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Original Paper

Efficiency of cryoprotectors for cryopreservation of two orchid species from Americas

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Abstract

The objective of this study was to evaluate the efficiency of cryoprotective solution (PVS2) combined with phloroglucinol for the cryopreservation of seeds of two orchid species, *Encyclia cordigera* and *Epidendrum ciliare*. Seeds of *Encyclia cordigera* had 91.03% initial viability and 91.99% germination. The treatment of the seeds with PVS2 at 0 °C with 1% phloroglucinol for 60 min returned 93.79% viability and 91.01% germination after recovery from LN, consequently resulting in faster development of protocorms. For *Epidendrum ciliare,* seed viability was 85.65% and germination was 85.90%. Seed exposure to the PVS2 at 0 °C with 1% phloroglucinol for 180 min showed viability of 39.23% and germination of 37.88%. Despite lower germination, 78.90% of the protocorms reached stage P3 of development, when evaluated 45 days after sowing, not significantly different from the control 1, and showed normal development. These results indicate that PVS2 cryoprotective solution is efficient when combined with phloroglucinol for the cryopreservation and successful recovery of seeds of *Encyclia cordigera* and *Epidendrum ciliare.* The present study also indicates that response to cryopreservation and success of recovery after cold storage is species-specific and requires adjustments in exposure time to PVS2 at 0 °C prior to immersion in LN.

Key words: *Encyclia cordigera*, *Epidendrum ciliare*, germplasm conservation, Orchidaceae, PVS2.

Resumo

O objetivo deste estudo foi avaliar a eficiência da solução crioprotetora (PVS2) combinada com floroglucinol para a criopreservação de sementes de duas espécies de orquídeas, *Encyclia cordigera* e *Epidendrum ciliare*. Sementes de *Encyclia cordigera* apresentaram viabilidade inicial de 91,03% e germinação de 91,99%. O tratamento das sementes com PVS2 a 0 °C com 1% de floroglucinol por 60 min promoveu a recuperação de 93,79% da viabilidade e 91,01% da germinação após a recuperação do nitrogênio líquido, resultando consequentemente no desenvolvimento mais rápido dos protocormos. Para *Epidendrum ciliare,* a viabilidade inicial das sementes foi de 85,65% e germinação de 85,90%. E quando suas sementes foram tratadas com solução PVS2 a 0 °C com 1% floroglucinol por 180 min atingiram viabilidade de 39,23% e germinação de 37,88%. Apesar de observada menor porcentagem de germinação para o referido tratamento, 78,90% dos protocormos atingiram estágio de desenvolvimento P3, quando avaliados 45 dias após a semeadura, não apresentando diferença significativa comparada ao controle 1, e apresentando protocormos com desenvolvimento normal. Esses resultados indicam que a utilização da solução crioprotetora de PVS2 combinado ao floroglucinol 1%

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foi eficiente na recuperação de sementes criopreservadas de *Encyclia cordigera* e *Epidendrum ciliare*. O presente estudo também indica que a resposta à criopreservação após o armazenamento a frio é específica da espécie e requer ajustes no tempo de exposição ao PVS2 a 0 °C antes da imersão em nitrogênio líquido.
Palavras-chave: *Encyclia cordigera*, *Epidendrum ciliare*, conservação de germoplasma, Orchidaceae, PVS2.

Introduction

Orchidaceae is the most diverse group of plants within the angiosperms (Swarts & Dixon, 2009) comprising a large family with more than 27,000 species (Zotz 2013), and known for its wide flower diversity and global distribution (Merritt *et al*. 2014).

The genus *Encyclia* has about 250 species distributed between epiphytic and lithophytic habitats. *Encyclia cordigera* Dresser is one of the most desired genera due to the beautiful and intoxicating fragrance of its pink-lipped flowers, which smell like roses. This species can be found in Mexico, Central and North America, and possibly in Brazil (Lavarack *et al*. 2002).

The genus *Epidendrum* has about 100 species that can be epiphytic, terrestrial or lithophytic. The species *Epidendrum ciliare* L. can be found from southern Mexico to the northern part of South America. It grows on trees or rocks, usually in full sun (Lavarack *et al*. 2002).

E. cordigera and *E. ciliare* L. have been used in the creation of intergeneric hybrids, due to the beauty of its flowers and the generation of novelty plant material for the market of ornamental plants.

Biotechnology offers two techniques for the conservation of plant species, including *in vitro* conservation and cryopreservation (Vendrame & Khoddamzadeh 2017). Through cryopreservation, long-term seed banks can be created, which represent one of the pillars of *ex situ* biodiversity conservation (Seaton *et al*. 2010) and a promising alternative to other conservation approaches (Meritt *et al*. 2014; Popova *et al*. 2016).

The use of cryopreservation for the long-term seed storage, in a seed bank, consists in storing plant material in liquid nitrogen at -196 °C, or in the vapor phase of nitrogen at around -150 °C, for long time span and with a low risk of genetic or physiological variation (Reed 2008; Vendrame & Khoddamzadeh 2017).

Most cryopreservation protocols utilize vitrification solutions as a form of cryoprotection to prevent ice nucleation and cell damage during the process of storage in liquid nitrogen. The plant vitrification solution 2 (PVS2) has been one of the most common cryoprotectant solutions used (Sakai *et al*. 1990). However, efficient recovery of plant material after cryopreservation can be low due to oxidative stress and toxicity of certain components in vitrification solutions. Phloroglucinol (PG - 1,3,5-trihydroxybenzene) is a derivative of phenol, from the group of phenolic compounds, which occurs naturally in plants. PG can be isolated from other sources such as seaweed and microorganisms (Teixeira da Silva *et al*. 2013) and it is known to protect cells against oxidative stress, inflammation, and damage caused by free radicals (Kang *et al*. 2006; Kim & Kim 2010). Phloroglucinol has been successfully used in cryopreservation of some orchid hybrids and species (Galdiano *et al*. 2012, 2013; Vendrame and Faria 2011).

Cryopreservation of orchid seeds of the genera Encyclia and Epidendrum has been previously studied for *Encyclia pygmea* and *Encyclia odorantissima* (Pardo-Alvarez and Ferreira 2006); *Encyclia cochleata* Lemée (Nikishina *et al*. 2001); *Epidendrum quitensium* Rchb.f. and *Epidendrum anderssonii* Hágsater & Dodson (Cerna *et al*. 2018); *Encyclia tampensis, Epidendrum amphytomum, Epidendrum nocturnum* and *Epidendrum rigidum* (Hughes & Kane 2018).

The objective of this study was to evaluate the efficiency of the PVS2 cryoprotective solution combined with phloroglucinol for the cryopreservation of *Encyclia cordigera* and *Epidendrum ciliare* seeds.

Material and Methods

Seed material

Mature seeds of *E. cordigera* and *E. ciliare* were obtained through artificial pollination. Seeds from one fruit of each species were harvested at dehiscence stage and stored in a refrigerator for three months (*E. cordigera*) and fifteen months (*E. ciliare*). These propagules were obtained from greenhouse grown plants located at the Tropical Research and Education Center (TREC), University of Florida, in Homestead, FL, USA. Seed size, measured by length (mm) x width (mm), was obtained for 50 seeds from each species, with the aid of an electron microscope and the Spot Basic

software. The seeds were stored in small paper envelopes placed in a plastic pot, and maintained in a refrigerator at a temperature of 10 ± 2 °C. Before the cryopreservation procedures, which were performed at the laboratory of Ornamental Horticulture and Biotechnology at TREC, seed moisture content was adjusted in an oven at 105 ± 3 °C for 24 hours, according to the Rules for Seed Analysis - RAS (Brazil 2009), with one repetition for *E. cordigera* and two repetitions for *E. ciliare,* whereby 0.02 g of seeds were used per repetition, representing an average of 2500 seeds per repetition.

Seed viability and germination prior to cryopreservation

Initial seed viability was evaluated using the 2,3,5-triphenyl tetrazolium chloride (TTC) test (Hosomi *et al*., 2012). Seeds were counted using a Leica MZ 12.5 stereomicroscope (Leica Microsystems, Buffalo, NY, USA). Seeds that stained pink-red were considered viable as per the TTC test.

To validate the TTC viability tests, germination tests were performed in petri dishes containing 25 ml of half-strength semi-solid culture medium (½ MS) (Murashige and Skoog, 1962), supplemented with 15 g L^{-1} sucrose, solidified with 7 g L^{-1} agar and pH adjusted to 5.7, prior to autoclaving at 121 °C and 20 psi for 20 minutes. The seeds were disinfested in 70% ethanol (1 min), 0.8% sodium hypochlorite (5 min), and rinsed three times with autoclaved distilled water (1 min each) under a laminar flow chamber prior the sowing in Petri dishes, and maintained at 27 ± 2° C; under 60 µmol m^{-2} s^{-1}; and 18-hours photoperiod, provided by two 9A Philips® fluorescent light bulbs. The germination was evaluated 45 days after sowing by visualizing the protocorm formation, counted using a Leica MZ 12.5 stereomicroscope (Leica Microsystems, Buffalo, NY, USA) and germination percentage was calculated.

Cryopreservation procedures and treatments

For each species, 0.038 g of seeds were selected, subdivided into small portions and placed in 2-ml cryovials, followed by the addition of 1 ml of a cryoprotectant solution, composed of 2.0 M glycerol and 0.4 M sucrose. Cryovials containing the seed and cryoprotectant solution were maintained at room temperature (27 ± 2 °C) for 30 min. Subsequently, the first solution was replaced by 1 ml of plant vitrification solution 2 (PVS2), with or without 1% phoroglucinol (PG) for additional 60, 120 and 180 min, at 0 °C (pre-freezing, through partial immersion of cryotubes in ice) prior to immersion in liquid nitrogen (LN). The PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) in of half-strength semi-solid culture medium (½ MS) 0.4 M sucrose, with pH adjusted to 5.7 (Sakai *et al*. 1990). Two controls were included, consisting of direct *in vitro* germination without cryoprotectants and without immersion into LN (control 1) and direct immersion of the seeds in LN without cryoprotectants. The different treatments are described below, as follows:

C1 – control 1: direct *in vitro* germination, no LN

C2 – control 2: direct immersion in LN, no cryoprotectants

T1 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 60 min

T2 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 120 min

T3 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 180 min

T4 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 60 min

T5 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 120 min

T6 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 180 min

Post-cryopreservation procedures

Cryovials were maintained in LN for 72 hours, followed by rapid thawing in a water bath at 40 °C for 1.5 min. Cryoprotectant solutions were removed from cryovials, and 1 ml of 1.2 M sucrose solution prepared in MS culture medium was added. After 20 min, the sucrose solution was removed and seeds were washed twice with autoclaved distilled water, followed by disinfestation in 70% ethanol (1 min), 0.8% sodium hypochlorite (5 min), and rinsed three times with autoclaved distilled water (1 min each) under a laminar flow chamber.

Three cryovials were used as replications for the controls, and five cryovials were used for each treatment. After removal from LN, all seeds (controls and treatments) were placed for germination in Petri dishes with ½ MS culture medium with an average of 120 seeds per plot. The entire experiment was repeated.

Seed viability, germination and protocorm development after cryopreservation

Seed viability and germination percentages, and protocorm development were evaluated 45 days after sowing. A total of 5 petri dishes were evaluated per treatment, using a Leica MZ 12.5 stereomicroscope (Leica Microsystems, Buffalo, NY, USA). Seed viability was evaluated using the 2,3,5-triphenyl tetrazolium chloride (TTC) test and germination was evaluated by observing protocorm formation, as described previously.

Protocorm development was evaluated according to a methodology adapted from Suzuki *et al*. (2009), considering the following developmental classes: stage 1 (P1): chlorophylled swelled protocorms; stage 2 (P2): seedlings exhibiting first leaf; stage 3 (P3): seedlings with two or three leaves.

Experimental design and statistical analysis

The experimental design was completely randomized with 8 treatments, 5 replicates per treatment (T1 thought T6) and 3 replicates for the control treatment. Data were submitted to analysis of variance, and data with a percentage value were transformed using arcsine (x/100) √ ½. Means were compared by the Tukey test at 5% probability. The analyzes were performed using the statistical program AgroEstat (Barbosa & Maldonado Jr 2015).

Results

Seed characteristics, viability and germination

Seed characteristics, viability, germination and water content for both species are shown in Table 1. Seed size, as measured by length (mm) × width (mm) was 0.43 mm × 0.11 mm and 0.42 mm × 0.10 mm on average for *E. cordigera* and *E. ciliare,* respectively. The water content was 8% for *E. cordigera* and 20% for *E. ciliare* (Tab. 1). Seed shape and size were similar for both species (Fig. 1).

Under control 1 (C1), seeds of *E. cordigera* had 91.03% initial viability and 91.99% germination. For *E. ciliare,* seed viability was 85.65% and germination was 85.90% (Tab. 2). However, under control 2 (C2), where seeds were directly immersed in LN with no PVS2 and no PG, seeds of *E. cordigera* still showed some viability (26.6%) and germination (30.6%), while for *E. ciliare*, no germination occurred as seeds were not viable (Tab. 2).

After recovery from cryopreservation, *E. cordigera* seeds showed viability and germination for all treatments that were similar to the initial viability and germination values (C1). Although no significant differences were observed among treatments and control 1, treatments 3 (T3) and 4 (T4) had highest viability and germination percentages (Tab. 2). Under T3 (2.0 M glycerol + 0.4 M sucrose for 30 min + PVS2 for 180 min), viability and germination were 91.0% and 91.6%,

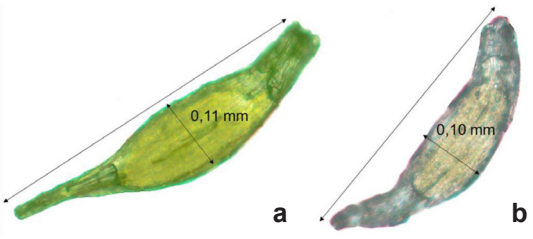

Figure 1 – Seed of *Encyclia cordigera.* (A) and seed of *Epidendrum ciliare* (B), length (mm) and width (mm).

Table 1 – Characterization of the seeds of *Encyclia cordigera* and *Epidendrum ciliare*: biometric characterization, storage time and water content.

Characteristics of seeds	*Encyclia cordigera*	*Epidendrum ciliare*
Seed length (mm)[a]	0.43 ± 0.066[a]	0.42 ± 0.023
Seed width (mm)[a]	0.11 ± 0.002[a]	0.10 ± 0.071
Storage time	3 months	15 months
Water content	8 (%)	20 (%)

[a]Average of 50 seeds.

Table 2 – Seed viability (%) and germination (%) of *Encyclia cordigera* and *Epidendrum ciliare* after recovery from cryopreservation using different treatments (T1-T6) with PVS2 solution with or without 1% of phloroglucinol (PG).

	Encyclia cordigera		*Epidendrum ciliare*	
Treatment*	**Viability (%)****	**Germination (%)*****	**Viability (%)****	**Gemination (%)*****
C1	91.03ab	91.99a	85.65a	85.90a
C2	26.56c	30.59c	0.00f	0.00g
T1	89.83ab	90.96a	24.87c	25.50cd
T2	89.02ab	88.24ab	14.22d	14.29e
T3	91.00ab	91.57a	5.38e	5.83f
T4	93.76a	91.02a	22.69cd	22.64d
T5	87.36ab	88.43ab	28.03c	27.05bc
T6	81.53b	77.99b	39.23b	37.88b
CV%	5.39	5.98	7.93	9.46

* C1 = control 1: direct seed germination, no PVS2, no phloroglucinol (PG), no NL; C2 = control 2: no PVS2, no (PG), direct immersion in NL; T1 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 60 min; T2 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 120 min; T3 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 180 min; T4 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 60 min; T5 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 120 min; T6 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 180 min.
** Values are the means of 3 repetitions with 100 seeds per repetition.
*** Values are the means of 5 repetitions with 600 seeds per treatment, being 5 repetitions.
Means followed by the same letter within columns are not significantly different by the Tukey's test at 5% probability.

respectively. Under T4 (2.0 M glycerol + 0.4 M sucrose for 30 min + PVS2 + PG 1% for 60 min), viability and germination were 93.8% and 91.0%, respectively (Tab. 2).

Viability and germination percentages were significantly lower than the control 1 for *E. ciliare* under all treatments (Tab. 2). The best results were under treatment 6 (2.0 M glycerol + 0.4 M sucrose for 30 min + PVS2 + PG 1% for 180 min) with 39.2% viability and 37.9% germination (Tab. 2).

Protocorm development

Protocorms from both species showed healthy growth into the different developmental stages with no morphological abnormalities (Fig. 2). The first developmental stage (P1) was characterized by a swelled chlorophylled protocorm, followed by P2 with seedlings showing one leaf and P3 where a larger seedling showed two or more leaflets (Fig. 2). Protocorm development was similar for both *E. cordigera* and *E. ciliare* (Fig. 2).

Protocorm development 45 days after sowing following cryopreservation showed variation for both species under all treatments (Tab. 3). For *E. cordigera*, although most protocorms reached seedling stage with first leaf under C1, only T1 (2.0 M glycerol + 0.4 M sucrose for 30 min + PVS2 for 60 min) and T4 (2.0 M glycerol + 0.4 M sucrose for 30 min + PVS2 + PG 1% for 60 min) showed protocorms in stage P3 (seedlings with two or three leaves). The percentage of protocorms in stage P3 was 9.8% for T4, significantly higher than T1 (4.9%), but significantly lower than C1 (15.2%). No protocorms reached stage P3 under C2, T2, T3, T5, and T6 (Tab. 3).

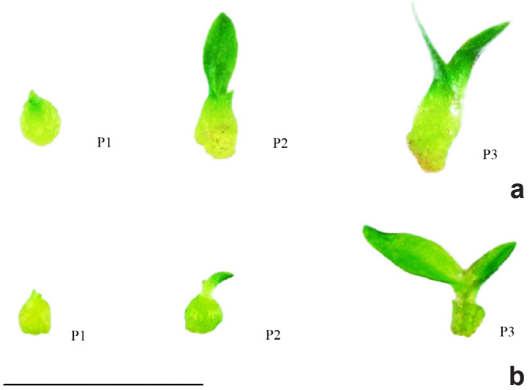

Figure 2 – Development of *Encyclia cordigera* protocorms (A). Development of *Epidendrum ciliare* protocorms (B), photographs of the morphological differences, classified by the development stages: P1 – chlorophylled swelled protocorms, P2 – seedlings exhibiting first leaf, and P3 - seedlings with two or three leaves (Suzuki *et al.* 2009).

Table 3 – Protocorm development for *Encyclia cordigera* and *Epidendrum ciliare* at 45 days after sowing following immersion in LN. The different morphological classes and stages of development are chlorophylled swelled protocorms (P1), seedlings exhibiting first leaf (P2), and seedlings with two or three leaves (P3).

	Encyclia cordigera			***Epidendrum ciliare***		
Treatment	**P1(%)****	**P2(%)****	**P3(%)****	**P1(%)****	**P2(%)****	**P3(%)****
C1*	15.1 ± 1.00d	69.7 ± 1.15a	15.2 ± 0,81a	2.09 ± 0.89cd	22.5 ± 1.47cd	75.4 ± 1.62a
C2	43.9 ± 1.73c	56.1 ± 1.73b	0.00d	0.00d	0.00e	0.00d
T1	23.6 ± 1.91d	71.5 ± 1.49a	4.9 ± 1.24c	60.5 ± 3.71a	39.5 ± 3.71b	0.00d
T2	57.4 ± 1.98b	42.6 ± 1.98c	0.00d	18.9 ± 0.88b	81.1 ± 0.88a	0.00d
T3	50.5 ± 1.22bc	49.5 ± 1.22bc	0.00d	56.8 ± 3.53a	43.2 ± 3.53b	0.00d
T4	42.7 ± 1.56c	47.5 ± 1.39bc	9.8 ± 1.09b	15.4 ± 1.07b	42.6 ± 0.66b	42.0 ± 0.30c
T5	41.9 ± 1.42c	58.1 ± 1.42b	0.00d	3.6 ± 1.01c	37.0 ± 2.37bc	59.5 ± 2.45b
T6	72.8 ± 2.27c	27.2 ± 1.27d	0.00d	3.7 ± 1.80c	17.5 ± 1.10d	78.9 ± 1.23a
CV%	8.42	7.08	22.13	20.41	13.87	9.46

* C1 = control 1: direct seed germination, no PVS2, no phloroglucinol (PG), no NL; C2 = control 2: no PVS2, no (PG), direct immersion in NL; T1 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 60 min; T2 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 120 min; T3 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 180 min; T4 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 60 min; T5 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 120 min; T6 – (2.0 M glycerol + 0.4 M sucrose 30 min) + PVS2 + PG 1% 180 min.

** Values are the means of 5 repetitions with 600 seeds per treatment, being 5 repetitions.

Means followed by the same letter within columns are not significantly different by the Tukey's test at 5% probability.

For *E. ciliare*, no protocorms developed under any of the different stages for C2 (Tab. 3). Protocorms reached stage P3 under T4 (42.0%), T5 (59.5%), and T6 (78.9%), while C1 had 75.4% protocorms in P3 (Tab. 3). The percentage of protocorms in P3 for T6 was not significantly different from C1 (Tab. 3).

Discussion

When developing protocols for orchid seed cryopreservation, an assessment of seed characteristics is important, particularly due to the extensive variability within the Orchidaceae. The values for seed length assessed for both *E. cordigera* (0.43 mm) and *E. ciliare* (0.42 mm) in this study fall within the range cited by Arditti and Ghani (2000), whereby the length of the orchid seeds can range from 0.05 to 6.0 mm.

In addition, the assessment of percent seed viability (TTC test) and how it relates to percent germination is of great relevance for the proper evaluation of success in cryopreservation protocols. In this study, the TTC test proved to correlate closely with germination.

Viability of seeds is also affected by the period of seed storage and depends on their intrinsic characteristics, associated with biotic and abiotic components of their environment (Merritt *et al*. 2014). In this study, the 3 months storage on refrigerator for *E. cordigera* seeds resulted in higher viability and germination, as compared to 15 months storage for *E. ciliare* seeds (Tab. 1).

E. cordigera seeds cryopreserved with phloroglucinol 1% in PVS2 solution for 60 min (T4) had the highest percentages for viability and germination after recovery from LN. However, they were not significantly different from other treatments, except for T6 (2.0 M glycerol + 0.4 M sucrose 30 min; PVS2 + PG 1% 180 min), which had significantly lower viability and germination. The reduced viability and germination of seeds under T6 may be indicative of the toxicity due to longer exposure of seeds (180 min) to the cryoprotectant solution. Cryoprotectant solutions, such as PVS2 may be toxic and cause osmotic stress, leading to cell death or morphological changes in the seedlings (Sakai 1995). It is also important to note that, although viability and germination percentage values for *E. cordigera* were lower for T6, they can still be considered successful as both viability and germination after LN represented about 89% and 85% of the initial viability and germination, respectively (Tab. 2).

In our study, *E. ciliare* seeds showed best viability and germination under the longest period of exposure to PVS2 (T6) prior to immersion in

LN, in comparison to *E. cordigera* seeds. Viability and germination were significantly lower for all treatments as compared to the initial viability and germination values. The best values under T6 represented 45.8% and 44.1% of the initial viability and germination. For *Dendrobium* hybrid seeds with 12% water content, the highest germination (58%) was observed for seeds under 180 min of PVS2 exposure (Galdiano Jr. *et al*. 2014). Similarly, *Vanda coerulea* seeds with 33% water content reached 67% germination when submitted to PVS2 for 70 min (Thammasiri & Soamkul 2007). The results observed for *E. ciliare* seeds (20% water content) submitted to cryopreservation reinforce the positive effect of dehydration provided by the vitrification solution PVS2 during the period of 180 min. Exposure of seeds to PVS2 for a period of time provides sufficient dehydration and consequently cryoprotection for vitrification when immersed in LN, allowing good seed germination after cryopreservation (Vendrame *et al*. 2007).

In contrast, when seeds of both species were immersed directly in LN without cryoprotectant (C2), viability and germination were significantly lower than all treatments. Particularly for *E. ciliare*, seeds did not survive direct immersion in LN without PVS2, showing that the use of vitrification solutions for this species, such as PVS2 prior to cryopreservation is therefore a needed approach for the success of seed germination, as demonstrated by Sakai *et al*. (1990). This result can be attributed to the water content of *E. ciliare* seeds. The lethality of seeds immersed in liquid nitrogen without cryoprotection was also observed in *Vanda coerulea*, with 33% of water content (Thammasiri & Soamkul 2007).

The species-specificity is also evident by the results from different studies. Seeds cryopreserved from nine species of Florida native orchids recovered and germinated successfully after direct immersion in LN without exposure to cryoprotectants (Hughes & Kane 2018).

In our study, the direct immersion of *E. cordigera* seeds in LN resulted in about 27% viability and 31% germination, and the means of viability and germination were significantly higher for the treatments that exposed the seeds to PVS2 prior to cryopreservation. Similar results were reported for *Angraecum magdalenae* seeds, which still showed 40% germination after direct immersion to LN, compared to 92% germination when pre-treated with PVS2 for 30 min prior to cryopreservation (Schofield *et al*. 2018). Although the values of viability and germination percentages in cryopreservation of seeds without cryoprotection (C2) were significantly lower than the treatments with cryoprotection, results are considered satisfactory. This is because a single orchid capsule can contain hundreds of thousands to millions of seeds (Arditti & Ghani 2000), thus smaller percentages (20–30%) still represent large numbers of seeds.

In this study, phloroglucinol (PG) provided significantly higher viability and germination when combined with PVS2 for both species. The use of PG in cryopreservation protocols has been reported to improve recovery, germination and survival in several orchid species and hybrids, such as *Dendrobium nobile* protocorms (Vendrame and Faria, 2011)*, Dendrobium* hybrid seeds and protocorms (Galdiano Jr. *et al*. 2012), *Oncidium flexuosum* seeds (Galdiano Jr. *et al*. 2013), *Cattleya walkeriana* seeds (Galdiano Jr. *et al*. 2017) and *Catasetum atratum* seeds (Suzuki *et al*. 2018). Phloroglucinol also has antioxidant properties that may have contributed to reducing oxidative stress in cells of germinating seeds (Benson & Bremmer 2004).

Although protocorms of both species were morphologically similar and normal in appearance, differences in development were observed. Protocorms of *E. cordigera* showed best development under T4, likely because it was the best cryopreservation treatment returning the highest viability and germination percentages. Protocorms in T4 went through all three stages of development, showing the highest percentage of seedling formation (P3). The treatment 1 (T1), showed half of seedlings in stage P3, compared to T4. No other treatment reached stage P3. Considering the only difference between T1 and T4 was the addition of 1% PG to the PVS2 solution, this result reinforces the positive effect of PG in improving not only germination after LN, but also subsequent development of protocorms into seedlings, and such benefits have been reported previously. Vendrame and Faria (2011) showed significantly improvement in protocorm development in *Dendrobium nobile* protocorms when 1% PG was added to PVS2. Similarly, Suzuki *et al*. (2018), showed the addition of 1% PG to PVS2 for 10 min improved germination and development of cryopreserved seeds of *Catasetum atratum.*

The development of *E. ciliare* protocorms 45 days after sowing into stage P3 was observed for

treatments T4, T5 and T6, with higher percentage of protocorms developing into seedlings (42 to 79%) as compared to *E. cordigera* (4.9 to 9.8%). The effect of PG added to PVS2 for cryopreservation of seeds was more evident for *E. ciliare* than for *E. cordigera*, as only treatments with PG resulted in protocorm development into stage P3 for *E. ciliare*. Therefore, use of PG was essential for the maintenance of seed viability and for the development of normal protocorms. These results confirm the benefits that PG can confer on plant seeds and tissues, such as protecting cells from oxidative stress, inflammation, and damage from free radicals (Kim and Kim, 2010). It also confirms that results can be species specific, as indicated by Hughes & Kane (2018), since despite the presence of PG in PVS2 for cryopreservation of *E. cordigera* seeds, not all treatments with PG resulted in recovery and development into P3, contrasting the results with *E. ciliare.*

The success of cryopreservation processes involves rigorous control in the dehydration processes, permeability of the cryoprotectant solution and prevention of lesions caused by osmotic stress and toxicity of chemical components of cryoprotective solutions during dehydration (Vendrame *et al*. 2014, 2018). Vitrification is known to provide a suitable transition of intracellular water to an amorphous glass state that prevents ice crystallization and consequently cell damage (Kulus & Zalewska 2014). In the present study, the process of vitrification using cryoprotectants was essential for the cryopreservation and successful recovery of seeds from both orchid species. The evaluation of different exposure times to PVS2 and the assessment of the addition of PG to PVS2 provided the elements for the determination of a cryopreservation protocol for both orchid species.

Our results indicate that the use of PVS2 combined with PG for 60 min should be selected for cryopreservation of *Encyclia cordigera* seeds, while PVS2 with PG for 180 min is the best approach for *Epidendrum ciliare* seeds.

The differences observed between species confirm that protocols for cryopreservation of orchid seeds are species specific and this is an important factor to be considered when developing cryopreservation protocols for other orchid species.

Recovery from cryopreservation requires close observation to the elements involved in the process, including proper dehydration and combination of cryoprotectants, as well exposure to such cryoprotectants to ensure success in seed germination and protocorm development.

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