BRIEF REPORT

Cryopreservation of orchid seeds through rapid and step freezing methods

[version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Ecuador has a great variety of climatic regions that potentiate biodiversity. The family *Orchidaceae* constitutes one of the most important of the country, having identified about 4032 species with a high degree of endemism, therefore the development and research of alternative methods of storage and conservation of species is a strategy of primary interest for researchers and for society in general. In cryopreservation, temperatures reach below -190°C in order to paralyze the chemical reactions and keep the plant material viable for long periods. The present research focuses on the development of protocols for cryopreservation of seeds, aimed at the preservation of biodiversity, focusing on the family *Orchidaceae*, for the subsequent generation of a seed bank. The assays were performed on seeds of *Epidendrum quitensium, Sobralia rosea*, and *Epidendrum anderssonii*. Two freezing rates were tested: rapid freezing at -196°C; and step freezing at -22°C, -60°C to 196°C, further analyzed four combinations from Dimethylsulfoxide DMSO, glycerol and sucrose (DMSO 1M; DMSO 1M + glycerol 1M; DMSO 1M + sucrose 1M; DMSO 1M + glycerol 0,5M + sucrose 0,5M). The best results were obtained both in rapid and stepped freezing without the use of cryo-protective substances, by introducing the seeds directly into liquid nitrogen. Species of the genus *Epidendrum* presented a more efficient response in comparison to *Sobralia*. The viability of the seeds was evaluated by the tetrazolium test.

Keywords

Orchidaceae, Epidendrum, Sobralia, seeds, cryoconservation, liquid nitrogen, Tetrazolium.

Open Peer Review

Approval Status ? ? ✓

	1	2	3
version 1 20 Feb 2018	? view	? view	✓ view

1. **Alzbeta Novotna**, University of Gdańsk, Gdańsk, Poland
2. **Song-Jun Zeng**, Chinese Academy of Sciences, Guangzhou, China
3. **Éva Borbélyné Hunyadi**, Research Institute for Organic Agriculture (ÖMKi), Budapest, Hungary

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: Cerna M: Conceptualization, Investigation, Methodology, Writing – Original Draft Preparation; **Valdivieso P**: Investigation, Methodology; **Cella R**: Investigation, Methodology, Writing – Original Draft Preparation; **Mátyás B**: Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; **Aucapiña C**: Investigation, Methodology, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

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Introduction

The Republic of Ecuador is located on the South American continent. From north to south the country is crossed by the Andes mountain range and has four climatic regions: Coast, Andes, Amazon and the Insular region[1]. Its position in the middle of the world, the luminous intensity, the ocean currents and the different altitudes produce 82 types of ecosystems (see Ministry of Environment document on ecosystems in Ecuador) There is a great variety of climatic regions that have an important effect in the diversification of plant formations[2]. Concerning the *Orchidaceae* family, in Ecuador as of 2010, 4032 species of orchids have been identified, of which 1714 (42.5%) are endemic[3]; 4.5% of the orchids of the planet are found in Ecuador. Seed banks allow the conservation of the biodiversity *ex situ* and prioritize species used for food, medicine and those in danger of extinction. *Orchidaceae* is a large family with many endangered species and all of them are included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) I and II[4]. Cryopreservation is an efficient strategy to safeguard these species, but unfortunately, orchid seeds have short lifetimes[5]; the longevity depends on the moisture content and storage temperature, so it is necessary to experiment with efficient storage systems for each species[5]. The advantages of cryopreservation are: storage for an indefinite period, genetic stability of the individuals, reduced infrastructure, can have independent energy and the stored genetic material does not require manipulation[6].

Therefore, the objective of this research was to define protocols for cryopreservation of orchid seeds, in order to install a seed bank that promotes the conservation of vulnerable species.

Methods

Collection of biological material

The collection of plant material was made through the authorization of the Ministry of Environment of Ecuador No. 17-2011-Investigación-B- DPMS/MAE,FloraX, N0. 08−2013−0869−*I C*$_{\mu}$*AU−F LO−DAPI −UNO−MAE* and the Botanical Garden "Orquídeas de Sarina" patent No. 006-2015- FLO-DPAP- MA.

The cryopreservation tests were developed with the seeds of 3 species: *Epidendrum quitensium Rchb.f.*, *Sobralia rosea Poepp. & Endl.* and *Epidendrum anderssonii Hágsater & Dodson* (Figure 1). The cryopreservation tests were developed with 3 species:

- 2392 Epidendrum quitensium Rchb.f., (0° 17'52.1"N 78° 22'33.3"W 3200 msnm)
- 2420 Sobralia rosea Poepp.& Endl. (0°52'11.8"N 78° 26'53.8"W 600 msnm)
- 2706 Epidendrum anderssonii Hágsater&Dodson (0° 50'36.2"N 78° 25'01.5"W 1200 msnm)

The species pertain to three different altitudes and were selected from many sources and have capsules with viable seeds. The seeds collected from the forest were stored in an absorbant paper bag with respective codes for the plant, after they were stored in a Ziplock bag with rice of 12% humidity.

Freezing speed

Two types of freezing were tested, suggested according to Mroginski *et al*[7]. The sample units had 0.2 g of seeds stored in cryo tubes (091.11.102, ˙ISOLAB, Wertheim, Germany) of 2 ml. Steps of freezing: freezing was carried out in the following sequence, 0°C for 1 hour by placing the samples in an refrigerator (Electrolux, Stockholm, Sweden), -22°C for 1 hour placing the seeds in a freezer (Selecta Templow, Barcelona, Spain), - 60°C for 1 hour inserting the seeds in an ultra low temperature freezer (New Brunswick Scientific, Edision, NJ, USA), then the seeds were held at 196°C by submerging the samples in liquid nitrogen contained in a thermal container. Finally the samples were placed in racks and stored in a thermal tank (STATE-BOURNE biorack 5400, Washington, UK). Rapid freezing: the samples were placed directly in liquid nitrogen at 196°C by immersion using a procedure similar to that used in steps of freezing. In addition, four combinations of cryo preservatives were analyzed: 1- DMSO 1M (Fisher Scientific, Hampton, NH, USA); 2-DMSO 1M (Fisher) – glycerol 1M; 3- DMSO 1M (Fisher) – sucrose 1M; 4- DMSO 1M (Fisher) – glycerol 0.5M – sucrose 0.5M (Fisher) (Table 1).

Figure 1. Orchids used for cryopreservation tests. A) Epidendrum quitensium, **B**) Sobralia rosea, **C**) Epidendrum anderssonii.

Table 1. System design and freezing seed symbology used for cryoprotective substances and their concentrations. M: molar.

TYPE OF FREEZING			
GRADUAL (P)		Rapid (F)	
0° ___ -22° ___ -60° ___ -196		-196°	
CRYOPRESERVANTES	**SYMBOL**	**CONCENTRATION**	
NONE	N		
DMSO	D	1M	
GLYCEROL	G	1M	0,5M
SUCROSE	S	1M	0,5M
COMBINATION OF CRYOPRESERVANTES			
NONE			N
DMSO 1M			D
DMSO 1M	GLYCEROL 1M		DG
DMSO 1M	SUCROSE 1M		DS
DMSO 1M	GLYCEROL 0,5M	SUCROSE 0,5M	DGS

Seed viability

Seed viability was tested after freezing. Briefly, 5mg of seeds was added to 1.5 ml of 10% sucrose solution and left at 25° C for 24 hours, the seeds were washed with water and 1ml of triphenyl tetrazolium chloride solution (TTC, 1%) (Sigma-Aldrich, St Louis, MI, USA) was added, and then incubated at 40° C for 24 hours. Finally, the seeds were washed with sterilised water and observed under the microscope with a 4x lens (MC100Led, MI-CROS, St. Veit/Glan, Austria). The process for calculating the TTC method was carried out as follows: -Observe the seeds in microscope using lense 4X. -Identify viable seeds and non viable seeds. -Use cross multiplication to determine the average of viability of all seeds.

Statistical analysis

The experimental design 2x5 with three repetitions was applied to analyse the freezing methods (Table 2). The results were analyzed by unidirectional ANOVA with 95% confidence. To determine the best treatments the Duncan test was used. This analysis was carried out with RStudio 3.1 (package: Agricolae).

Results

The seeds were considered viable when red coloration of the embryo was observed[8] (Figure 2).

According to the data obtained (Table 3, Figure 3), there is a significant difference in the results when comparing the data between the species and between the treatments. According to the Duncan test, the best treatments were rapid freezing and step freezing without the use of cryopreservatives. The least efficient treatment was step freezing with the use of DMSO as a cryopreservant (Table 4). The species *Epidendrum quitensium* and *Epidendrum anderssonii* showed better results (Figure 4).

Dataset 1. TTC-stained seeds subjected to the "Rapid" cryopreservation process: Epidendrum quitensium

http://dx.doi.org/10.5256/f1000research.13609.d194564

Dataset 2. TTC-stained seeds subjected to the "Rapid" cryopreservation process: Sobralia rosea

http://dx.doi.org/10.5256/f1000research.13622.d194234

Dataset 3. TTC-stained seeds subjected to the "Rapid" cryopreservation process: Epidendrum anderssonii

http://dx.doi.org/10.5256/f1000research.13622.d194235

Dataset 4. Percentage for seed viability calculations

http://dx.doi.org/10.5256/f1000research.13622.d194236

Table 2. Experimental design, testing orchid seeds cryopreservation - design 2x5 with three repetitions, Symbols (N: none, D: DMSO, G: glycerol, S: sucrose, P: Freeze steps, R: Rapid).

	STEP (P)			FAST (R)		
	P1	P2	P3	R1	R2	R3
N	PN1	PN2	PN3	RN1	RN2	RN3
D	PD1	PD2	PD3	RD1	RD2	RD3
DG	PDG1	PDG2	PDG3	RDG1	RDG2	RDG3
DS	PDS1	PDS2	PDS3	RDS1	RDS2	RDS3
DGS	PDGS1	PDGS2	PDGS3	RDGS1	RDGS2	RDGS3

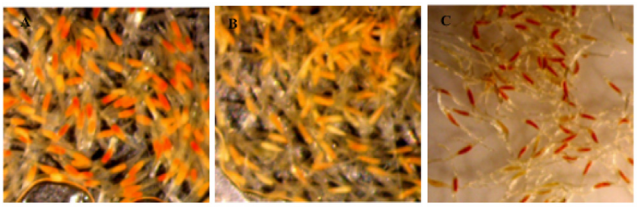

Figure 2. TTC-stained seeds subjected to the "stepped" cryopreservation process without any cryopreservation substances. Viable seeds (dark red embryos) and non-viable (pale embryos). A) *Epidendrum quitensium*, B) *Sobralia rosea*, C) *Epidendrum anderssonii*.

Table 3. Cryopreservation of orchid seeds. Values represent percentage of viability assessed by the TTC method, N: cryo preservative; D: DMSO; S: sucrose; G: glycerol.

		Step				
CI	**Species**	**N**	**D**	**DG**	**DS**	**DGS**
2392	*Epidendrum quitensium*	83.20	54.87	83.20	46.36	47.01
2420	*Sobralia rosea*	55.93	12.29	10.72	19.34	18.88
2706	*Epidendrum anderssonii*	93.50	40.88	51.81	62.84	55.94
		Rapid				
2392	*Epidendrum quitensium*	74.79	48.29	62.87	52.79	51.73
2420	*Sobralia rosea*	65.60	50.15	55.04	52.72	52.52
2706	*Epidendrum anderssonii*	84.49	18.57	41.07	63.08	60.10

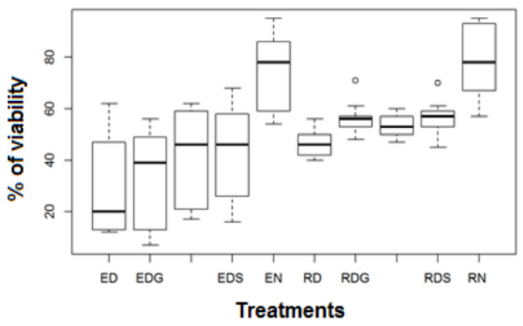

Figure 3. Seed cryopreservation: variability by treatment. Results obtained using the Tukey test.

Table 4. Duncan test groups obtained after cryopreservation test. Results are given as orchid seed viability percentage; a, b, c and d, indicate groups with statistical significance. Classification was made under an alpha of 0.01, and 78 degrees of freedom for error. Symbols (treatment): N: none, D: DMSO, G: glycerol, S: sucrose. Symbols (types of freezing) P: Freeze steps, R: Rapid.

#	Treatment	Mean					
1	RN	78.00	a				
2	PN	74.66	a				
3	RDG	56.66		b			
4	RDS	56.00		b			
5	RDGS	53.33		b	c		
6	RD	46.55		b	c		
7	PDS	43.00			c	d	
8	PDGS	41.88			c	d	
9	PDG	33.33				d	e
10	PD	28.55					e

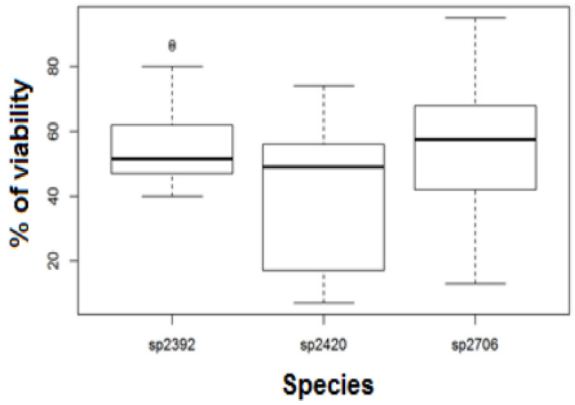

Figure 4. Cryopreservation of seeds: variability by species. Results obtained using the Duncan test, sp2706 (T57.2), sp2392 (T 57.03), sp2420(T39.37).

Discussion

Currently, cryopreservation is a safe and cost-effective option for the conservation of endangered species[9]. In the present investigation, a protocol was developed for cryopreservation of orchid seeds that provides a high percentage of viability, is easy to apply and economical. The seeds of orchids frozen at -196°C can be kept alive with a moisture content of 12% and do not require cryo-protective substances, confirming what is described by Iriondo *et al.* and others[10,11]. The use of cryopreservatives is recommended for seeds with a high moisture content, as stated by Reed and others[12–14]. Furthermore, Harding[15] states that it is necessary to demonstrate the genetic stability of plants regenerated from cryopreserved plant material to approve their release and reintroduction into the environment; but to date, there have been no reports showing changes at the phenotypic, biochemical, chromosomal or molecular levels attributed to storage systems by cryoconservation[14]. The cryoconservation method that gave the best results was the "Rapid" freezing without the addition of any cryopreservative substance.

Data availability

Dataset 1: TTC-stained seeds subjected to the "Rapid" cryopreservation process: *Epidendrum quitensium* 10.5256/f1000research.13622.d194233[15]

Dataset 2: TTC-stained seeds subjected to the "Rapid" cryopreservation process: *Sobralia rosea* 10.5256/f1000research.13622.d194234[16]

Dataset 3: TTC-stained seeds subjected to the "Rapid" cryopreservation process: *Epidendrum anderssonii*. 10.5256/f1000research.13622.d194235[17]

Dataset 4: Percentage for seed viability calculations 10.5256/f1000research.13622.d194236[18]

Competing interests

No competing interests were disclosed.

Grant information

The author(s) declared that no grants were involved in supporting this work.

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PubMed Abstract

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Data Source

16. Cerna M, Valdiviezo P, Cella R, *et al.*: **Dataset 2 in: Cryopreservation of orchid seeds through rapid and step freezing methods.** *F1000Research.* 2018.
Data Source

17. Cerna M, Valdiviezo P, Cella R, *et al.*: **Dataset 3 in: Cryopreservation of orchid seeds through rapid and step freezing methods.** *F1000Research.* 2018.
Data Source

18. Cerna M, Valdiviezo P, Cella R, *et al.*: **Dataset 4 in: Cryopreservation of orchid seeds through rapid and step freezing methods.** *F1000Research.* 2018.
Data Source

Open Peer Review

Current Peer Review Status: ? ? ✓

Version 1

Reviewer Report 11 July 2018

https://doi.org/10.5256/f1000research.14799.r33998

✓ **Éva Borbélyné Hunyadi**
Research Institute for Organic Agriculture (ÖMKi), Budapest, Hungary

In the paper entitled "Cryopreservation of orchid seeds through rapid and step freezing methods" written by Marco Cerna *et al*. two types of freezing technique were tested by the seeds of 3 species:
Epidendrum quitensium Rchb.f., Sobralia rosea Poepp. & Endl. and Epidendrum anderssonii Hágsater & Dodson.

The authors present step freezing and rapid freezing methods for cryopreservation purposes. There was a significant difference in the results between the species and between the treatments. The cryoconservation method that provided the best results was the "Rapid" freezing, without the addition of any substance.

The introduction is well structured, helping the readers to understand the issue the method tries to solve. Considering that it is a research note the methodology is very detailed.

Nevertheless, in my opinion it is important to highlight the viability of the developed method on a wider spectrum if possible. In the current form of the Discussion, only general statements can be found regarding the viability of the developed method.

A more concrete concluding sentence is missing that could answer the following question:

For what kind of other plants, could this method be useful?

If the developed method can be applied "only" for orchid seeds, a concluding sentence should be placed in the Discussion, stating the limiting factors (in terms of species) of the method. Although in this study only 3 species were examined, I believe that its important to inform the readers about the authors' recommendation regarding the potential usefulness in other species.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 April 2018

https://doi.org/10.5256/f1000research.14799.r32906

?

Song-Jun Zeng
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The authors described a protocol for cryopreservation of seeds of three tropical orchids in liquid nitrogen (LN). They tested rapid and progressive cooling with the controlled temperature at 0°C, -22°C, -60°C and -196°C. They also included the application of three different cryoprotectants such as DMSO, glycerol and sucrose. The viability of tested seeds was checked using tetrazolim test. The protocol might have potential for the cryopreservation of these orchids. However, the manuscript cannot be accepted to publish at present and need major revision for the following main reasons:.

1. Abstract: Authors could not bring the conclusion 'Species of the genus *Epidendrum* presented a more efficient response in comparison to *Sobralia*.', because only one species was be tested in the this manuscript.

2. Introduction: Orchidaceae is a large family with many endangered species and all of them are included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) I **and** II.
And should be revised as 'or'.
Current research of orchid Cryopreservation should be introduced in the section.

3. Methods: DPMS/MAE,FloraX the behind of ',' should have a blank.

4. *Epidendrum quitensium* ***Rchb.f.***, *Sobralia rosea* ***Poepp. & Endl.*** and *Epidendrum anderssonii* Hágsater & Dodson
Rchb.f., Poepp. & Endl. and *Hágsater & Dodson* should not be italic.

5. 2392 **Epidendrum quitensium** Rchb.f., 2420 **Sobralia rosea** Poepp.& Endl., **Epidendrum anderssonii** Hágsater & Dodson
Epidendrum quitensium, Sobralia rosea, Epidendrum anderssonii should be italic.

6. Figure 1: Epidendrum quitensium, Sobralia rosea, Epidendrum anderssonii should be italic.
7. Figure 2: A) *Epidendrum quitensium*, B) *Sobralia rosea*, C) *Epidendrum anderssonii*
A, B, C should be labelled in Photos.

8. Figure 3: What is ED, EDD.....what is the meaning of “E”?

9. sp2706 (T57.2), sp2392 (T 57.03), sp2420(T39.37), should be replaced by Latin name of orchids species.

10. Discussion: The discussion should be rewritten. There were few relevant literatures of orchid Cryopreservation were cited in this manuscript. There have lots of related literatures for orchid Cryopreservation at present, which should be discussed and what is the innovation of the manuscript?

11. All the source data should be executed by statistical analysis.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 28 March 2018

https://doi.org/10.5256/f1000research.14799.r31828

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Alzbeta Novotna
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The authors describe in the presented manuscript titled “Cryopreservation of orchid seeds through rapid and step freezing methods” a new protocol for cryopreservation of seeds of three tropical orchids in liquid nitrogen (LN). They tested rapid and progressive cooling with the controlled temperature at 0°C, -22°C, -60°C and -196°C. They also included the application of three different cryoprotectants such as DMSO, glycerol and sucrose. The viability of tested seeds was checked using tetrazolim test. Red coloured embryos were considered as the main indicator of the preserved viability of the tested seeds. I suppose that this work brings a novelty to a cryoscience research, especially due to the geographical origin of the studied material. Neotropical Orchidaceae should be prioritized in various research studies for their ecological vulnerability and/or a shortage of knowledge. Especially, the research achievements from such country as Ecuador (the orchid biodiversity hotspot), are important for science. However, numerous modifications and a deep correction of the text should be accomplished.

1. English correction is strongly recommended throughout the whole text. There are many errors in English grammar in the current form of the manuscript, which cause huge difficulties in text reading.
2. Abstract: The authors should be focused on the topic of cryopreservation. The first two

sentences in the abstract are out of topic. Moreover, the number of the orchid species (provided number 4032) is not correct. The authors should consider to check the work of D. Neill published in 2015 (¿Cuantas especies nativas de plantas vasculares hay en Ecuador?; Revista Amazónica Ciencia y Tecnología) and Cataloque of the Vascular Plants of Ecuador available on www.tropicos.org, for example.

3. Abstract, sentence “In cryopreservation, temperatures reach below -190°C” should be rephrased because the cryostorage is a long-lasting preserving of studied material at temperature of -80°C, -130°C upto -196°C, which is the temperature of the liquid nitrogen.
4. Abstract, the authors should rephrase the sentence “The present research focuses on……” because of repetition of the verb to focus. Instead of “…for a subsequent generation of a seed bank” to use “for future seed collection” for example.
5. Abstract, “the use of cryo-protective substances”, better to use the term „cryoprotectants“.
6. Introduction; The first half of the abstract is out of the topic of cryopreservation. The authors should be focused on this topic. Moreover, the authors should provide much more citations regarding this topic and discuss shortly the history of cryopreservation of seeds with more interest in orchid seeds. There are many available papers regarding this topic.
7. Methods; The first paragraph should be included in “Acknowledgement” at the end of the paper, not in methods. However, each part of the Methods should be provided with a number, e.g. 1. 1 Collection of the seed material. The sentence “The cryopreservation test were developed with 3 species” is repetition of the first sentence of this paragraph.
8. The authors should use "a.s.l."as the abbreviation for above sea level; the provided abbreviation "msnm" is not understandable. The part of the sentence” The species pertain to three different altitudes….. „ is a repetition of the information written before. Authors write “…..were selected from many sources“, what does it mean? The authors should provide more detailed information about the sources. Then, the part of the sentence …“and have capsules with viable seeds” does not bring the clear information. Were the capsules opened during collection or not? When did the authors collect the seeds (the date)?
9. Methods; Freezing speed. Better to rewrite the title, e.g. Cryopreservation of the seeds
10. This part should be shorten. Besides, the citation of the first sentence Mroginski et al. does not correspond with the number 7. Totally different citation is written under this number. The authors should check the citations of provided literature in the text. Moreover, the authors should provide the information about **duration** of cryostorage. This very important information is missing.
11. In the paragraph “Freezing speed” the authors provide information about placing the tested orchid seeds under the temperature 0°C in a refrigerator. However, this fact is not written in the abstract.
12. The authors should eliminate “…., contained in a thermal container”.
13. The sentence “Finally the samples were places in racks and store in thermal tansk……” should be eliminated, because it is not understandable.
14. Freezing speed; In addition,….should be eliminated because the application of cryoprotectants is one of the main objectives of this research paper. It is not something additional.
15. Why did the authors not test application of sucrose and glycerol individually?
16. The authors should clearly demonstrate, which treatment is considered as a control.
17. Table 1 should be deleted, because all the information given in this table is written in the text.
18. Figure 1; Title should be improved, e.g. “Orchid species used in this study”.

19. The paragraph of “Seed viability” should be eliminated. Instead just one sentence with a citation of the work, where this method was used as first, should be placed at the end of the previous paragraph.
20. Statistical analysis; The authors should explain better of the meaning “The experimental design 2x5”. It is not clear from the written text.
21. Results; The sentence “The seeds were considered viable when red.....” is actually the part of Material and methods.
22. Results; this part should be re-written. The information should be provided more clearly.
23. Table 3; What is CI? Better to use term “Gradual” instead of “step”. The abbreviations of each treatment are already provided in Material and Methods.
24. Figure 3; Authors should provide information about the meaning of “E” (in e.g. ED, EDG) and “R” (in e.g. RD, RDG).
25. Figure 4; Authors should provide information about the meaning of sp2392, sp2420 and sp2706. It is not clear.
26. Discussion; First, the obtained main result should be provided and discussed with much more available literature. Especially, the authors should compare their findings with achievements in other studies. The provided references are very limited. The works dealing with cryostorage of seeds of other plant families should be included. The given number of cited paper in discussion does not correspond to the number provided in the References.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Isolation, molecular determination and cryopreservation of orchid mycorrhiza fungi and associated bacteria. Experienced with orchid seed banking techniques.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.