

Chapter 27

Conservation Strategies of New Fungi Samples in Culture Collections: *Piriformospora indica* Case

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Abstract Conservation and maintenance strategies for microorganisms are essential to develop the biotechnology and microbiology of twenty-first century. It is necessary to develop methodologies to keep stable and safely each strain isolated or developed in a laboratory and to be able to keep the microbes exactly in the same conditions that they were found or selected. In the case of fungi, especially in non-sporulated, conservation and maintenance are more complicated than other microbes, due to the difficulty of lyophilizing it. Other times, it is necessary to consider that there are laboratories where it is not possible to lyophilize the samples or keep them in safety freezer due to the lack of facilities. That is why here we presented a simply methodology to find the best conservation method for a new fungus, according to the conditions of laboratories with different budgets and facilities. Along the chapter, a methodology to be followed by a researcher who has to find the more suitable method to conserve new fungus, adapting the method to the fungi characteristic and laboratory restrictions, is presented. *Piriformospora indica*, a recently discovered and promising fungus from the point of view of plant-microbe interaction biotechnology, is taken as a successful conservation case.

27.1 Introduction

One of the keystones of twenty-first century biotechnology is microbiology; nowadays, it is applied to almost every area of our lives, from agriculture to medicine. Indeed, the vast majority of living organisms in the planet are microbes, in both diversity and number (Hug et al. 2016). The complete information about the amount of microbes that inhabit our planet is still unknown, but it might be almost infinite (García-Villaraco et al. 2013). It has been shown that a single environmental sample

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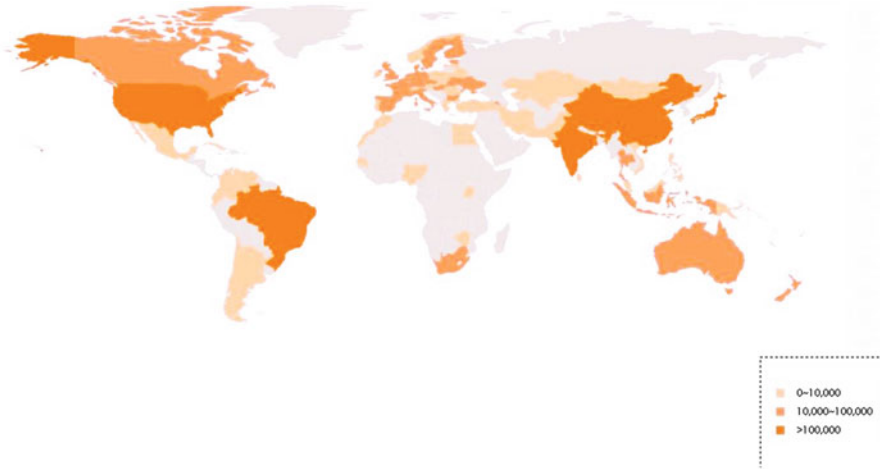


Fig. 27.1 Distribution map of preserved items in different countries and regions. The three different colors from shallow to deep on this map show the corresponding number of preserved cultures: 0–10,000; 10,000–100,000; above 100,000 items

of DNA from marine water encodes more than 1.2 million previously unknown genes from 1800 predicted genomic species (Venter et al. 2004).

The use of non-properly conserved biological material is a real problem in research and industrial processes. Well-established collections and conservation methods are necessary to provide strains and services of high quality (Homolka 2013).

Today, there is a strong worldwide interest to search and conserve the information stored in the microbial samples and individual microbe strains. The first recorded service culture collection was the Král Collection established in 1890 at the German University of Prague, Czech Republic, where cultures of microbes were made commercially available (Sette et al. 2013). However, the first independent center to endeavor to preserve and supply a wide range of microbes was the Centraalbureau voor Schimmel cultures (The Netherlands CBS), established in 1904 (Hawksworth 2004). There are 705 Culture Collections around the world, distributed in 72 countries, with 2.5 million microorganisms (among them, 1 million Bacteria, 0.7 million Fungi, and 37,787 Virus) (Juncai et al. 2014). Also, it is possible to observe that the collections are widely distributed around the world (Fig. 27.1).

The increase of the registered cultures in the World Federation for Culture Collections shows the increasing interest in this topic (Fig. 27.2). Hence, this evolution confirms the importance to develop new strategies to optimize microbe conservation techniques (Homolka 2013).

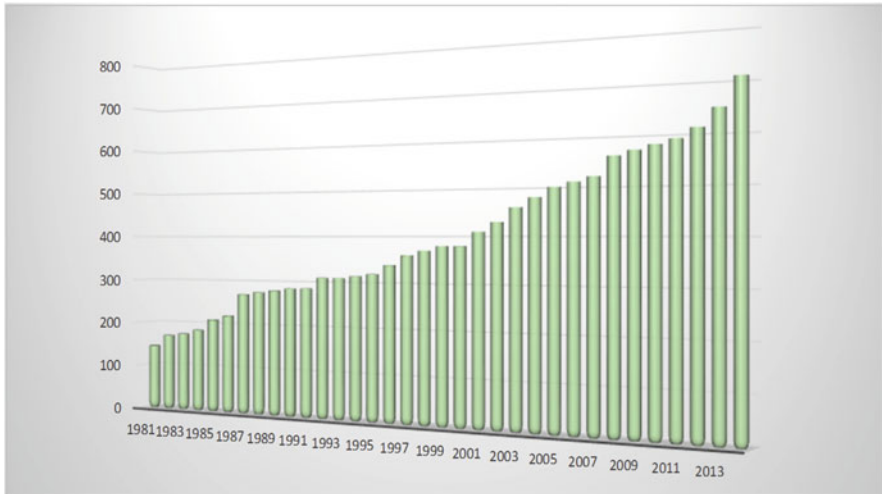


Fig. 27.2 Culture collection registered until 2014 (Juncai et al. 2014)

27.2 Classification of Microbes Conservation Methods

The correct conservation of the strain should be done by keeping it alive, pure, and genetically stable. There are a lot of conservation methods classified into two main groups: hypobiotic state (where the cells are reduced to minimum vital activity) and anabiotic state (where the growth of cells has been stopped by freezing or freeze-drying):

1. **Anabiotic state:** are the most suitable methods. The risk of contamination or alteration of the strain is minimal; however, not all the strains are compatible with these methods.
 - (a) **Cryopreservation or freezing:** Cell growth is standby because the free water is frozen, but cells are alive. In this method, the temperature should be under -40°C , but it is more convenient around -80°C or lower in the case of liquid nitrogen. In addition, cryoprotectant agents should be used to protect cells from damage during freezing.
 - (b) **Lyophilizing or freeze-drying:** Cell growth is standby because the cells have been dehydrated softly by sublimation. It is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying). Likewise, cryoprotectant agents should be used to protect cells during freezing.
2. **Hypobiotic state:** there are several strains complicated to conserve following the methods described above (like genus *Spirillum* or *Rhodospirillum*). In these cases, several methods have been developed:

- (c) **Periodic transference or subcultivation:** This is a very simple and low-cost technique that allows subcultures live for a longer time, reaching to a survival of 2–30 years depending on the strain. Although some strains can adapt well the subsequent transfer, the drawback of this method is that, because the cells are alive, genetic, morphological, and physiological changes are quite common (without forgetting the risk of contamination in each subcultivation) (Castellani 1939).
- (d) **Mineral oils:** The method essence is covering the well-grown culture on liquid or agar nutrient medium with sterile nontoxic mineral oil. The most common oil used is paraffin with layer thickness 1–2 cm. The aim is to limit the oxygen access that reduces the microorganisms' metabolism and growth, as well as to restrict the cell drying during preservation in freezing conditions. Following this method the microbes conservation is possible without subcultivation up to 12 years (Uzunova-Doneva & Donev 2005; Lima Freire et al. 2016).
- (e) **Water or water-salt solutions:** The cells are placed in liquid medium with a compound to avoid the osmotic shock. Although this method is recommended for short-term storage at 4–8 °C for 1 week to 12 months, it can be used for some fungi, where it is demonstrated that 80% of strains survived up to 20 years at room temperature (Bueno & Gallardo 1998).
- (f) **Drying:** The cultures are mixed with carriers as sterile soil, mud, active carbon, sawdust, synthetic balls, or polymers. This method takes advantage of the natural properties of the microbes to survive in dry environments until the conditions are appropriate.

The last two methods are not usually used for big collections, but they are commonly used for agro-biotechnology companies to store the biofertilizers or biopesticides with high shelf life.

27.3 Conservation of Fungi Collections

Although the properties of fungi have been exploited for thousands of years, mainly in brewing and baking, with the last years “biotechnology boom,” the interest in fungi has increased, resulting in the introduction of the term “myco-technology” (Bennett 1998). Nowadays, fungal strains can be applied to many social-economic areas, including the production of a wide range of commercially interesting compounds, such as biofertilizers, biostimulants or biopesticides, enzymes, antibiotics, pigments, vitamins, alcohols, organic acids, pharmaceuticals, cosmetics, among others. The main issue to develop and apply these fungi is the problem related with strain conservation.

There is a rich literature on the preservation of fungal cultures, and this chapter will not review all possible techniques. The aim of this chapter is to design a protocol to conserve a new fungus for which conservation method has not been optimized yet (Fig. 27.3).

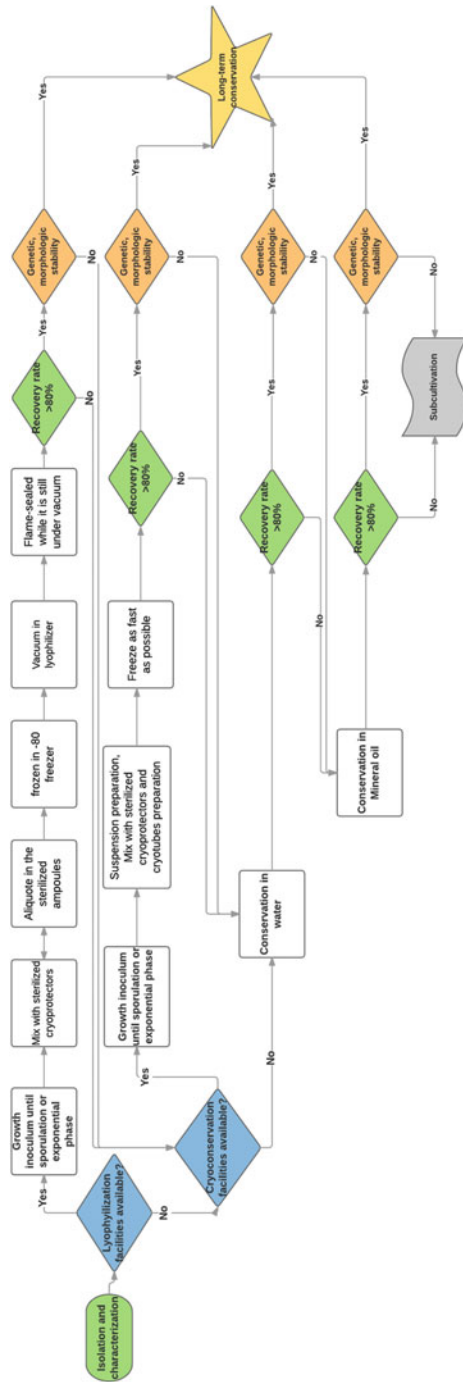


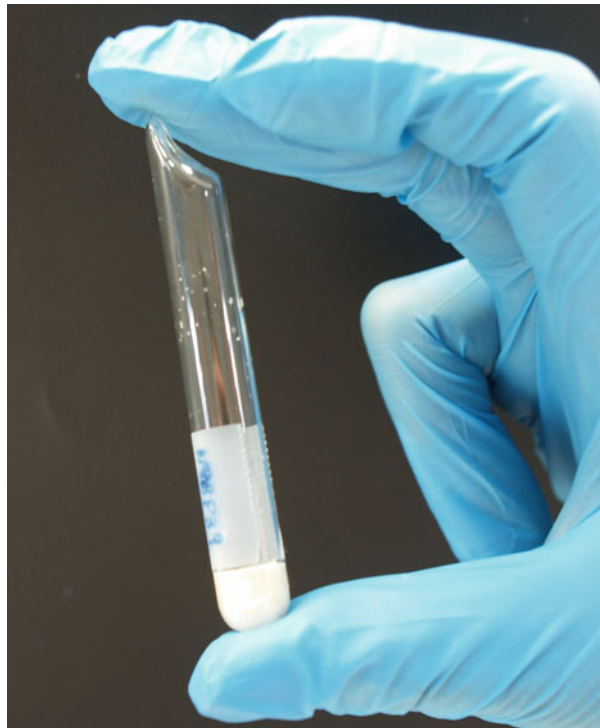
Fig. 27.3 Flowchart of proposed methodology to find the best conservation method

27.3.1 Lyophilization

The more suitable way for long-term conservation of fungi is lyophilization (Fig. 27.4). This method should be the first to check if the laboratory has the needed facilities. This method has several benefits; cultures can be stored in little space at room temperature during long-term conservation and are easily sent elsewhere. It is effective for a lot of conidial fungi as ascomycetes and basidiomycetes. It is not usually useful for fungi with very “watery” cells with large vacuolar volumes but may succeed if the spores are not heavily vacuolated (e.g., the sporangiospores of many zygomycete fungi). Also, it has to be considered that, in general, spores can tolerate the most of conservation methods better than vegetative hyphae, and lyophilizing is not the exception. Large-scale processes use bottles dried on shelves in a large vacuum chamber and sealed under vacuum by the lowering of a pressure plate onto the partially seated rubber stoppers (Humber 2012).

There are two main problems associated with this method: first of all, the availability of the lyophilizer. The second issue is that there are a lot of fungi that it is not possible to recover, especially the ones that cannot sporulate (Homolka

Fig. 27.4 Example of lyophilized sample



2013). For example, in our lab we had problems in conservation by lyophilization of several fungi, so, we had to utilize cryopreservation techniques that after several tests worked perfectly in long-term conservation.

In these cases, we can choose two kinds of methods depending on the conditions of the lab: if a -80°C freezer or a liquid nitrogen freezer is available, freezing is the most recommended method. In the case it is not available or the electricity is not assured, we should try “low-cost” methods, like Mineral oil or Water or water-salt solutions.

27.3.2 Freezing

It was introduced in 1960 in ATCC (American Type Culture Collection) for long-term storage of large numbers of fungal species with very good results (Homolka 2013; Hwang 1960). Nowadays, it is the main conservation technique in the world, with lyophilizing.

Agar block with the fungal mycelium and spores immersed in an appropriate cryoprotectant is the traditional and simplest method. However, other methods exist as polystyrene beads or porous ceramic beads (Chandler 1994; Palagyi et al. 1997).

The two main factors are freezing and thawing conditions. In general, there are two possibilities: slow or controlled freezing or fast, with pros and cons in both of them. Although each microbe is different, generally too low freezing rates cause extreme dehydration and concentration of the solution, leading to cell damage. On the other hand, too fast freezing causes insufficient dehydration and formation of abundant ice crystals.

Other important factor is the cryoprotectant applied. Protective compounds or cryoprotectants are found to eliminate most of the multiple destructive factors during freezing of biological structures. The main cryoprotectants are dimethylsulfoxide (Me_2SO), glycerol, blood serum or serum albumin, skimmed milk, yeast extract, saccharose, glucose, methanol, peptone, polyvinylpyrrolidone (PVP), sorbitol, and malt extract.

Cryoprotectants can be classified in various ways, such as either low-MW or high-MW (Molecular Weight) additives or depending on the rate of penetration (Hubalek 2003); those that penetrate quickly, around 30 min, include methanol, ethanol, ethylene glycol (EG), 1 propylene glycol (PG), dimethylformamide, methylacetamide, and Me_2SO . Also, Glycerol is among the most important ones, which penetrates more slowly. Nonpenetrating or nonpermeating are polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyethylene oxide (PEO), or polyvinyl alcohol, mono-, oligo-, and polysaccharides, mannitol, sorbitol, dextran, hydroxyethyl starch (HES), methyl cellulose, albumin, and gelatin.

The most usual cryoprotectants are Me_2SO and glycerol. Dimethylsulfoxide was firstly used to cryoprotect red blood cells (RBC) and spermatozoa (Lovelock &

Bishop 1959). The optimum Me₂SO concentration varies widely, from 1 to 32% (median around 10%). *Neurospora crassa*, *Sclerospora sorghi*, certain *Pezizales*, *Volvariella volvacea*, and other basidiomycetes have been conserved with dimethylsulfoxide (Hubalek 2003; Barnhart & Terry 1971; Challen & Elliot 1986; Homolka 2013). Many fungi do not usually tolerate high concentrations of Me₂SO, but no marked toxicity of Me₂SO to filamentous fungi has been described (Hubalek 2003). Among alcohols, Glycerol (1,2,3-propanetriol) and Polyethylene glycol (PEG) have been the most widely used in fungi. Also, glycerol allows intermediate methods between freezing and room temperature conditions. For example, *Aspergillus*, *Penicillium*, or *Trichoderma* have been conserved up to 30 months, in glycerol 50% (Shankar Paul et al. 2015).

27.3.3 Water or Water–Salt Solutions

Sometimes, lyophilization or freezing is not possible because of the fungi or the lab conditions. It was demonstrated that conservation under pure sterile water or water-salt condition can be a cheap alternative and low space-demanding technique. Cells are placed in liquid medium, and they approach a hypobiotic state. The suspension density, the presence of Ca²⁺ ions in the medium, the solution composition, pH, and the preservation temperature influence the quantity and protection of the cells. Some fungi can endure up to 20 years in sterile water (De Capriles et al. 1989), but the danger is that some fungi lose viability much sooner. In fact, distilled water was the most appropriate form of preservation of endophytic microorganisms in recent published work (Lima Freire et al. 2016). Some problems for this technique are easily avoided: too much inoculum for the volume of water may threaten the ability of the fungus to withstand long-term storage (the water volume should be around 40 times greater than the inoculum blocks) (Humber 2012). Too much medium in the vial can contain too much nutrients that shorten the longevity of the stored fungus.

27.3.4 Mineral Oil

Storing culture under a layer of sterile mineral oil is an approach still widely used when the methods above are not possible. The oil prevents dehydration as well as it reduces gas exchange, reducing fungal metabolism to a very low level. If there are spaces available to store racks of tubes, this is a common alternative for the storage. Cultures under mineral oil may remain viable for decades (Silva et al. 1991; Dasilva et al. 1994).

A comparative study was conducted on long-term fungal culture preservation methods. A total of 112 isolates of several phytopathogenic fungi (*Alternaria alternata*, *A. solani*, *Fusarium oxysporum* f. sp. *lentis*, *F. oxysporum* f. sp. *capsici*,

Rhizoctonia solani, *Lasiodiplodia theobromae*, *Colletotrichum gloeosporioides*, and *Curvularia lunata*) were stored up to 2 years by using five preservation methods. Although the stability and regeneration rate of mineral oil method was not the best, it produced good results. In all cases, there was more than 50% of percentage of revival, so, it could be suitable in some lab conditions (Aliya et al. 2015).

27.4 Example of a Problematic Case: Conservation of *Piriformospora*

Piriformospora indica (a mycorrhiza like endophytic Agaricomycetes fungi) has received great attention over the last few decades, due to its exceptional ability to efficiently promote plant growth, protection, and stress tolerance (Gill et al. 2016; Varma et al. 1999; Varma and Kost 2013). Due to the possibility of *P. indica* to be cultivable axenically and its versatility for colonizing/hosting a broad range of plant species, it has a great potential for biotechnological applications (Fig. 27.5).

In fact, in 2016 a molecular ecology study revealed the hyperdiversity of Sebaciniales and their evolutionary diversification into two sister families, *Sebacinaceae* (that forming basidiomes, isolation into pure culture so far only for early-diverging saprotrophic species, and endophytic, ectomycorrhizal, and orchid mycorrhizal in partially or fully mycoheterotrophic plants) and *Serendipitaceae* (basidiomes never observed until now, frequently isolated into pure cultures from orchid roots, frequently endophytic, orchid mycorrhizal in green species, ericoid mycorrhizal, symbiotic of liverworts, and ectomycorrhizal in some lineages). And it is proposed to transfer the endophytic cultivable species *P. indica* and *P. williamsii* to the genus *Serendipita* (Weiß et al. 2016).

Fig. 27.5 *Piriformospora indica* growing in liquid medium



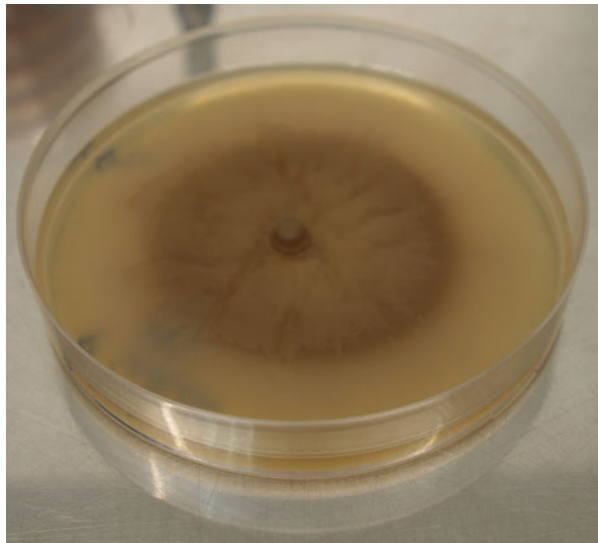
Because of the interest in developing biofertilizers and biostimulants based on *Piriformospora*, it is vital to develop a method that can ensure the conservation of the strain without any single genetic or morphologic modification.

One of the first problems is that *Piriformospora* spp. loses its root colonization efficiency (or endophytic capacity) after repeated subculturing on synthetic medium. To maintain this efficiency, the fungus must be periodically inoculated to the roots of host plants (in vitro or in soil) and re-isolated from the internally colonized roots (Schedel et al. 2012; Johnson et al. 2011). Therefore, it was really important to find an anabiosis method to conserve the strain stable and in perfect conditions.

As it is explained above, the first candidate technique for conservation was lyophilizing. We tried it and studied the regeneration rate and stability after several time points:

1. Culture preparation: Because this method requires fungi spores, once the inoculum was sporulated (Fig. 27.6), cultures were mixed with sterilized skim milk solution (20%). Others media are serum, peptone, various sugars etc.
2. In parallel, ampoules were closed with small cotton plugs and autoclaved.
3. Then, the mix was aliquoted in the sterilized ampoules. The preparations were frozen in -80°C freezer.
4. Once frozen, the ampoules were inserted in a strong vacuum to the lyophilizer (Fig. 27.7).
5. As soon as it is lyophilized, it is necessary to check that the sample is completely dried.
6. Ampoules were flame-sealed while it is still under vacuum.
7. Conservation. Although the lyophilized samples can be kept at room temperature, it is recommended to keep at 4°C .

Fig. 27.6 *Piriformospora indica* growing in solid medium



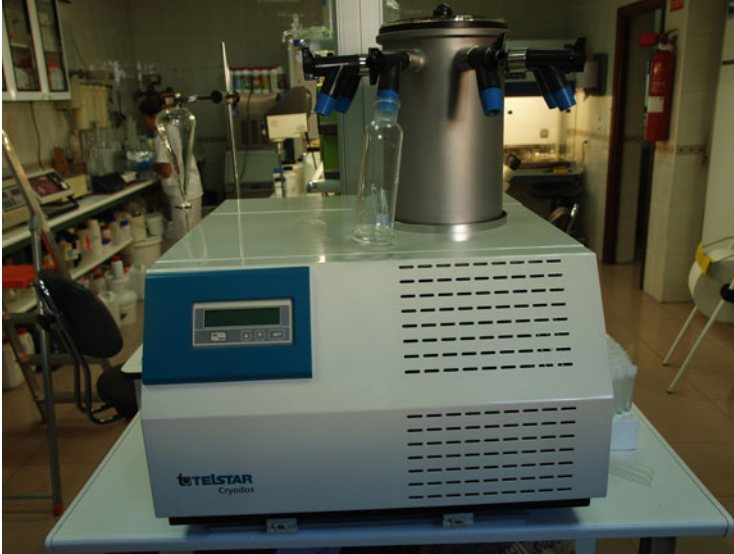


Fig. 27.7 Typical laboratory lyophilizer

8. Monitoring. To study the stability and recovery rate, the recovery of the culture was analyzed each month during 2 years. To recover the culture, it was necessary just to sterilize the surface of the ampoule with ethanol or similar, open it scoring the neck, and add sterile water or liquid medium to the freeze-dried contents to reconstitute the culture.

The results of this study were not satisfactory because the recovery rate were under 10% after 6 months, which is far from being adequate for a long-term collection.

As noted above, we tried the second method, the cryo-conservation. We chose it because conserving the samples below -80°C will ensure their long-term integrity.

Cryo-conservation protocol:

1. Culture preparation: it is better to use the spores mixed with hyphae. If it is not possible, we should use the culture in the exponential growth phase.
2. Suspension preparation: If the culture is liquid, it should be centrifuged gently to remove part of the nutrients and concentrate it, increasing the number of cells in the final preparation. It is necessary to check the total and viable unit colonies forming. Then, it is necessary to use a protective media to keep the microorganism viability that the researcher has to choose among some of the listed above (it has to be chosen empirically). It must be nontoxic, have good water solubility and low eutectic temperature, prevent salt hyperconcentration in the suspension, stabilize hydrogen connections in the crystal lattice, and prevent large crystal

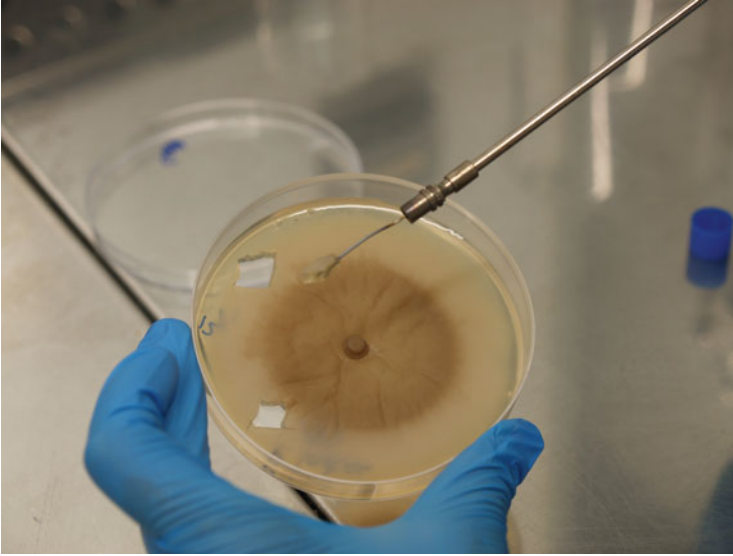


Fig. 27.8 Cutting the cubes of agar with *Piriformospora indica* to cryopreservation

forming. In a new fungus always it is appropriate to check several cryoprotectors. After several tests, we decided to use glycerol to conserve *P. indica* (Fig. 27.8). The common method is to apply 500 μ l glycerol 30% to 500 μ l of the culture. However, we checked diverse methods, concluding that the best is to add 4 cubes of 0.5 cm \times 0.5 cm approx. of agarized medium with the *P. indica* to a dilution of glycerol 10% (Fig. 27.9).

3. Cryotubes preparation: Once best conservant is decided, it is necessary to autoclave the cryotubes containing 1 ml of glycerol 10%.
4. Freeze as fast as possible. If it is possible, sink in liquid nitrogen before to keep in the -80 $^{\circ}$ C freezer or preserve directly in 80 $^{\circ}$ C freezer.
5. Monitoring: Always it is recommended to check the stability of the culture periodically to ensure a long-term conservation of a new fungus. Our results (Fig. 27.10) show a monthly monitoring. We found a recovery rate close to 100% after 2 years.

When we are working with fungi that interact with other organisms, it is very recommendable to check that it is keeping this capacity of interaction. In our study, after 2 years of cryoconservation (as it was explained in the protocol above), we tested the root colonization efficiency of *P. indica* under controlled conditions. The cocultivation experiments were carried out with the model plant *Arabidopsis thaliana* in Petri dishes and with *Solanum lycopersicum* plants in growth chamber).

For *Arabidopsis thaliana* assay, cocultivation of *Piriformospora* was done as described by JM Johnson et al. (2011), with modifications. Colonized and uncolonized roots were cut after 27 and 20 days of cocultivation. The roots were

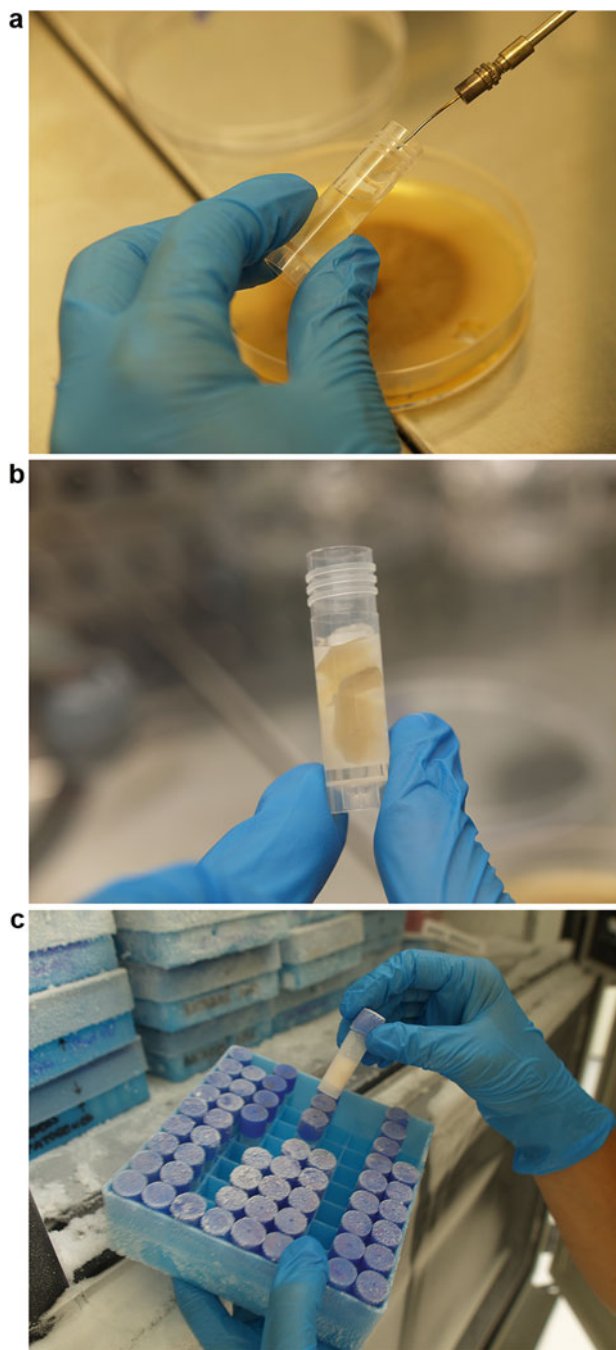


Fig. 27.9 Procedure to keep the samples by cryopreservation. (a) Immersion of the agar cubes in cryoprotectors. (b) Cubes in cryotubes. (c) Cryotubes stored in -80°C

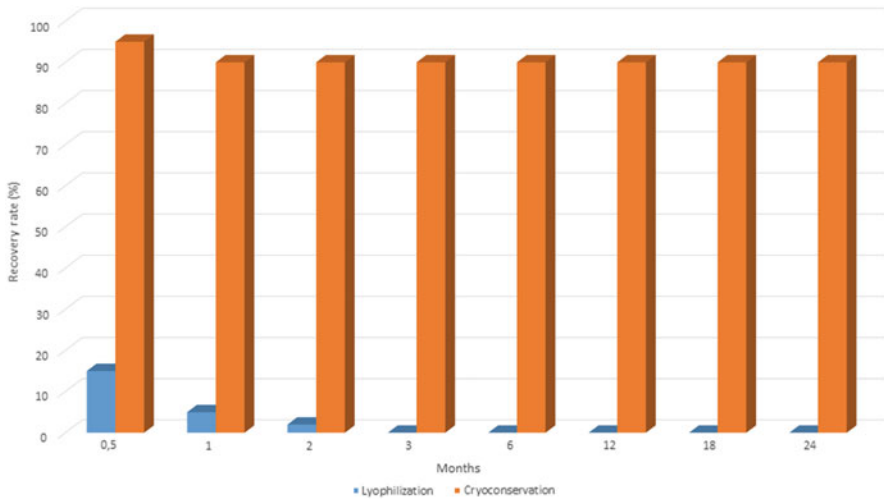


Fig. 27.10 Recovery rate of *P. indica* in the two methods tested along 2 years monitoring it

treated with 10% KOH and incubated overnight at room temperature in Petri dishes. After incubation, roots were water washed and incubated in 1% HCl for 3 min. Finally, the roots were washed again and dyed using lactophenol blue solution, in order to observe the preparation under 10X objective in the microscope.

To re-isolate the fungus, roots were surface cleaned with 0.1% sodium hypochlorite for 2 min, water washed, and dried in flow chamber. In order to observe the fungus growth along the root pieces, they were placed on Hill and Kaefer plates and incubated 5–7 days at 30 °C in the dark. The fungus was then immediately subcultured by taking the hyphal tip to fresh Hill and Kaefer plates.

For the tomato bioassay (var. “Marmander”), tomato seedlings (one- to two-true-leaf stage) grown in Brown 25 W peat (Projar, Valencia, Spain) were transplanted into each pot with PS Potplant substrate (Projar, Valencia, Spain). Plants were irrigated as needed and fertilized with Hoagland nutrient solution twice per week. The assay was carried out in a growth chamber at 25 °C, photosynthetically active radiation (PAR) intensity 280 $\mu\text{E}/\text{m}^2 \text{ s}$, photoperiod 16 h light and 8 h dark, and 60% relative humidity.

The endophyte *Piriformospora* was propagated on solidified Hill and Kaefer medium, at 30 °C. Fungal plugs of 5 mm diameter were made with cork borer and transferred to the flasks with 250 ml Kaefer liquid medium. The flasks were further incubated at 30 °C and 140 rpm for 2 weeks (Adya et al. 2013). Mycelium and spores were mixed with a blender for 2 min at lowest speed in sterile distilled water. Chlamyospore total number was examined in a Thoma chamber, and the number of viable propagules was determined by plating on solid Hill and Kaefer media (Fakhro et al. 2010). The suspensions were adjusted with sterile distilled water to a

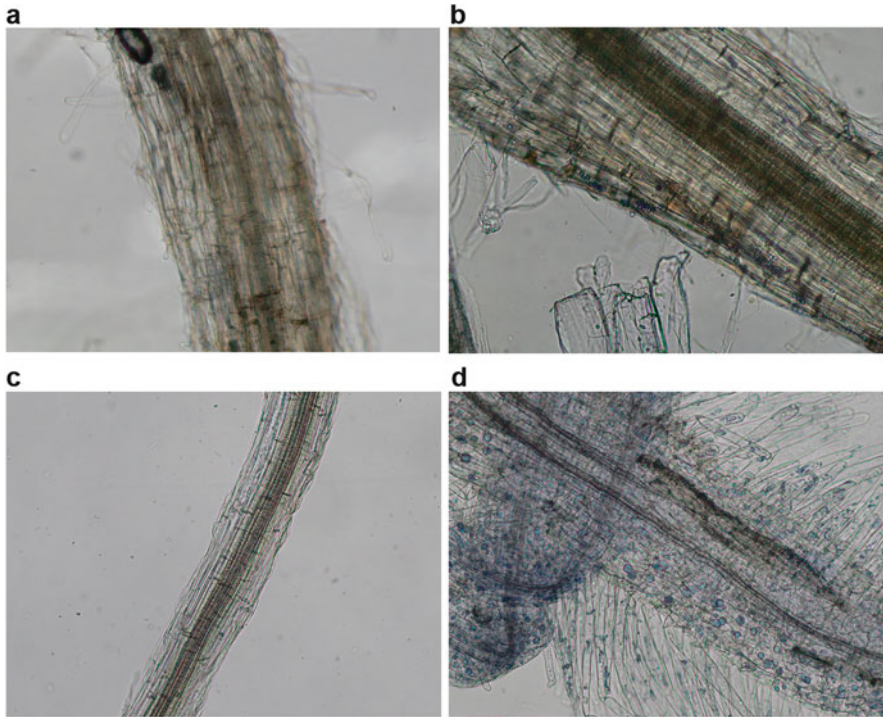


Fig. 27.11 *Piriformospora indica* in a root colonization efficiency test, in tomato ((a) *Solanum lycopersicum* root control; (b) *S. lycopersicum* root inoculated with *P. indica*; (c) *Arabidopsis thaliana* root control; (d) *A. thaliana* root inoculated with *P. indica*)

concentration of 10^4 CFU/ml, not exceeding 5×10^4 CFU per plants (Fakhro et al. 2010).

Piriformospora was applied to the tomato plants at 7, 14, and 21 days post-transplant through irrigation. The assays were established with twelve plants per treatment, distributed in four randomized blocks.

At the end of the bioassay, to check *Piriformospora* “root colonization efficiency” persistence, the roots were prepared as explained above (Johnson et al. 2011) and examined under the microscope (Fig. 27.11).

27.5 Concluding Remarks

The conservation strategies are one of the twenty-first century biotechnology keystones. Traditional subculture methods to keep alive fungus are not desirable in the “new generations” biotechnology. When these traditional methods are long-term applied, they can trigger mutations and unnatural selections. It is essential to be able to keep the fungi exactly in the same conditions that it was isolated or

selected. Sometimes, it is difficult to find the best method to conserve fungi, especially with non-sporulated fungus, that are not possible to keep them lyophilized, and it is necessary to search alternative methods to keep them stable. In this chapter, we present a simple methodology to find the best conservation method for a new fungus, according to the conditions of laboratories with different budgets and facilities.

References

- Adya A, Gautam A, Zhang L, Varma A (2013) Characterization of *Piriformospora indica* culture filtrate. In: Varma A, Kost G, Oelmüller R (eds) *Piriformospora indica*. Springer, Berlin, pp 345–375
- Aliya T, Naz F, Rauf CA, Irshad G (2015) Long term and least expensive preservation methods for various fungal cultures. *Pak J Phytopathol* 27:147–151
- Barnhart EE, Terry CE (1971) Cryobiology of *Neurospora crassa*. *Cryobiology* 8:323–327. doi:10.1016/0011-2240(71)90125-8
- Bennett JW (1998) Mycotechnology: the role of fungi in biotechnology. *J Biotechnol* 66:101–107
- Bueno L, Gallardo R (1998) Filamentous fungi preservation in distilled water. *Rev Iberoam Micol* 15:166–168
- Castellani A (1939) The viability of some pathogenic fungi in sterile distilled water. *J Trop Med Hyg* 42:225–226
- Challen MP, Elliot TJ (1986) Polypropylene straw ampoules for the storage of microorganisms in liquid nitrogen. *J Microbiol Methods* 5:11–22
- Chandler D (1994) Cryopreservation of fungal spores using porous beads. *Mycol Res* 98:525–526
- Dasilva AMM, Borba CM, Deoliveira PC (1994) Viability and morphological alterations of *Paracoccidioides brasiliensis* strains preserved under mineral oil for long periods of time. *Mycoses* 37:165–169
- De Capriles CH, Mata S, Middelveen M (1989) Preservation of fungi in water (Castellani): 20 years. *Mycopathologia* 106:73–79
- Fakhro A, Andrade-Linares DR, von Bargen S et al (2010) Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. *Mycorrhiza* 20:191–200
- García-Villaraco A, Bonilla A, Garcia-Seco D, Algar E (2013) Metagenomics of plant-microorganism interaction: source of novel recombinant genes for biotechnological application. In: Rodelas B (ed) *Beneficial plant-microbial interactions: ecology and applications*. CRC Press, Hoboken
- Gill SS, Gill R, Trivedi DK, Anjum NA, Sharma KK, Ansari MW, Ansari AA, Johri AK, Prasad R, Pereira E, Varma A, Tuteja N (2016) *Piriformospora indica*: potential and significance in plant stress tolerance. *Front Microbiol* 7:332. doi:10.3389/fmicb.2016.00332
- Hawksworth DL (2004) Fungal diversity and its implications for genetic resource collections. *Stud Mycol*:9–17
- Homolka L (2013) Methods of cryopreservation in fungi. In: Gupta VK et al (eds) *Laboratory protocols in fungal biology: current methods in fungal biology*. Springer, New York, pp 9–15
- Hubalek Z (2003) Protectants used in the cryopreservation of microorganisms. *Cryobiology* 46:205–229
- Hug LA, Baker BJ, Anantharaman K et al (2016) A new view of the tree of life. *Nat Microbiol* 1:16048. doi:10.1038/nmicrobiol.2016.48
- Humber RA (2012) Preservation of entomopathogenic fungal cultures. In: *Manual of techniques in invertebrate pathology*. pp 317–328

- Hwang S-W (1960) Effects of ultra-low temperatures on the viability of selected fungus strains. *Mycologia* 52(3):527. doi:10.2307/3755974
- Johnson JM, Sherameti I, Ludwig A, Nongbri P, Sun C, Lou B, Varma A (2011) Protocols for *Arabidopsis thaliana* and *Piriformospora indica* co-cultivation—a model system to study plant beneficial traits. *J Endocytobiosis Cell Res* 21:101–113
- Juncal M, Hideaki S, Desmeth P (2014) World directory of culture collections (Sixth version, 2014). Database. <http://www.wfcc.info/ccinfo/home/>
- Lima Freire AK, dos Santos BA, Lima Sampaio I, Moura de Lima A, Botineli L, Coelho da Rocha L, Braga De Souza J, Souza É (2016) Availability and morphological characteristics of endophytic fungi held in different methods of preservation. *Sci Res Essays* 11:76–79
- Lovelock KE, Bishop MWH (1959) Prevention of freezing damage to living cells by dimethylsulphoxide. *Nature* 183:1394–1395
- Palagyi Z, Nagy A, Vastag M, Ferenczy L, Vagvölgyi CS (1997) Maintenance of fungal strains on cryopreservative-immersed porous ceramic beads. *Biotechnol Tech* 11:249–250
- Schedel S, Camehl I, Oelmüller R (2012) Long-term interaction between *Arabidopsis* and *Piriformospora indica* on expanded clay. *Endocyt Cell Res* 22:1–5
- Sette LD, Pagnocca FC, Rodrigues A (2013) Microbial culture collections as pillars for promoting fungal diversity, conservation and exploitation. *Fungal Genet Biol* 60:2–8
- Shankar Paul J, Tiwari KL, Jadhav SK (2015) Long term preservation of commercial important fungi in glycerol at 4 °C. *Int J Biol Chem* 9:79–85
- Silva M, Lima D, Cavalcanti M (1991) Induction of the sporulation in *Colletotrichum gloeosporioides* Penz, preserved under mineral oil. *Pesq Agrop Brasileira* 1(11):12
- Uzunova-Doneva T, Donev T (2005) Anabiosis and conservation of microorganisms. *J Cult Collect* 4:17–28
- Varma A, Kost GOR (2013) *Piriformospora indica*—Sebacinales and their biotechnological application. Springer, Berlin. isbn: 978-3-642-33802-1
- Varma A, Verma S, Sahay N (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Appl Environ Microbiol* 65:2741–2744
- Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen J, Wu D, Paulsen I, Nelson K, Nelson W, Fouts D, Levy S, Knap A, Lomas M, Nealsen K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers Y, Smith H (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304:66–74
- Weiß M, Waller F, Zuccaro A, Selosse M-A (2016) Sebacinales—one thousand and one interactions with land plants. *New Phytol* 211:20–40