact PLBs pre-treated in 2% sucrose for 24 h were placed in LN for 1 h, 1 day, 1 week, 1 month or 1 year to assess the impact of cryopreservation period on the same survival and growth parameters.

2. Desiccation method and duration

After pre-culture of encapsulated intact PLBs under optimal conditions determined in TEIXEIRA DA SILVA (2013c) (liquid TC, 2% sucrose for 24 h), alginate beads were rapidly surface-dried by rolling them, in an air-flow cabinet, on two layers of sterile filter (Whatman No. 1) paper to remove any remaining liquid preculture TC medium and were subjected to dehydration by evaporation at room temperature. Two desiccation methods were tested at 25 °C: (1) dehydration under the air current of a laminar air flow cabinet; (2) dehydration in sealed (with a single strip of Parafilm® (Pechiney Plastic Packaging Co., Chicago, USA) Petri dishes (9 cm in diameter) containing 50 g of dry silica gel (Wako). Alginate beads from both dehydration methods were maintained in this state for 0 (control), 1, 6 or 12, 24 or 48 h. Processing of alginate beads (i.e., adding to LN for the same periods and post-cryostorage treatments) was identical to the preculture trials. PLB moisture content was determined as explained next.

Vitrification protocols

PLBs derived from growth on TC medium were prepared using the same protocol for the encapsulation of PLBs [TEIXEIRA DA SILVA, 2012a], except that PLBs were not encapsulated. Two protocols were followed. In protocol 1, unencapsulated PLBs were immersed in PVS2 [SAKAI & al. 1990, 2000] for 30, 60, 90 or 120 min. PLBs were then placed in 2-ml polypropylene tubes, 5/tube, and plunged directly into LN for 1 h, 1 day, 1 week, 1 month or 1 year. Protocol 2, originally established for *Rubus* [KOVALCHUK & al. 2010], was followed with modifications. Unencapsulated PLBs were submerged in loading solution (TC medium with 2 M glycerol and 0.4 M sucrose) for 20 min and then transferred to 2-ml cryovials on ice with 2 ml PVS2 for 80 min. Vials were then submerged in LN for 1 h, 1 day, 1 week, 1 month or 1 year, resulting in vitrified PLBs. All subsequent steps (rewarming and PLB regeneration) followed the alginate beads cryopreservation protocol described above. Controls were pretreated, exposed to PVS2 alone (protocol 1) or to loading solution plus PVS2 solution (protocol 2), then rinsed with liquid TC medium and plated on recovery medium solidified with agar. To assess the effectiveness for transgenic material, transgenic PLBs derived from particle bombardment containing the pWI-GUS plasmid vector [TEIXEIRA DA SILVA & TANAKA, 2009, 2011] were cryopreserved using vitrification Protocol 1 for the same period of time and compared.

Flow cytometry

This protocol was used previously for the same cultivar in TEIXEIRA DA SILVA & TANAKA (2006) and TEIXEIRA DA SILVA & al. (2005, 2006a, 2006b, 2007), although described with some modifications here, including the lack of an internal standard, which was barley in previous studies. Nuclei were isolated from about 0.5 cm^2 of control and treatment *neo*-PLBs by chopping in a few drops of Partec Buffer A (2 µg/ml 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton-X, pH 7.5; MISHIBA & MII, 2000). Nuclear fluorescence was measured using a Partec® Ploidy Analyser (Partec GmbH, Munich, Germany) after filtering the nuclear suspension through 30 µm mesh size nylon filter (CellTrics®, Partec GmbH) and adding five times of Buffer A for 1 min. Three samples were measured, and relative fluorescence intensity of the nuclei was analyzed when the coefficient of variation was < 4%. A minimum of 2500 nuclei were counted for any sample.

Morphological and photosynthetic parameters assessed

For all treatments, the number of *neo*-PLBs that formed from explants (intact PLBs, half-PLBs, PLB lTCLs) on TC medium was observed at 2 and 4 weeks, but scored at 4 weeks (30 days). The fresh weight (mg) of PLB explants + *neo*-PLBs that formed on them were also assessed at 30 days.

Statistical analyses

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment (i.e., each medium and/or explant type). All experiments were repeated three times (n = 90, total sample size per treatment). Data was subjected to analysis of variance with mean separations by Duncan's multiple range test (DMRT) using SAS® ver. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were presented at the level of $P \leq 0.05$.

Results

Following the preparation of three different explant types (Fig. 1) and treatment with two cryopreservation protocols, this study has two main sets of novel findings: 1) the quantitative outcome of *neo*-PLBs that can be achieved through cryopreservation using two techniques, as outlined in TEIXEIRA DA SILVA (2013c); 2) the viability of *neo*-PLBs as assessed by flow cytometry.

Intact PLBs produced significantly more *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 1). The same trend was observed for fresh weight of *neo*-PLBs. Similarly, within each cryopreservation method tested (vitrification protocol 1 or 2, or encapsulation-dehydration), intact PLBs produced significantly more *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 1).

The addition of 2% sucrose significantly improved the number of *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 2). All other osmotic agents (at any concentration) resulted in a significant decrease in the number of *neo*-PLBs and fresh weight of *neo*-PLBs than half-PLBs or PLB lTCLs, independent of the path (1 or 2) (Tab. 2). However, the length of exposure to sucrose did not significantly affect the number of *neo*-PLBs and fresh weight of *neo*-PLBs in path 1, except for 48 h exposure (Tab. 3). The trend was different, however, for path 2, in which there was a significantly negative effect on the number of *neo*-PLBs and fresh weight of *neo*-PLBs (Tab. 3).

In general, the method of desiccation (air current or silica gel) negatively affected the number of *neo*-PLBs and fresh weight of *neo*-PLBs in both paths (Tab. 4).

The length of cryopreservation had a negative impact on the number of *neo*-PLBs and fresh weight of *neo*-PLBs, with most severe negative effects being registered after 1 year cryopreservation period (Tab. 5). A similar trend was observed for transformed tissue, but the negative impact was even more pronounced (Tab. 5).

Cryopreserved *neo*-PLBs showed high levels of endopolyploidy relative to non-cryopreserved PLBs (Fig. 3), but shoots that regenerated from all *neo*-PLBs (cryopreserved, transformed, or not) showed no endopolyploidy (data not shown).

Tab. 1. Effect of explant type during encapsulation (i.e., alginate bead) of hybrid *Cymbidium* Twilight Moon 'Day Light' on number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants

		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
		Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control 1	Intact PLB	9.6 a	814 a	n/a	n/a
	Half-PLB	8.2 b	521 c	n/a	n/a
	PLB lTCL	2.2 e	186 e	n/a	n/a
Control 2	Intact PLB	7.9 b	763 b	6.4 a	718 a
	Half-PLB	5.0 d	486 cd	3.4 b	421 b
	PLB lTCL	0.4 f	156 ef	0.3 c	131 d
VP 1	Intact PLB	7.8 b	416 d	n/a	n/a
	Half-PLB	3.0 e	371 d	n/a	n/a
	PLB lTCL	0.2 f	91 f	n/a	n/a
VP 2	Intact PLB	6.5 c	456 cd	n/a	n/a
	Half-PLB	2.0 e	386 d	n/a	n/a
	PLB lTCL	0.1 f	108 f	n/a	n/a
E-D	Intact PLB	8.4 b	516 c	6.1 a	407 b
	Half-PLB	5.2 d	401 d	3.9 b	298 c
	PLB lTCL	0.4 f	126 ef	0 c	63 e

E-D, encapsulation-dehydration; lTCL, longitudinal thin cell layer; n/a, not applicable (because the vitrification protocols do not involve explant encapsulation as an alginate bead); PLB, protocorm-like body; VP = vitrification protocol

* see details of paths 1 and 2 in Fig. 1

[1,2] Controls: 1 = non-cryopreserved and unencapsulated state on TC medium [TEIXEIRA DA SILVA, 2012b]; 2 = non-cryopreserved but encapsulated state (i.e., alginate bead according to TEIXEIRA DA SILVA, 2012a) on TC medium.

Different letters within each column represent significant differences across treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 2. Effect of osmotic agent (24-h exposure) on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs[1] as assessed by number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants

Treatment	Concentration % (w/v)	Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
		Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 b	763 b	6.4 a	718 a
Sucrose	1	6.8 c	681 c	4.6 b	554 b
	2	8.8 a	874 a	6.1 a	699 a
	3	4.9 d	504 d	3.3 c	412 c
	4	2.2 f	238 f	1.7 ef	184 e
Mannose	1	3.4 e	381 e	2.6 cd	281 d
	2	3.0 e	314 ef	2.1 d	201 de
	3	1.1 g	109 g	0.9 e	106 f
	4	0 h	56 h***	0 f	58 g***
PEG-6000	1	0.3 h	71 gh	0.1 f	66 g
	2	0.8 f	99 g	0.2 f	68 g
	3	0.2 h	68 gh	0 f	61 g***
	4	0 h	57 h***	0 f	57 g***
DMSO	1	2.5 ef	271 ef	1.6 ef	173 e
	2	2.0 f	221 f	1.2 e	124 ef
	3	0.9 f	106 g	0.3 f	68 g
	4	0.2 h	63 h	0 f	54 g***

[1] Results from Table 1 indicated that intact PLBs were the best explant for cryopreservation

DMSO, dimethylsulfoxide; PEG, polyethylene glycol 6000; PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1

** Control = non-cryopreserved but encapsulated state (i.e., alginate bead according to TEIXEIRA DA SILVA, 2012a) on TC medium [TEIXEIRA DA SILVA 2012b]

*** The weight is not 0, even though no new explants formed. The average weight of the basal explant is 54 mg (n = 10; data not shown). Different letters within each column represent significant differences across treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 3. Effect of length of exposure to 2% sucrose[1] (osmotic agent) on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs[2] as assessed by number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants

		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Duration (h)	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 a	763 a	6.4 a	718 a
	12	7.7 a	741 a	6.1 a	699 ab
	24[1]	7.8 a	756 a	5.6 ab	631 b
	36	7.6 a	749 a	5.2 b	608 b
	48	7.5 a	721 b	4.3 c	501 c

[1] Results from Table 2 indicated 2% sucrose to be the best osmotic treatment for cryopreservation

[2] Results from Table 1 indicated intact PLBs to be the best explant for cryopreservation

PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1

** Control = non-cryopreserved state on TC medium [TEIXEIRA DA SILVA, 2012b]. Different letters within each column represent significant differences between treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 4. Impact of desiccation method on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs as assessed by number of neo-PLBs formed and fresh weight assessed 30 days after replating explants

		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Duration (h)	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 a	763 a	6.4 a	718 a
Air current	6	6.1 b	518 b	6.2 a	689 ab
	12	3.8 d	263 e	5.8 ab	651 b
	24	2.9 e	198 f	5.2 b	482 c
	36	1.1 f	104 g	3.8 cd	291 d
Silica gel	6	5.6 bc	472 c	4.1 c	701 a
	12	5.2 c	436 c	3.9 c	693 ab
	24	4.4 cd	318 d	3.7 cd	481 c
	36	2.9 e	206 f	3.3 d	388 d

PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1.

** Control = non-cryopreserved state on TC medium [TEIXEIRA DA SILVA, 2012b]. Different letters within each column represent significant differences across treatments within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

Tab. 5. Effect of cryopreservation period on efficiency of encapsulated PLB pre-treatment using intact hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs[1] following osmopriming in 2% sucrose[2] for 24 h[3] as assessed by number of *neo*-PLBs formed and fresh weight assessed 30 days after replating explants.

		Untransformed tissue			
		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Cryopreservation period	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	7.9 a	763 a	6.4 a	718 a
	1 week	7.6 a	731 ab	4.1 b	501 b
	1 month	7.4 a	716 b	3.0 c	298 c
	1 year	6.8 b	621 c	1.1 d	146 d
		Transformed tissue***			
		Explant removed from alginate bead (path 1)*		Explant re-grown from alginate bead (path 2)*	
	Cryopreservation period	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Control**	0	6.4 a	598 a	5.2 a	614 a
	1 week	6.1 a	571 ab	3.9 b	482 b
	1 month	6.0 a	556 b	2.6 c	238 c
	1 year	4.9 b	473 c	0.8 d	106 d

[1] Results from Table 1 indicated intact PLBs to be the best explant for cryopreservation

[2] Results from Table 2 indicated 2% sucrose to be the best osmotic treatment for cryopreservation

[3] Results from Table 3 indicated osmopriming with 2% sucrose for 24 h to be the best treatment for cryopreservation

PLB, protocorm-like body

* see details of paths 1 and 2 in Fig. 1.

** Control = non-cryopreserved state on TC medium [TEIXEIRA DA SILVA, 2012b]

*** Transformed PLBs containing pWI-GUS obtained following particle bombardment according to TEIXEIRA DA SILVA & TANAKA (2009, 2011) and transgenic tissue confirmed by GUS assay and growth on selective medium containing 100 mg/l kanamycin sulphate; Vitrification Protocol 1 was used after it was established that this was the superior protocol.

Different letters within each column represent significant differences between periods within each tissue (transformed vs untransformed) within each path (1 or 2) according to DMRT at $P \leq 0.05$ (n = 30 (10 replicates × 3) for each treatment).

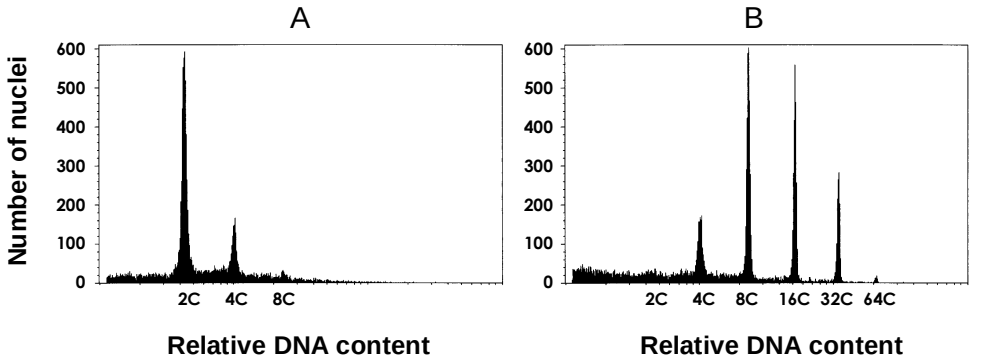

Fig. 3. Flow cytometric analyses of control and cryopreserved PLBs. (A) Young (30 days old), actively dividing *neo*-PLBs from control, non-cryopreserved half-PLB growing on TC medium. (B) Older (120 days old) *neo*-PLBs from which shoots and roots have formed derived from cryopreserved (pooled from encapsulation-dehydration and vitrification protocols) half-PLBs under optimized conditions indicated by this study and TEIXEIRA DA SILVA (2013a, 2013b).

Discussion

Cymbidium in the wider perspective of orchid cryopreservation

The cryopreservation of orchid tissue serves as an important biotechnological tool for the preservation of rare [e.g., GOGOI & al. 2012] or important tissue [e.g., TEIXEIRA DA SILVA, 2013c]. However, storage alone serves no purpose if the material is unable to regenerate into morphologically stable tissue after thawing. In an earlier study [TEIXEIRA DA SILVA, 2013c], an attempt was made to optimize cryopreservation parameters step by step for the same hybrid *Cymbidium* tissue. The cryopreservation of "raw" tissue, without appropriate osmotic protection can result, understandably, in near necrosis of tissue and extremely poor, if any, regeneration after thawing [TEIXEIRA DA SILVA, 2012a]. With this premise in hand, at first, encapsulation of hybrid *Cymbidium* PLBs and alginate bead production was optimized according to TEIXEIRA DA SILVA (2012a). Of note is the fact that TC medium was employed in the calcium alginate bead and also included CW. Most, if not all orchid studies on cryopreservation and alginate bead do not include CW [HOSSAIN & al. 2013]. Then, using this optimized alginate bead medium, factors such as explant source, choice and concentration of osmoticant, method of dehydration and vitrification protocol were all tested and optimized [TEIXEIRA DA SILVA, 2013c]. By priming PLB alginate beads by culture in liquid sucrose-free TC medium supplemented with 2% sucrose for 24 h positively impacted cryostorage [TEIXEIRA DA SILVA, 2013c and Tab. 2, 3, this paper]. This "basal" level of sucrose in the alginate bead may have provided an osmotic buffer against damage by cryostorage (personal hypothesis) although desiccation or air-drying following exposure to silica gel, which had different rates of moisture loss, negatively impacted *neo*-PLB survival (Fig. 2 in TEIXEIRA DA SILVA, 2013c) but did not negatively impact the number of *neo*-PLBs (Tab. 3, this study). This counters the claims by other scientists [e.g., KHODDAMZADEH & al. 2011] who stated that desiccation of *Phalaenopsis* PLB tissue improved explant survival. Although the constant presence of the alginate bead was a negative influence on *neo*-PLB formation (Tab. 1, 2 in TEIXEIRA DA

SILVA, 2013c and Tab. 3, this study), it appeared to afford some protection (or buffering effect) against the negative impacts of desiccation (Tab. 4 in TEIXEIRA DA SILVA 2013c and Tab. 4, this study). In that study, only two treatments were comparable to the control (i.e., regular *in vitro* culture of PLBs without the formation of alginate bead): preculture of half-PLBs left on TC medium for 2 weeks (Fig. 1B), and the addition of CW at 10% (v/v) into the sodium alginate solution (Tab. 1 in TEIXEIRA DA SILVA, 2013c). In the former, most likely the PLBs that were used had already survived any injury from explant preparation and had already initiated the development of *neo*-PLBs while in the latter, CW tends to include a large number of unknown substances, including PGRs, which may have stimulated the growth of PLBs even further, as found for other orchids [ZENG & al. 2012; TEIXEIRA DA SILVA, 2013b]. Even though the use of intact PLBs resulted in higher fresh weight of PLB-alginate bead + *neo*-PLBs, the actual number of PLBs/explant was low, so it was only given a mildly effective code (Tab. 1 in TEIXEIRA DA SILVA, 2012a). In a separate study on an epiphytic orchid, *Cymbidium eburneum*, encapsulation-vitrification was superior to vitrification in the cryopreservation of protocorms, with both techniques requiring the use of PVS2 [GOGOI & al. 2012]. In this study, vitrification (either protocol 1 or 2) was superior to encapsulation-dehydration (Tab. 1).

Cymbidium longifolium synthetic seeds or 'synseeds' were formed after the encapsulation of protocorms [SINGH, 1988]. *Cymbidium* PLB synseeds embedded in a fungicide, and cocooned in chitosan resulted in a 35% germination rate when sown directly on non-sterilized substrate [NHUT & al. 2005]. Protocorms 3-4 mm in diameter were suitable for optimal conversion frequency of encapsulated *Cymbidium giganteum* PLBs, but smaller PLBs could not withstand encapsulation or required a long time to emerge out of the capsule [CORRIE & TANDON, 1993]. High germination frequencies of encapsulated *C. giganteum* PLBs [SAIPRASAD & POLISETTY, 2003] or *C. longifolium* protocorms [CHETIA & al. 1998] occurred when they were stored at 4 °C.

The encapsulation-dehydration and vitrification protocols used in this study for hybrid *Cymbidium* Twilight Moon 'Day Light' were developed by TEIXEIRA DA SILVA (2013c). The following 13 main findings (some written *verbatim*) could be observed in that study: 1) the percentage of explants forming *neo*-PLBs was always significantly lower than the percentage of surviving explants, except for the control. 2) Encapsulation negatively impacted explant survival. 3) Intact PLBs had a significantly higher re-growth and percentage survival than half-PLBs or PLB lTCLs. 4) For all three types of explants, Vitrification Protocol 1 was significantly better than Vitrification Protocol 2. 5) Even though explant survival from encapsulation-dehydration tended to be higher than other protocols, the percentage of explants forming *neo*-PLBs and re-growth percentage was always significantly lower. 6) Any treatment involving the removal of the explant from alginate beads showed higher survival than when explants were left encapsulated. Pre-treatment of PLBs with sucrose and mannose significantly negatively affected PLB viability, but sucrose, especially 2%, had the most positive influential impact on growth parameters. 7) The percentage of explants forming *neo*-PLBs was always significantly lower than the percentage of surviving explants and encapsulation had a negative effect on explant survival. 8) Mannose, PEG-6000 and DMSO negatively impacted explant survival and alginate bead re-growth (relative to sucrose). 9) Hyperhydricity was not observed in any treatment. 10) Preculture of PLBs in stationary liquid sucrose-free TC medium supplemented with 2% sucrose for 24 h improved viability and survival while mannose had

a negative impact on both these parameters. 11) When alginate beads were desiccated on a laminar flow bench, there was a much greater loss of relative moisture content than when silica gel was used, and even though the regeneration of *neo*-PLBs was better in the latter, both methods were in most cases inferior to the control. 12) The survival of half-PLBs decreased significantly as the storage period increased for transformed or untransformed tissue, although the latter showed lower values, possibly because of explant damage caused by the biolistic treatment. Even so, considerable control and transgenic material could be recovered which, if replated onto TC medium, was able to regenerate. 13) PLBs from cryopreservation treatments or *neo*-PLBs that formed from encapsulated PLBs were morphologically normal, as assessed by light microscopy and SEM while shoots from *neo*-PLBs derived from cryopreservation and alginate bead-derived treatments were also normal. Except for point 10), where no significant differences were found between most treatments in paths 1 and 2 (Tab. 3), the trends are the same for number of *neo*-PLBs and *neo*-PLB fresh weight in this study.

Some hyperhydricity was observed in *neo*-PLBs (Tab. 1 in TEIXEIRA DA SILVA 2013c), but the frequency was low. RADY & HANAFY (2004) also observed hyperhydricity in regenerating *Gypsophila paniculata* (non-orchid) plantlets following alginate bead germination. In this study, using optimized conditions, hyperhydricity was not observed in *neo*-PLBs or in regenerated shoots (data not shown).

One of the possible reasons for the low level of germination (i.e., percentage of alginate beads forming *neo*-PLBs in Tab. 1 in TEIXEIRA DA SILVA, 2013c), in which germination is not the traditional formation of a shoot, but rather the *neo*-formation of PLBs or the formation of *neo*-PLBs, may be because half-PLBs were used rather that intact PLBs, suggesting that tissue injury may have negatively impacted the regeneration capacity. The reader is reminded that PLBs are in fact considered to be (i.e., synonymous with) somatic embryos in orchids [TEIXEIRA DA SILVA & TANAKA, 2006], and would thus represent the ideal form of a dipolar propagule for alginate beads, even though half-PLBs and PLB-derived TCLs can also be used.

Why use alginate beads?

Alginate beads (synonymous with synseeds; SHARMA & al. 2013) present a hypothetically excellent way to cryostore orchid material for weeks to months, even years, while maintaining the clonal stability of material. Alginate beads in orchid biotechnology have particular relevance considering that they produce tiny, non-endospermic seeds. CORRIE & TANDON (1993) encapsulated *Cymbidium giganteum* protocorms and healthy plantlets formed after synseeds were transferred either to nutrient medium or directly to sterile soil with a 100% conversion frequency *in vitro*. As described in the introduction, PLB encapsulation is well documented in several orchids.

The principle of desiccation (or air-drying) is to reduce the water content to a sufficiently low level and to induce an intrinsic tolerance to desiccation by triggering the genes responsible for desiccation tolerance. In hybrid *Cymbidium* (Fig. 2 in TEIXEIRA Da SILVA 2013c), and also for *Phalaenopsis* [KHODDAMZADEH & al. 2011], silica gel allowed a slower decrease in moisture content than laminar air flow, providing, most likely, an opportunity for PLBs to adapt to dehydration stress. If PLBs are not sufficiently dehydrated, injury after immersion in LN can occur due to intracellular ice formation but if over over-dehydrated, osmotic stress can cause damage [BIAN & al. 2002], this being the

most likely explanation for the extremely low survival (6%) of encapsulated *Cymbidium* PLBs following immersion in LN without a tissue-drying pre-treatment [TEIXEIRA DA SILVA, 2012a]. Incidentally, in *Dendrobium candidum* PLBs, dehydration tolerance was also induced by treatment with abscisic acid (ABA) for three days and optimal water content for cryopreservation was estimated to be 11-33% [BIAN & al. 2002]. Exogenous ABA treatment of the PLBs induced accumulation of soluble sugars, heat-stable proteins, and dehydrins [BIAN & al. 2002]. The synthesis of dehydrins was also enhanced by ABA treatment in *Spathoglottis plicata* protocorms [WANG & al. 2003]. Dehydrins are involved in the reactions of plants against drought, salinity, and dehydration and their accumulation during cell dehydration suggests their involvement in protective reaction, including in membrane stabilization, preventing denaturation and aggregation of macromolecules, detoxification of salt ion, and stabilization of transcription machinery [CLOSE, 1997; ALLAGULOVA & al. 2003; HANIN & al. 2011]. Thus it is effective for the acquisition of dehydration tolerance to accumulate dehydrins in PLBs. When the moisture content of encapsulated plant tissue lies between 20 and 40%, this ensures the highest survival after cooling in LN because this level is roughly the amount of un-freezable water in a plant cell, e.g., 38% in *Vanda coerulea* [JITSOPAKUL & al. 2007], or 30 or 43.5% in silica gel dehydration *versus* laminar-flow dehydration, respectively for *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011]. When membranes are excessively desiccated, they undergo structural changes and proteins denature [HOEKSTRA & al. 2001], this being a possible explanation why viability was much lower at 12 and 24 h dehydration than at 6 h (optimal) (Tab. 4 in TEIXEIRA DA SILVA, 2013c), while excessive moisture in tissues would result in the production of extracellular ice crystals [POPOV & al. 2006], also resulting in reduced viability, although this trend was not observed for *Cymbidium* tissue (Tab. 4 in TEIXEIRA DA SILVA, 2013c). Bucking this trend and logic, low levels of crystallizable water within poplar tissue ensured high levels of recovery percentages after cryostorage [LAMBARDI & al. 2000].

The alginate bead-cryopreservation interface: factors influencing successful regeneration

For seeds, synseeds or alginate beads to be successfully cryopreserved, seed moisture is an important factor because the presence of unbound water in seeds considerably reduces their germinability causing seeds to perish because of the formation of ice crystals in their cells during freezing in LN [ZHANG & al. 2001]. Most orchid cryo-researchers apply deep-freezing to seeds with a moisture content of less than 13% (i.e., vitrification-based protocols) while only scarce work has dealt with direct freezing of orchid seeds. In orchids, PRITCHARD (1984) (then later PRITCHARD & al. 1999) first cryopreserved terrestrial and epiphytic orchid seeds with a moisture level below 11%, and seed germinability did not change after cryoconservation for most species examined. In seeds of *Dendrobium candidum*, a high survival rate (about 95%) was also obtained when the desiccated seeds at 8-19% water content with silica gel were directly plunged into liquid nitrogen [WANG & al. 1998]. ISHIKAWA & al. (1997) successfully cryopreserved the zygotic embryos of *Bletilla striata* by vitrification. NA & KONDO (1996) described a cryopreservation protocol for preservation of tissue-cultured shoot primordia from shoot apices of cultured *Vanda pumila* protocorms following preculture in ABA and desiccation, which reduced relative humidity from 40-45% to 24%, with a 65.0 ± 7.5% survival rate and

no reported abnormalities in chromosome number or cell structure. NIKISHINA & al. (2001) found that cryopreservation of the mature seeds of eight orchid genera had no negative effect on the duration of germination, growth and development of protocorms or juvenile plants, although 24% water content for *Encyclia cochleata* was necessary. After cryopreservation by desiccation, protocorms derived from cryopreserved seeds of hybrid *Bratonia* developed faster than protocorms from non-treated seeds during the first 45 days [POPOV & al. 2004]. HIRANO & al. (2005a) applied three cryogenic procedures, namely (1) direct plunging into LN, (2) vitrification, and (3) vitrification with 3-day preculture on medium supplemented with 0.3 M sucrose to preserve immature seeds (2–4 months after pollination) of *Bletilla striata* with a 81-92% survival rate and development of seedlings into normal plantlets *in vitro*. In *Phaius tankervilleae*, when the seeds were dehydrated to 5% water content and preserved at a low temperature (4 °C), there was no decrease in viability or germinability after 3 months but after storage for 6 months, the seeds showed a drastic decrease (9%) in germinability, while cryopreservation of seeds by the vitrification method showed no deterioration after 12 months of storage [HIRANO & al. 2009].

Sucrose and mannose can serve as osmotic protectants, with sucrose accumulating within cells when exposed to sucrose in the surrounding medium, and while 0.75 M sucrose pretreatment resulted in maximum viability in *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], and while 0.3 M sucrose resulted in a maximum of 30% survival in *Digitalis thapsi* after cryopreservation [MORAN & al. 1999], or 0.1 M sucrose for 7 days in *Doritaenopsis* [TSUKAZAKI & al. 2000], a high sucrose concentration was detrimental for hop [MARTINEZ & al. 1999], indicating that the response to and success of pretreatment is species-specific. High concentrations of sucrose or the use of other osmoticants (Tab. 2 in TEIXEIRA DA SILVA, 2013c), as well as the excessive exposure to an osmoticant (Tab. 3 in TEIXEIRA DA SILVA, 2013c) all had a negative impact on the survival of hybrid *Cymbidium*. Sucrose can reduce the freezable water through osmosis, protecting the cytoplasm by entering a vitrified state, although excessively high sucrose concentrations can lead to over dehydration of the cell, resulting in plasmolysis and membrane rupture [POPOV & al. 2006]. VERTUCCI & ROOS (1991) found that drying plant samples with silica gel enhanced the effect of their desiccation before immersion in LN. Since the -OH groups of sucrose are able to replace water and interact with membrane phospholipids, and subsequently stabilize cellular membranes and protect cells from damage during exposure to LN [TURNER & al. 2001], optimization of the preculture period is essential to minimize cell injuries during cryopreservation [TOUCHELL & al. 2002].

Cryopreservation offers other advantages relative to other available storage approaches, including the stability of phenotypic and genotypic characters, minimal storage space and maintenance requirements. Classical cryopreservation procedures include the following successive steps: pregrowth of samples, cryoprotection, slow cooling (0.5-2.0 °C/min) to a determined prefreezing temperature (usually around −40°C), rapid immersion of samples in LN, storage, rapid thawing and recovery which are generally complex since they require the use of sophisticated and expensive programmable freezers. Increasingly, improved and advanced cryopreservation procedures such as vitrification-based techniques are employed. To date, seven different vitrification-based techniques have been developed and applied: (i) encapsulation–dehydration, (ii) a procedure actually termed vitrification, (iii) encapsulation–vitrification, (iv) dehydration, (v) pregrowth, (vi) pregrowth–dehydration and (vii) droplet–vitrification [ENGELMANN, 2011]. Vitrification

involves the treatment of samples with cryoprotective substances, dehydration with highly concentrated vitrification solutions, rapid cooling and rewarming, removal of cryoprotectants and recovery. In total, three cryopreservation techniques have been applied to orchid species, including desiccation (air-drying), vitrification and encapsulation-dehydration [HIRANO & al. 2006; KHODDAMZADEH & al. 2011]. The desiccation technique involves direct dehydration of naked PLBs which are very sensitive to dehydration, as was observed by TEIXEIRA DA SILVA (2012a), while the vitrification technique uses high concentrations of chemicals which can be toxic, possibly explaining the differences in vitrification protocols used for *Cymbidium* (Tab. 1 in TEIXEIRA DA SILVA, 2013c). Encapsulation-dehydration may be the most suitable method as it results in a high survival frequency after cryogenic storage. The encapsulation of explants in alginate beads for cryopreservation has some benefits 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 [MANEERATTANARUNGROJ & al. 2007]. A limited number of studies have reported on the cryopreservation of orchid PLBs, including *Geodorum densiflorum* [DATTA & al. 1999], *Doritaenopsis* [TSUKAZAKI & al. 2000], *Dendrobium* Walter Oumae [LURSWIJIDJARUS & THAMMASIRI, 2004], *Dendrobium candidum* [YIN & HONG, 2009], *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], and *Aerides odorata* [HONGTHONGKHAM & BUNNAG, 2014]. Only one other study in the *Cymbidium* genus, *C. eburneum*, exists [GOGOI & al. 2012]. Seeds of different orchids have also been successfully cryopreserved (reviewed in HOSSAIN & al. 2013).

The aim of vitrification is to increase the solute concentration inside the cells to avoid ice crystal formation [MATSUMOTO & al. 1994; HARDING, 2004; SAKAI & ENGELMANN, 2007]. This is progressively done by first exposing the explants to a constant source of carbon, which helps to stabilize the cell and to exchange inner water with the cryogenic liquids [UCHENDU & REED, 2008; VARGHESE & al. 2009]. In *Doritis pulcherrima*, when the seeds were osmotically dehydrated by PVS2 for 50 min, about 62% of the seeds thus treated survived after plunging into LN [THAMMASIRI, 2000]. 82-86% of *Bletilla striata* seeds were successfully cryopreserved by vitrification following the TTC test [HIRANO & al. 2005a], as were the immature seeds of *Ponerorchis graminifolia* var. *suzukiana* with 85-88% survival but 48-52% germination level [HIRANO & al. 2005b].

Survival, which, depending on the situation, could be synonymous with re-growth, has been variable in different orchid species following cryopreservation: 13.3% in *Dendrobium* Walter Oumae [LURSWIJIDJARUS & THAMMASIRI, 2004], 30% in *Phalaenopsis bellina* [KHODDAMZADEH & al. 2011], 37% in *Dendrobium virgineum* [MANEERATTANARUNGROJ & al. 2007], or 40% in *Vanda coerulea* [JITSOPAKUL & al. 2007]. In this study, a wide range of PLB re-growth (assessed by explant survival and regeneration ability, i.e., *neo*-PLB formation) was possible, but this depended on the choice of explant and treatment, the highest value reached for any parameter being 21% (Tab. 1-5 in TEIXEIRA DA SILVA, 2013c). Such variability would depend on the physiological status of the explants, including its oxidation state [VERLEYSEN & al. 2004], or even on the cryopreservation protocol employed [REED & al. 2001]. Even though lTCLs yield much lower number of *neo*-PLBs/explant than intact or half-PLBs [TEIXEIRA DA SILVA, 2013a], once the Plant Growth Factor is employed [TEIXEIRA DA SILVA &

DOBRÁNSZKI, 2011], then the true number of *neo*-PLBs that can be derived from a PLB lTCL is in fact considerably higher than conventional PLB explants while the timing of the assessment of PLB development can also impact the conclusions drawn [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013]. Consequently, in this study, even though PLB lTCLs performed relatively poorly – relative to intact or half-PLBs – in terms of cryopreservation ability, the fact that lTCLs occupy a significantly smaller surface area and volume, and the fact that – depending on the cultivar – more explants can be derived from a single PLB than the number of explants from intact or half-PLBs [TEIXEIRA DA SILVA & TANAKA 2006], indicates that lTCLs should not be ignored as a potentially viable explant for future *Cymbidium* and orchid cryopreservation studies.

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. Each stage plays an important role in determining the survival of tissue upon re-warming. Since somatic embryos and PLBs in orchid biotechnology are synonymous [TEIXEIRA DA SILVA & TANAKA, 2006], and since it is possible to generate alginate beads from PLBs [TEIXEIRA DA SILVA, 2012a], the modest success achieved in this study on the cryopreservation of PLBs, or related tissue, bodes well for the long-term preservation of *Cymbidium* germplasm, although conditions will almost inevitably need to be optimized for different cultivars and species.

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