Viability and sporulating capability of Coelomycetes preserved under a range of different storage regimes

Cintia de Moraes Borba and Katia F. Rodrigues

Departamento de Micologia do Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brasil.

Summary

The viability and sporulating capability of 45 Coelomycetes strains were evaluated. Strain subcultures were maintained under mineral oil, in soil and on agar slant for different periods of time lasting as long as 50 years, 39 years and 2 years, respectively. Of the 34 strains preserved under mineral oil, 20 maintained their viability but lost the sporulating capability with exception of one strain of *Pestalotiopsis guepinii*. Of the 16 strains also preserved in soil only one was viable and it was not able to sporulate. All 12 endophytic strains, 11 preserved on agar slant and one under mineral oil remained viable; however, the strain preserved under mineral oil lost its sporulating capability, while the strains on agar slant were only able to sporulate after culturing on sterilized alfalfa twigs. The results demonstrate that routine monitoring, and the use of different preservation methods, specially with the addition of sterilized plant tissue on the culture media for promoting conidiomata formation, is necessary for the success of the Coelomycetes long-term preservation.

Key words

Coelomycetes, Culture collection, Endophytic fungi, Long-term preservation, Viability

Viabilidad y capacidad de esporulación de Coelomycetes mantenidos bajo diferentes regímenes de almacenamiento

Resumen

Se evaluaron la viabilidad y la capacidad de esporulación de 45 cepas de Coelomycetes. Los subcultivos se mantuvieron en aceite mineral, en suelo y en agar por diferentes periodos de tiempo: 50, 39 y 2 años, respectivamente. De las 34 cepas conservadas en aceite mineral, 20 mantuvieron su viabilidad pero perdieron su capacidad de esporulación, con la excepción de una cepa de *Pestalotiopsis guepinii*. De las 16 cepas también preservadas en suelo sólo una fue viable pero incapaz de esporular. Las 12 cepas endofitas, 11 de ellas conservadas en agar y una en aceite mineral, permanecieron viables; sin embargo, la cepa en aceite mineral perdió su capacidad de esporular, mientras que las conservadas en agar fueron capaces de esporular únicamente tras cultivo en alfalfa estéril. Los resultados demuestran que la conservación exitosa de Coelomycetes durante un amplio periodo de tiempo requiere el control rutinario y el uso de diferentes métodos de conservación, especialmente añadiendo tejidos vegetales estériles a los medios de cultivo para promover la formación de conidias.

Palabras clave

Coelomycetes, Colección de cultivos, Hongos endofitos, Conservación a largo plazo, Viabilidad

Coelomycetes fungi are composed of approximately 9,000 species being found as saprophytic or parasitic on vascular plants, other fungi, lichens, vertebrates [1,2] and in human disease [3]. They are mitosporic, asexual fungi that form conidia within cavities known as conidiomata.

Coelomycetes constitute an economically important group of plant pathogenic fungi, with increasing number of studies on their biochemistry. Therefore, their *ex-situ* conservation for a long period of time has become of great importance to the scientific community. These organisms have been considered of easy preservation in water, showing 2 to 10 years of successful storage by this means [4,5].

Dirección para correspondencia:
Dr. Cintia de Moraes Borba
Dpto. de Micología, FIOCRUZ/IOC,
Av. Brasil, 4365, RJ 21045-900, Brasil
Tel.: +55 21 598 4356; Fax: +55 21 590 3545
E-mail: cborba@gene.dbbm.fiocruz.br

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Currently, there are 1,700 strains of filamentous fungi included in the Culture Collection of the Oswaldo Cruz Institute, comprising 45 Coelomycetes strains preserved under a range of different storage regimes: mineral oil, in soil and on agar slant. Preservation of fungi under the above storage regimes has been used in many laboratories worldwide, presenting different results depending upon the fungal group [5].

The method of fungal preservation under sterile mineral oil was first employed by Sherf [6] and its effectiveness has been reported in many studies for different groups of fungi [7-15].

The method of fungal preservation in sterile soil, described by Bakerspigel [16,17] have been useful in the *Fusarium* sp. preservation, but Windels *et al.* [18] described that storage in soil carries a risk of mutation which can result in loss of morphological characters and, sometimes, pathogenicity.

The method of preservation on agar slant is a classical method to store fungi. It consists in transferring the culture at frequent intervals, to suitable solid substrate depending on the microorganisms and the room conditions.

The objective of the present study was to evaluate the viability and sporulating capability of Coelomycetes strains preserved under mineral oil, in soil and on agar slant at Culture Collection of the Oswaldo Cruz Institute (IOC).

MATERIALS AND METHODS

A total of 45 Coelomycetes strains, most of them isolated from plant tissues, representing 27 species within 18 genera, were preserved under different storage regimes at IOC. From these, 34 were under mineral oil and among them 16 also were in soil, at room temperature, and 11 strains preserved on agar slants at 4°C. The strains stored at IOC since 1920's were kept on agar slant at room temperature until 1940's and after that they were preserved under mineral oil and in soil.

The storage periods changed from 4 to 50 years without a change of medium for strains under mineral oil; 32 to 39 years for those preserved in soil and 2 years for those on agar slant. Each strain preserved under mineral oil was distributed among a maximum of 5 tubes with different dates, whereas each of the strains preserved in soil and on agar slant was represented by 1 and 2 sample, respectively.

Thirty-four strains preserved on PDA (potato dextrose agar, Difco) under mineral oil and the 16 strains also preserved in soil were grown on PDA and incubated for 30 days. The viable strains were cultured in Petri dishes containing PDA, MEA (malt extract agar, Difco) and CMA (corn meal agar, Difco) and incubated for further studies of their morphology and sporulating capability. After 60 days of growth, strains which showed no sporulation were transferred to Petri dishes and tubes containing WA (water agar, 2% Bacto agar, Difco) with the addition of sterilized plant tissues, i.e. banana leaves, and alfalfa twigs, respectively.

Strains of endophytic fungi preserved on CMA slants were grown in Petri dishes with CMDA (corn meal dextrose agar, Difco). Strains that failed to sporulate were transferred onto tubes containing WA with alfalfa twigs and incubated.

Culture incubation was conducted under the same conditions for all fungal strains, i.e. a time of 12-h on/off cool white fluorescent light cycle under 21°C, according to the procedure shown in figure 1.

Figure 1. General flow-scheme used during evaluation of sporulation capability of fungal strains preserved in the culture collection.
PDA = potato dextrose agar
CMA = corn meal agar
MEA = malt extract agar
CMDA = corn meal dextrose agar
WA = water agar

RESULTS

Thirty-three of the 45 fungal strains studied were viable for a maximum of 45 years and a minimum of 2 years from the date of preservation. Of the 34 strains preserved under mineral oil, 20 were viable, and only one strain of *Pestalotiopsis guepinii* was able to sporulate after 3 years covered by 0.3-cm mineral oil

Of the 16 strains preserved in soil only *Septoria lycopersici* strain was viable but not able to sporulate.

Eleven endophytic strains, including *Colletotrichum gloeosporioides*, *Pestalotiopsis guepinii*, *Pestalotiopsis palmarum* and *Phomopsis* spp., which were preserved on CM agar slants maintained their viability and sporulating capability for 2 years, except for *Phomopsis* strains which were only able to sporulate after being cultured on sterilized alfalfa twigs. The only endophytic strain, a species of *Phomopsis*, stored under mineral oil, was viable after 8 years of storage but failed to sporulate after several attempts.

Table 1 shows the data concerning the viable strains preserved over different periods of time, which also gives the dates of entry in the Culture Collection (IOC) and the oil depth used for the storage. The following taxa showed no viability: *Colletotrichum lindemuthianum*, *Coryneum longistipitatum*, *Diplodina affinis*, *Pestalotia* sp. (IOC 2283), *Pestalotia cuboniana*, *Phlyctema* sp., *Phlyctema linicola*, *Phoma betae*, *Phoma cirsii*, *Phoma jolyana*, *Sphaeropsis eriobotryae*, *Stenocarpella maydis* (data not shown).

Table 1. Viability and sporulating capability of Coelomycetes strains preserved over different periods of time.

Taxon	Strains number	Entry date	Preservation year / method	Oil depth (cm)	Viability	Sporulation
Colletotrichum gloesporioides	19	1921	1982/MO	1,0	+	-
			1977/MO	1,0	-	-
			1977/MO	2,0	+	-
			1977/MO	1,3	-	-
			u/MO	0,5	+	-
Colletotrichum gloesporioides	2106	1947	1966/MO	0,5	+	-
			u/MO	0,5	+	-
			u/S	-	-	-
Colletotrichum gloesporioides	4107	1996	1996/AS	-	+	+
Colletotrichum gloesporioides	4121	1996	1996/AS	-	+	+
Colletotrichum gossypii	3297	1954	u/MO	0,5		-
			1957/MO	0,6	+	-
			u/S	-	-	-
Colletotrichum nigrum	2470	1947	1966/MO	0,5	+	-
			1966/MO	0,5	+	-
			1966/MO	0,8	-	-
			1966/S	-	-	-
Coniothyrium conicola	3065	1953	1966/MO	0,5	+	-
			1953/MO	0,5	+	-
			u/S	-	-	-
Diplodia cajani	3145	1954	1966/MO	0,2	+	-
			u/MO	0,5	+	-
			u/MO	0,6	+	-
Diplodia cajani	3375	1957	1966/MO	d	-	-
			u/MO	0,2	+	-
			u/MO	0,2	+	-
			u/MO	0,3	+	-
Discula fraxinea	3835	1990	1990/MO	0,5	+	-
Fusicoccum amygdali	3612	1966	1986/MO	0,5	+	-
			1986/MO	0,7	+	-
			1986/MO	0,5	+	-
			1966/MO	0,4	+	-
			u/S	-	-	-
Fusicoccum bacillares	3092	1953	1966/MO	0,5	-	-
			u/MO	0,3	+	-
			u/MO	0,2	+	-
Hendersonula toluroidea	3774	1983	1984/MO	0,2	+	-
			1984/MO	0,1	+	-
			1984/MO	0,1	+	-
Lasiodiplodia theobromae	3146	1954	1966/MO	d	-	-
			1957/MO	0,5	+	-
			u/MO	0,5	+	-
Pestalotia sp.	2060	u	1948/MO	0,5	-	-
			u/MO	0,5	+	-
			u/S	-	-	-
Pestalotiopsis guepinii	4009	1994	1994/MO	0,3	+	+
			1994/MO	1,0	+	+
			1994/MO	0,3	+	+
Pestalotiopsis guepinii	KFR 96-03	1996	1996/AS	-	+	+
Pestalotiopsis palmarum	4116	1996	1996/AS	-	+	+
Pestalotiopsis versicolor	3699	1983	1983/MO	1,0	+	-
			1987/MO	0,1	+	-
Pestalotiopsis sp.	3850	1990	1990/MO	0,5	+	-
Pestalotiopsis herbarum	2380	1947	1955/MO	0,5	-	-
			1953/MO	0,2	+	-
			u/S	-	-	-
Phomopsis oblonga	3095	1953	1966/MO	0,5	+	-
Phomopsis sp.	3831	1990	1990/MO	2,0	+	-
			1990/MO	1,7	c	c
Phomopsis sp.	4112	1996	1996/AS	-	+	+
Phomopsis sp.	4114	1996	1996/AS	-	+	+
Phomopsis sp.	KFR 97-09	1997	1997/AS	-	+	+
Phomopsis sp.	KFR 97-07	1997	1997/AS	-	+	+
Phomopsis sp.	KFR 97-06	1997	1997/AS	-	+	+
Phomopsis sp.	KFR 97-11	1997	1997/AS	-	+	+
Phomopsis sp.	KFR 97-10	1997	1997/AS	-	+	+
Pyrenochaeta romeroi	3431	1947	1960/MO	0,5	+	-
			1960/MO	0,5	+	-
			u/S	-	-	-
Pyrenochaeta romeroi	3695	1982	1982/MO	0,2	+	-
			1982/MO	0,2	+	-
Septoria lycopersici	2058	u	u/MO	0,8	-	-
			u/S	-	+	-

c = contaminated; u = unknown; d = dehydration of preservation medium; MO = sterile mineral oil; S = soil; AS = agar slant

DISCUSSION

The Coelomycetes studied here remained for long period of time covered by mineral oil, under a microaerobiosis condition, consequently reducing the capability of the fungi to sporulate. Long-term storage under mineral oil may lead to morphological and physiological alterations such as loss of sporulation capability, attenuation or loss of virulence, and others.

The oil depth used during preservation has been shown to affect the viability of fungal species. An oil layer of 0.2-0.5 cm prevents evaporation of water and dryness of the culture medium with sufficient oxygen while 1-cm layer or more creates relatively anaerobic conditions causing cellular damage [19]. The 34 strains preserved under mineral oil presented different oil depths (Table 1). Most of the viable strains were under an oil depth between 0.2 to 0.5 cm, confirming the results previously reported [19].

A long-term storage period and the oil depth are determinant factors for the success of the preservation method. The only strain able to sporulate was maintained for 3 years covered by 0.3 cm of mineral oil. Organisms maintained in culture collections and subjected to frequent transfers may lose some of their original metabolic activity. In addition, an oil deeper than 10 mm has proven harmful [5, 9].

Longevity of microorganisms under mineral oil varies considerably depending upon the species, storage, temperature, and probably culture media [13]. Several taxonomical groups of fungi have shown different responses according to the preservation method used [20-22]. A study of 33 strains of *Sporothrix schenckii* (Hyphomycetes) preserved under mineral oil in our culture collection showed revivals after storage period greater than 40 years, with 85% viability and maintenance of macro- and microscopic characteristics [15].

In relation to soil culture method of preservation only one strain was viable. The use of soil culture for long-term storage has been discouraged, especially for *Fusarium* sp., because of the risk of mutation and contamination by other organism [18]. Similarly, our viable strain did not sporulate after several attempts, in agreement with early report [23] in which the difficulties of various fungi to maintain viable cells in dry soil at room temperature had been suggested. Conversely, other investigators [24] reported 100% of viability and retention of pathogenicity in 24 strains of *Septoria* stored in soil up to 1 year. It may be that this genus survives better in soil than under mineral oil. We assume that our strain of *Septoria* has lost the sporulating capability because remained for a long time in soil. The register number of our samples indicates that they have been preserved by this means in the 1940's.

The endophytic species of Coelomycetes preserved for 2 years on agar slant, although viable did not sporulate when subcultured on several media, particularly *Phomopsis* strains that were only able to sporulate after the addition of sterilized alfalfa twigs on water agar. Maintaining endophytic fungi in culture collection has been a difficult task, especially after 1 year, because they lose their ability to produce reproductive structures in culture. Recently, a method for storing strains of grass endophytes, as agar slants within Eppendorf tubes topped by mineral oil, has been reported [25]. However, there are no reports concerning the effect of different storage regimes in relation to the morphological, biochemical and genetic stability of endophytic fungi preserved in culture collections.

The results reported here demonstrate that preservation of Coelomycetes fungi in culture collections demand attention, particularly because they may easily lose their sporulating capability. We recommend the use of the following fungal preservation methods, besides periodic monitoring: storage under 0,5 cm mineral oil layer; agar slant with sterilized plant tissue; and water storage based upon modified Castellani method [26,27]. The latter, has been considered very effective, especially for plant pathogens [27].

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