

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Cryopreservation of orchid mycorrhizal fungi: A tool for the conservation of endangered species

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/71282> since 2016-08-05T10:06:05Z

Published version:

DOI:10.1016/j.mimet.2013.03.003

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in JOURNAL OF MICROBIOLOGICAL METHODS, 93(2), 2013, 10.1016/j.mimet.2013.03.003.

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), 10.1016/j.mimet.2013.03.003

The publisher's version is available at:

<http://linkinghub.elsevier.com/retrieve/pii/S016770121300095X>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/2318/71282>

Cryopreservation of orchid mycorrhizal fungi: A tool for the conservation of endangered species

Enrico Ercole^{a, 1}, Michele Rodda^{b, 1}, Matteo Molinatti^a, Samuele Voyron^a, Silvia Perotto^{a, c}, Mariangela Girlanda^{a, c, *}

Abstract

The effectiveness of cryopreservation at $-80\text{ }^{\circ}\text{C}$ on orchid mycorrhizal fungi was assessed by testing the symbiotic ability of ten fungal isolates following cryo-storage for 10–24 months. The results obtained prove the efficacy of the method, thus providing a valuable tool for *ex situ* conservation.

Keywords

- Orchid mycorrhiza;
- Orchid germination;
- *Serapias*;
- *Tulasnella*;
- *Ceratobasidium*

Orchidaceae are a focus plant group for conservation, with more orchid species listed as threatened on the International Union for Conservation of Nature (IUCN) Red List than species from any other plant family ([IUCN, 1999](#)). As a result of overexploitation and habitat loss and fragmentation, drastic losses in orchid populations and diversity have already been documented ([Sosa and Platas, 1998](#), [Cozzolino et al., 2003](#), [Coates and Dixon, 2007](#) and [Juillet et al., 2007](#)).

Conservation measures need to take into account the peculiar biology of orchids. These plants rely on pollinators for reproduction and symbiotic fungi for successful seedling development ([Waterman and Bidartondo, 2008](#)). Germination of the minuscule, dust-like orchid seeds, featuring minimal nutritional reserves, is dependent on colonization by fungal symbionts, which provide not only water and mineral nutrients, but also organic carbon compounds (“mycoheterotrophy”; [Leake, 1994](#)). Association with a compatible fungus is also crucial for the development of the protocorm, that in many orchids is subterranean ([Smith and Read, 2008](#)).

In addition to the efforts for *in situ* conservation, which is essential for the preservation of orchid pollinators ([Kearns et al., 1998](#) and [Vereecken et al., 2010](#)), *ex situ* conservation strategies can be achieved for both the orchids and their symbiotic fungi (e.g. [Pritchard and Seaton, 1993](#), [Cribb et al., 2003](#), [Seaton and Pritchard, 2003](#), [Sommerville et al., 2008](#) and [Swarts and Dixon, 2009](#)). Most of the latter fungi belong to Basidiomycetes, which are often unable to form resistant propagules in pure culture. These fungi, therefore, can be preserved by serial transfers on agar (a labor-intensive procedure that can increase the risk of variation or loss of morphological or physiological features), or by cryopreservation in liquid nitrogen. However, storage in liquid nitrogen is very laborious and expensive for routine use, and facilities are not always available.

We have assessed the effectiveness of cryopreservation at $-80\text{ }^{\circ}\text{C}$ on orchid mycorrhizal fungi, by testing their symbiotic ability after cryo-storage, and by comparing it with the symbiotic ability of the same isolates kept as actively growing cultures in test tubes.

Ten fungal isolates obtained from mycorrhizal roots of Mediterranean terrestrial orchids ([Liebel et al., 2010](#) and [Girlanda et al., 2011](#)) were preserved both as actively growing cultures on 2% Malt Extract Agar (MEA) at $4\text{ }^{\circ}\text{C}$, and cryopreserved at $-80\text{ }^{\circ}\text{C}$ for 10–24 months following [Voyron et al. \(2009\)](#). The ten tested isolates belong to the genera *Tulasnella* and *Ceratobasidium* ([Table 1](#)). Germination ability was tested on seeds of *Serapias vomeracea* collected in Liguria (Italy), dried at room temperature for 2–3 weeks and stored in glass vials on silica gel at $4\text{ }^{\circ}\text{C}$. Both the cryopreserved (C) and the actively growing form (T) of each of the isolates were tested for their ability to induce seed germination. Germination tests were carried out following seed surface sterilization in 1% sodium hypochlorite and 0.1% Tween-20. Sterilized seeds were placed on a piece of filter paper ($1 \times 1\text{ cm}$) positioned at the edge of a 6 cm Petri dish, which was centrally inoculated with a 3 mm \times 3 mm mycelial plug. Five replicates per isolate were set up. Control plates were left uninoculated. Plates were incubated at $20\text{ }^{\circ}\text{C}$ in full darkness and checked for germination after 30 and 45 days of incubation. The percentage of seeds reaching the stage either of swollen seed with rhizoids but without a well-defined apex, or fully developed protocorm (stages 2 and 3, respectively, according to [Clements et al. \(1986\)](#); [Otero et al. \(2004\)](#); [Fig. 1](#)) was calculated. Mann–Whitney U-tests were used to compare the germination ability of the two preservation forms of each isolate.

Table 1.

Fungal isolates tested in this study. Isolate code in the MUT (*Mycotheca Universitatis Taurinensis*) collection, host plants (*Serapias* spp.), collection year, geographical origin, duration of cryopreservation at the time of the seed germination assay, isolate identity as assessed by means of ITS sequence analysis ([Liebel et al., 2010](#) and [Girlanda et al., 2011](#)), and GenBank accession numbers are reported.

Code (MUT)	Host plant	Collection year	Geographical origin	Preservation	Isolate identity	GenBank accession number
4044	<i>S. vomeracea</i>	2006	Liguria	24 months	<i>Tulasnella calospora</i>	JF926513
4047	<i>S. vomeracea</i>	2005	Campania	24 months	<i>Tulasnella</i> sp.	JF926500
4048	<i>S. vomeracea</i>	2005	Liguria	24 months	<i>Tulasnella</i> sp.	JF926506
4049	<i>S. vomeracea</i>	2005	Campania	24 months	<i>Tulasnella</i> sp.	JF926505
4217	<i>Serapias lingua</i>	2007	Sardinia	11 months	<i>Tulasnella</i> sp.	KC525058
4229	<i>S. vomeracea</i>	2006	Liguria	10 months	<i>Tulasnella</i> sp.	JF926519
4233	<i>S. vomeracea</i>	2006	Liguria	10 months	<i>Ceratobasidium</i> sp.	JF912485
4238	<i>Serapias cordigera</i>	2007	Sardinia	10 months	<i>Tulasnella</i> sp.	KC525059

Code (MUT)	Host plant	Collection year	Geographical origin	Preservation	Isolate identity	GenBank accession number
4247	<i>Serapias nurrica</i>	2007	Sardinia	10 months	<i>Tulasnella</i> sp.	KC525057
4249	<i>S. cordigera</i>	2007	Sardinia	10 months	<i>Tulasnella</i> sp.	KC525060

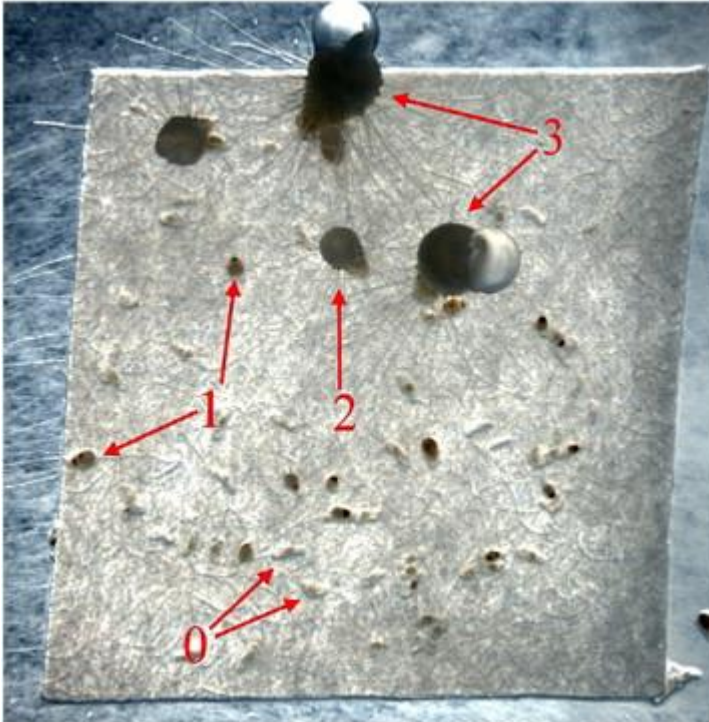


Fig. 1.

Germination stages of *S. vomeracea* seeds. The germination stages ([Clements et al., 1986](#) and [Otero et al., 2004](#)) are indicated as follows: (0) ungerminated seed; (1) swollen seed, without rhizoids; (2) swollen seed with rhizoids but without a well-defined apex; and (3) fully developed protocorm. (The filter paper square is 1 cm × 1 cm).

After 30 days from inoculation, all isolates (but 4049 T) promoted germination of *S. vomeracea* seeds up to stage 2 ([Fig. 2-I](#)). The only significant difference between preservation treatments was observed for isolate 4217, where the C form induced a higher germination rate than the corresponding T form ($16.6 \pm 4.5\%$ and $6.6 \pm 2.7\%$ of tested seeds, respectively). Eight isolates induced stage 3 development ([Fig. 2-II](#)). Significant differences between treatments were observed for isolate 4229 ($8.0 \pm 4.7\%$ and $2.3 \pm 2.1\%$ seeds for the C and T form, respectively) and isolate 4217 ($8.0 \pm 2.8\%$ and $1.5 \pm 1.2\%$ seeds for the T and C form, respectively), which exhibited an opposite influence of the preservation treatment.

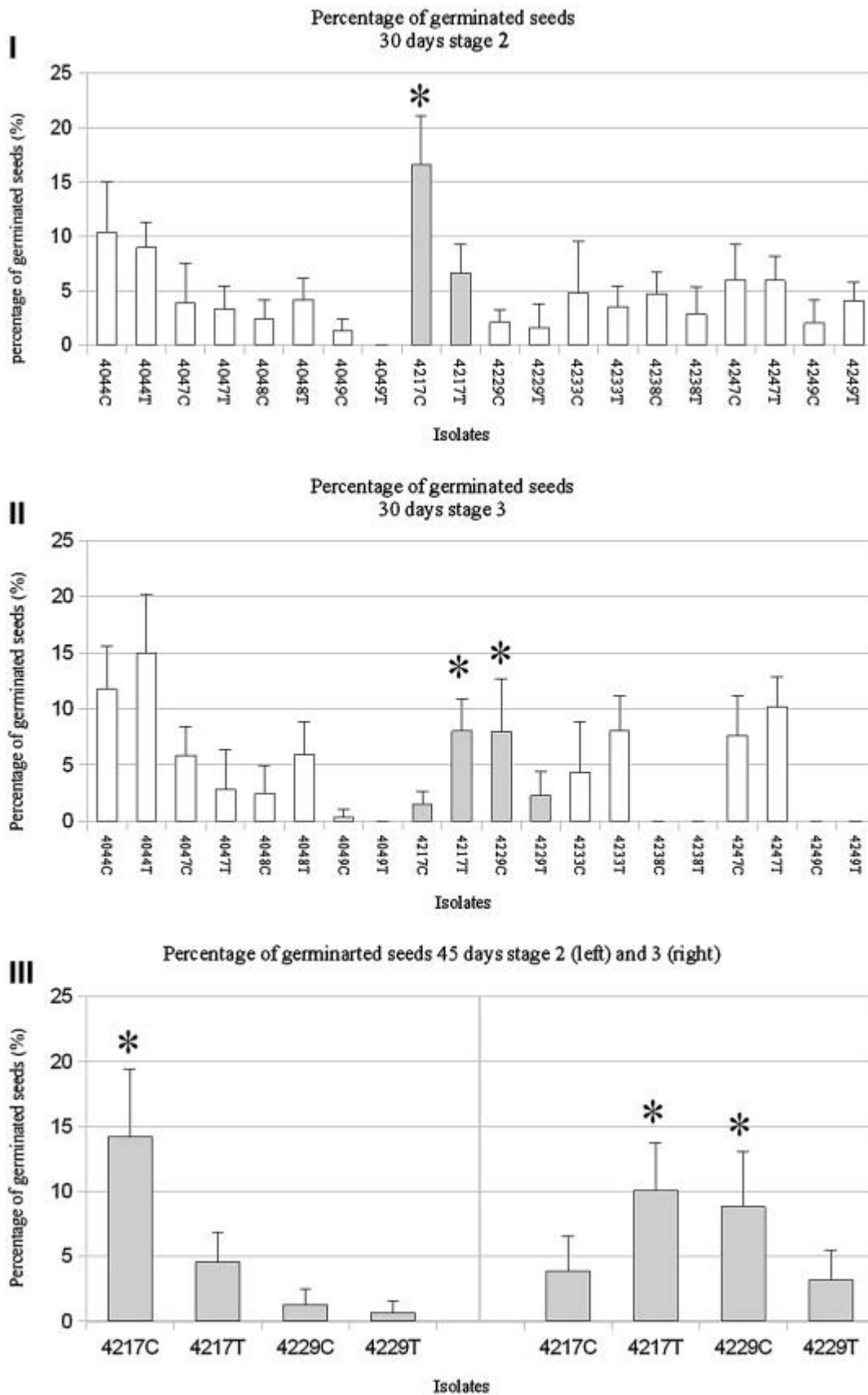


Fig. 2.

I: Percentages of seeds that reached germination stage 2 (swollen seed with rhizoids but without a well-defined apex) 30 days after inoculation with either the C or T form of each of the tested fungal isolates. II: Percentages of seeds that reached germination stage 3 (fully

developed protocorm) 30 days after inoculation. III: Percentages of seeds that reached stage 2 (on the left) and stage 3 (on the right) 45 days following inoculation with isolate 4217 and isolate 4229. C: cryopreserved form; T: actively growing form. Asterisks indicate a significant difference in germination ability between the C and T form of each isolate (Mann–Whitney U-tests, $p < 0.05$).

Given such differences, observations for the latter two isolates (4217 and 4229) were continued over 45 days (Fig. 2-III). At this time point, all forms promoted germination up to stage 2, with no significant differences between preservation treatments, the only exception being the C form of isolate 4217, which was more efficient than the corresponding T form. Similarly, all forms promoted germination up to stage 3. Significant, yet opposite differences between the two preservation methods were observed for both isolates, since the 4217 T form was more efficient than the corresponding C form ($10.1 \pm 3.7\%$ vs. $3.9 \pm 2.7\%$ seeds, respectively), while the C form of isolate 4229 was more efficient than the corresponding T form ($8.8 \pm 4.2\%$ vs. $3.2 \pm 2.2\%$) (Fig. 2-III).

These findings emphasize that the application of a preservation protocol does not always give consistent results on the totality of tested isolates, supporting the idea that the choice of a preservation protocol should be isolate-dependent (Smith and Onions, 1983, Smith and Onions, 1994 and Ryan et al., 2000). However, cryopreservation at $-80\text{ }^{\circ}\text{C}$ proved to be a suitable technique for medium-term storage of orchid mycorrhizal fungi, all cryopreserved isolates maintaining the ability to induce orchid seed germination. The absolute benefits of this methodology lie in the low cost and low labor needed to preserve a high number of isolates, which can be processed in a relatively short time. Compared with other studies, that have focused on the production of encapsulated seeds and compatible fungi for simultaneous preservation of the two partners of symbiosis (Sommerville et al., 2008), fungal cryopreservation is independent from the storage requirements of seeds and has also the advantage of allowing, after thawing, the germination of a range of orchid species.

References

1. M.A. Clements, H. Muir, P.J. Cribb A preliminary report on the symbiotic germination of European terrestrial orchids Kew Bull., 41 (1986), pp. 437–445
2. D.J. Coates, K.W. Dixon Current perspectives in plant conservation biology Aust. J. Bot., 55 (2007), pp. 187–193
3. S. Cozzolino, D. Cafasso, G. Pellegrino, A. Musacchio, A. Widmer Fine-scale phylogeographical analysis of Mediterranean *Anacamptis palustris* (Orchidaceae) populations based on chloroplast minisatellite and microsatellite variation Mol. Ecol., 12 (10) (2003), pp. 2783–2792
4. P.J. Cribb, S.P. Kell, K.W. Dixon, R.L. Barrett Orchid conservation: a global perspective K.W. Dixon, S.P. Kell, R.L. Barrett, P.J. Cribb (Eds.), Orchid Conservation, Natural History Publications, Kota Kinabalu (2003)
5. M. Girlanda, R. Segreto, D. Cafasso, H.T. Liebel, M. Rodda, E. Ercole, S. Cozzolino, G.P. Gebauer, S. Perotto Photosynthetic Mediterranean meadow orchids feature partial myco-heterotrophy and specific mycorrhizal associations Am. J. Bot., 98 (7) (2011), pp. 1148–1163
6. IUCN guidelines for the prevention of biodiversity loss due to biological invasion Species, 31–32 (1999), pp. 28–42
7. N. Juillet, S. Dunand-Martin, L.D. Gigord Evidence for inbreeding depression in the food-deceptive colour-dimorphic orchid *Dactylorhiza sambucina* (L.) Soð Plant Biol. (Stuttg.), 9 (1) (2007), pp. 147–151

8. C.A. Kearns, D.W. Inouye, N.M. Waser Endangered mutualisms: the conservation of plant-pollinator interactions *Annu. Rev. Ecol. Syst.*, 29 (1998), pp. 83–112
9. J.R. Leake The biology of myco-heterotrophic (saprophytic) plants *New Phytol.*, 127 (2) (1994), pp. 171–216
10. H.T. Liebel, M.I. Bidartondo, K. Preiss, R. Segreto, M. Stöckel, M. Rodda, G. Gebauer C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia *Am. J. Bot.*, 97 (6) (2010), pp. 903–912
11. J.T. Otero, J.D. Ackerman, P. Bayman Differences in mycorrhizal preferences between two tropical orchids *Mol. Ecol.*, 13 (8) (2004), pp. 2393–2404
12. H.W. Pritchard, P.T. Seaton Orchid seed storage: historical perspective, current status, and future prospects for long-term conservation *Selbyana*, 14 (1993), pp. 89–104
13. M.J. Ryan, D. Smith, P. Jeffries A decision-based key to determine the most appropriate protocol for preservation of fungi *World J. Microbiol. Biotech.*, 16 (2000), pp. 183–186
14. P.T. Seaton, H.W. Pritchard Orchid germplasm collection, storage and exchange K.W. Dixon, S.P. Kell, R.L. Barrett, P.J. Cribb (Eds.), *Orchid Conservation*, Natural History Publications, Kota Kinabalu (2003), pp. 227–258
15. D. Smith, A.H.S. Onions A comparison of some preservation techniques for fungi *Trans. Brit. Mycol. Soc.*, 81 (1983), pp. 535–540
16. D. Smith, A.H.S. Onions The preservation and maintenance of living fungi IMI Technical Handbooks, 1, CAB International, Wallingford (1994)
17. S.E. Smith, D.J. Read *Mycorrhizal Symbiosis* (3rd ed.) Academic Press, New York (2008)
18. K.D. Sommerville, J.P. Siemon, C.B. Wood, C.A. Offord Simultaneous encapsulation of seed and mycorrhizal fungi for long-term storage and propagation of terrestrial orchids *Aust. J. Bot.*, 56 (7) (2008), pp. 609–615
19. V. Sosa, T. Platas Extinction and persistence of rare orchids in Veracruz, Mexico *Conserv. Biol.*, 12 (1998), pp. 451–455
20. N.D. Swarts, K.W. Dixon Perspectives on orchid conservation in botanic gardens *Trends Plant Sci.*, 14 (11, Sp. Iss. SI) (2009), pp. 590–598
21. N.J. Vereecken, A. Dafni, S. Cozzolino Pollination syndromes in Mediterranean orchids—implications for speciation, taxonomy and conservation *Bot. Rev.*, 76 (2) (2010), pp. 220–240
22. S. Voyron, S. Roussel, F. Munaut, G.C. Varese, M. Ginestro, S. Declerck, V.F. Marchisio Vitality and genetic fidelity of white-rot fungi mycelia following different methods of preservation *Mycol. Res.*, 113 (2009), pp. 1027–1038
23. R.J. Waterman, M.I. Bidartondo Deception above, deception below: linking pollination and mycorrhizal biology of orchids *J. Exp. Bot.*, 59 (5) (2008), pp. 1085–1096