ReSEARCH NOTE

Cryopreservation of orchid seeds through rapid and step freezing methods [version 1; referees: awaiting peer review]

Marco Cerna¹, Paulina Valdivieso², Rino Cella³, Bence Mátyás ⁴, Cristina Aucapiña¹

¹Carrera de Biotecnología de los Recursos Naturales, Universidad Politécnica Salesiana, Quito, Ecuador
²Jardín Botánico “Orquídeas de Sarina”, Quito, Ecuador
³Laboratorio de biología molecular vegetal, University of Pavia, Pavia, Italy
⁴Grupo de Investigación Mentoría y Gestión del Cambio, Universidad Politécnica Salesiana, Cuenca, Ecuador

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.

Corresponding author: Bence Mátyás (bmatyas@ups.edu.ec)

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.

How to cite this article: Cerna M, Valdivieso P, Cella R et al. Cryopreservation of orchid seeds through rapid and step freezing methods [version 1; referees: awaiting peer review] F1000Research 2018, 7:209 (doi: 10.12688/f1000research.13622.1)

Copyright: © 2018 Cerna M et al. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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

**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. 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. Concerning the *Orchidaceae* family, in Ecuador as of 2010, 4032 species of orchids have been identified, of which 1714 (42.5%) are endemic; 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. Cryopreservation is an efficient strategy to safeguard these species, but unfortunately, orchid seeds have short lifetimes; the longevity depends on the moisture content and storage temperature, so it is necessary to experiment with efficient storage systems for each species. 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.

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 CAU-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 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. 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 (STATEBOURNE 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.](image)

<table>
<thead>
<tr>
<th>TYPE OF FREEZING</th>
<th>Rapid (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0° ___ -22° ___ -60° ___ -196</td>
<td>-196°</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CRYOPRESERVANTES</th>
<th>SYMBOL</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>D</td>
<td>1M</td>
</tr>
<tr>
<td>GLYcerol</td>
<td>G</td>
<td>1M, 0.5M</td>
</tr>
<tr>
<td>SUCROSE</td>
<td>S</td>
<td>1M, 0.5M</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>COMBINATION OF CRYOPRESERVANTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
</tr>
<tr>
<td>DMSO 1M</td>
</tr>
<tr>
<td>DMSO 1M GLYCEROL 1M</td>
</tr>
<tr>
<td>DMSO 1M SUCROSE 1M</td>
</tr>
<tr>
<td>DMSO 1M GLYCEROL 0.5M</td>
</tr>
<tr>
<td>DMSO 1M SUCROSE 0.5M</td>
</tr>
</tbody>
</table>
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 using 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 (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).

<table>
<thead>
<tr>
<th>Table 2.</th>
<th>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).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STEP (P)</td>
</tr>
<tr>
<td>P1</td>
<td>P2</td>
</tr>
<tr>
<td>N</td>
<td>PN1</td>
</tr>
<tr>
<td>D</td>
<td>PD1</td>
</tr>
<tr>
<td>DG</td>
<td>PDG1</td>
</tr>
<tr>
<td>DS</td>
<td>PDS1</td>
</tr>
<tr>
<td>DGS</td>
<td>PDGS1</td>
</tr>
</tbody>
</table>

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>
<thead>
<tr>
<th>Step</th>
<th>CI</th>
<th>Species</th>
<th>N</th>
<th>D</th>
<th>DG</th>
<th>DS</th>
<th>DGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid</td>
<td>2392</td>
<td>Epidendrum quitensium</td>
<td>83.20</td>
<td>54.87</td>
<td>83.20</td>
<td>46.36</td>
<td>47.01</td>
</tr>
<tr>
<td></td>
<td>2420</td>
<td>Sobralia rosea</td>
<td>55.93</td>
<td>12.29</td>
<td>10.72</td>
<td>19.34</td>
<td>18.88</td>
</tr>
<tr>
<td></td>
<td>2706</td>
<td>Epidendrum anderssonii</td>
<td>93.50</td>
<td>40.88</td>
<td>51.81</td>
<td>62.84</td>
<td>55.94</td>
</tr>
</tbody>
</table>

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

Table 3. Cryopreservation of orchid seeds. Values represent percentage of viability assessed by the TTC method. N: cryopreservative; D: DMSO; S: sucrose; G: glycerol.
Discussion
Currently, cryopreservation is a safe and cost-effective option for the conservation of endangered species. 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. The use of cryopreservatives is recommended for seeds with a high moisture content, as stated by Reed and others. Furthermore, Harding 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. 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.d19423
Dataset 2: TTC-stained seeds subjected to the “Rapid” cryopreservation process: Sobralia rosea 10.5256/f1000research.13622.d19423
Dataset 3: TTC-stained seeds subjected to the “Rapid” cryopreservation process: Epidendrum anderssonii 10.5256/f1000research.13622.d19423
Dataset 4: Percentage for seed viability calculations 10.5256/f1000research.13622.d19423

Competing interests
No competing interests were disclosed.

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

References

Reference Source

Reference Source

Reference Source

Reference Source

PubMed Abstract

PubMed Abstract

PubMed Abstract

PubMed Abstract

Data Source

Data Source

Data Source

Data Source
The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com