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

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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 °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 °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 °C, and cryopreserved at − 80 °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 °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 × 1 cm) positioned at the edge of a 6 cm Petri dish, which was centrally inoculated with a 3 mm × 3 mm mycelial plug. Five replicates per isolate were set up. Control plates were left uninoculated. Plates were incubated at 20 °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	*S. vomeracea*	2006	Liguria	24 months	*Tulasnella calospora*	JF926513
4047	*S. vomeracea*	2005	Campania	24 months	*Tulasnella* sp.	JF926500
4048	*S. vomeracea*	2005	Liguria	24 months	*Tulasnella* sp.	JF926506
4049	*S. vomeracea*	2005	Campania	24 months	*Tulasnella* sp.	JF926505
4217	*Serapias lingua*	2007	Sardinia	11 months	*Tulasnella* sp.	KC525058
4229	*S. vomeracea*	2006	Liguria	10 months	*Tulasnella* sp.	JF926519
4233	*S. vomeracea*	2006	Liguria	10 months	*Ceratobasidium* sp.	JF912485
4238	*Serapias cordigera*	2007	Sardinia	10 months	*Tulasnella* sp.	KC525059

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

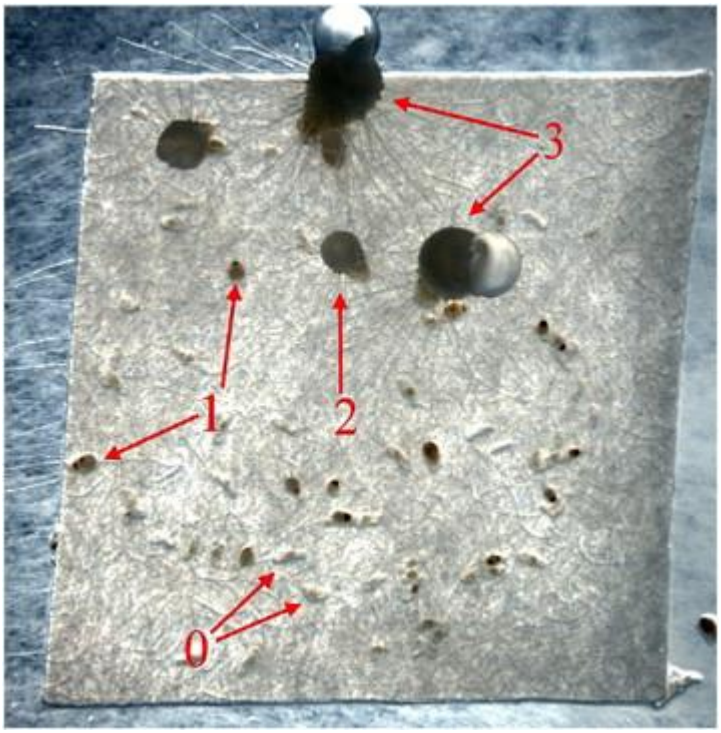

Fig. 1.

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 ± 4.5% and 6.6 ± 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 ± 4.7% and 2.3 ± 2.1% seeds for the C and T form, respectively) and isolate 4217 (8.0 ± 2.8% and 1.5 ± 1.2% seeds for the T and C form, respectively), which exhibited an opposite influence of the preservation treatment.

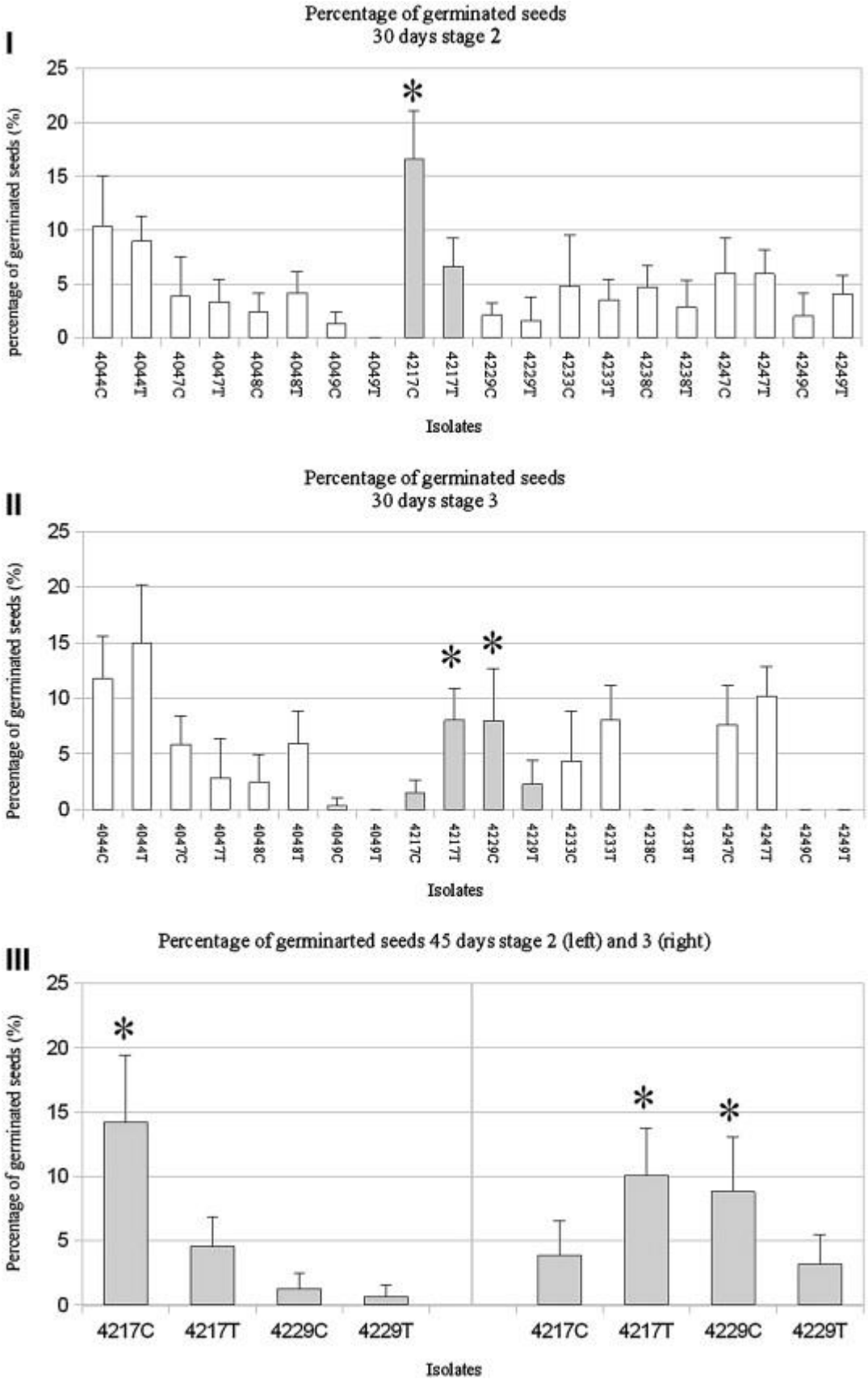

Fig. 2.

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 ± 3.7% *vs.* 3.9 ± 2.7% seeds, respectively), while the C form of isolate 4229 was more efficient than the corresponding T form (8.8 ± 4.2% *vs.* 3.2 ± 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 °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.

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