Viability, morphological characteristics and dimorphic ability of fungi preserved by different methods

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Summary

The viability, morphological characteristics and dimorphic ability of fungi were evaluated. Strain subcultures were maintained under mineral oil and in soil for different periods of time, ranging from 49 to eight years. Of the 16 *Blastomyces dermatitidis* strains, four maintained viability and were able to complete the dimorphic process to the M phase producing a large amount of conidia, but were unable to form Y cells at 36 °C. Of the 15 *Histoplasma capsulatum* var. *capsulatum* strains, only one was viable but it was impossible to check its identity because it lost sporulating and dimorphic ability. Of the 53 *Sporothrix schenckii* strains, 37 were viable, 28 able to sporulate and 12 of them completed the whole M ⇔ Y dimorphic process. All subcultures in soil became inviable. The results demonstrate that the preservation methods used here affected the morphology and sporulating and dimorphic ability of the strains. *B. dermatitidis* and *S. schenckii* were considered to be species that survive better than *H. capsulatum* var. *capsulatum* under mineral oil. Thus, it is necessary to establish routine monitoring and appropriate environmental and culture conditions, using less widely spaced transplants and choosing the exact time of intervention to induce growth and development restriction in each strain.

Key words

Dimorphic fungi, Long-term preservation, Viability, Morphological characteristics, Physiological characteristics, Culture collection

Viabilidad, características morfológicas y capacidad dimórfica de hongos conservados según diferentes métodos

Resumen

Se evaluó la viabilidad, las características morfológicas y la capacidad dimórfica de los hongos. Los subcultivos de las cepas se mantuvieron en aceite mineral y en suelo durante diferentes periodos de tiempo desde 49 hasta ocho años. De las 16 cepas de *Blastomyces dermatitidis* cuatro fueron viables y capaces de completar el proceso dimórfico hacia la fase M produciendo gran cantidad de conidios, pero incapaces de formar levaduras a 36 °C. De las 15 cepas de *Histoplasma capsulatum* var. *capsulatum* sólo una se mantuvo viable pero no fue posible comprobar su identidad porque perdió su capacidad dimórfica y de esporulación. De las 53 cepas de *Sporothrix schenckii*, 37 fueron viables, 28 capaces de esporular y 12 completaron el proceso dimórfico M ⇔ Y. Todos los subcultivos en suelo fueron inviables. Los resultados demuestran que los métodos de conservación utilizados afectaron a la morfología y a las capacidades de esporulación y de dimorfismo de las cepas. Se considera que *B. dermatitidis* y *S. schenckii* son especies que sobreviven mejor en aceite mineral que *H. capsulatum* var. *capsulatum.* Es por tanto necesario establecer un control de rutina con condiciones ambientales y de cultivo adecuadas, utilizando resiembras menos espaciadas en el tiempo y eligiendo el tiempo exacto de intervención en cada cepa para inducir restricciones de crecimiento y desarrollo

Palabras clave

Hongos dimórficos, Conservación a largo plazo, Viabilidad, Características morfológicas, Características fisiológicas, Colección de cultivos

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Blastomyces dermatitidis, *Histoplasma capsulatum* var. *capsulatum*, and *Sporothrix schenckii* are dimorphic fungi pathogenic to man. They are preserved in the Culture Collection of the Oswaldo Cruz Institute by different methods of storage for long periods of time.

Methods for the preservation of fungi under mineral oil and in soil have been used in many laboratories with results differing according to species [1-4]. However, some studies have been demonstrated that the storage of fungi *in vitro* for long periods of time may induce morphological changes [5], alterations in cell wall components [6], and loss or attenuation of virulence [7].

The preservation of fungi pathogenic to man and animals is important for research and biotechnology. Thus, the determination of the correct preservation method for each fungal species and periodical monitoring to check their morphology, pathogenicity and genetic stability has become a requirement, and studies are necessary to understand the problems related to storage conditions in order to select the best preservation method and to isolate avirulent strains [8] of great value for biochemical and molecular research.

The objective of the present study was to evaluate the viability, morphology and dimorphic ability of fungal strains preserved under mineral oil and in soil at the Culture Collection of the Oswaldo Cruz Institute (IOC).

MATERIALS AND METHODS

A total of 84 dimorphic fungal strains (16 *Blastomyces dermatitidis*, 15 *Histoplasma capsulatum* var. *capsulatum*, and 53 *Sporothrix schenckii* strains) isolated from humans and animals were first preserved at IOC by serial transfers and maintained at room temperature. In the 1940 decade the strains were transferred to potato dextrose agar medium (PDA) under mineral oil. Each strain preserved under mineral oil was distributed among a maximum of seven tubes with different dates, whereas each of the strains preserved in soil was represented by one sample.

Samples of the strains submitted to the different preservation methods were grown on PDA (Difco, USA) and incubated for 60 days at room temperature to evaluate viability. The viable strains were examined for morphology and sporulating ability on Sabouraud agar and PYG agar media (0.5% peptone, 0.5% yeast extract, 2% dextrose, and 1.5% agar, Difco). After 60 days of growth, strains which showed no sporulation were transferred to tubes containing malt extract agar (MEA, Difco) and corn meal agar (CMA, Difco). Microcultures of *S. schenckii* were done to observe the characteristic of the conidiomata without damage.

The M ⇒ Y dimorphic process was evaluated by subculturing *B. dermatitidis* and *H. capsulatum* var. *capsulatum* strains on PYG agar and SABHI agar (50 ml brain heart infusion, Oxoid; 50 ml Sabouraud, Difco, and 1.5% agar, Difco) and incubating at 36 °C in a model 347 stove (FANEM, São Paulo, Brasil). *S. schenckii* and *B. dermatitidis* strains were cultured in BHI broth (3.7% brain heart infusion, Oxoid) and SABHI broth, respectively, at 36 °C in a shaker at 120 oscillations/min (Edmund Bühler model SM-30 control, Germany) and subcultures were done each seven days to obtain the yeast phase. Passages were done three times and the cells prepared in Amann lactophenol-cotton blue [5] were monitored with a model Axiophot Zeiss light microscope (Germany).

Figure 1 shows the procedure followed to take the strains from the preservation methods and their evaluation.

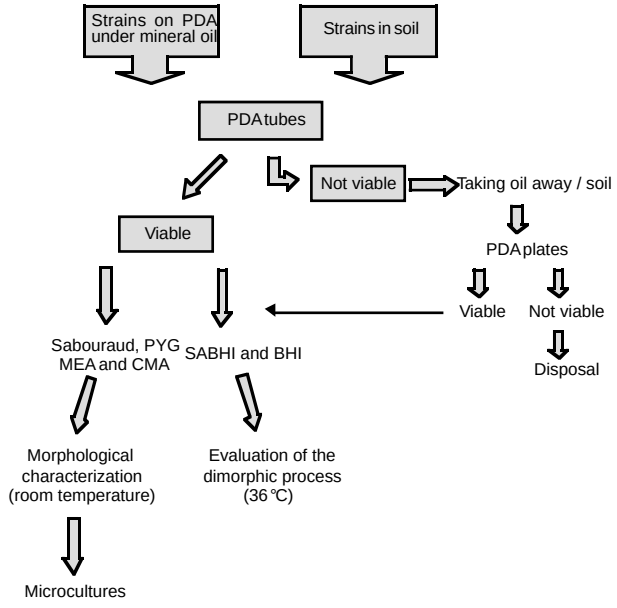

Figure 1. Schematic representation of the evaluation of viability, morphological characterization and dimorphic ability of fungal strains preserved in a culture collection. PDA = potato dextrose agar; MEA = malt extract agar; CMA = corn meal agar; PYG = peptone, yeast extract and glucose; SABHI = Sabouraud and brain heart infusion; BHI = brain heart infusion.

RESULTS

Of the total number of *B. dermatitidis* strains (16), maintained under oil, only four were viable (25%) for a maximum period of 37 years and for a minimum period of 21 years. Of the 15 *H. capsulatum* var. *capsulatum* strains preserved from 48 to 15 years, only one was viable and after 15 years under oil. Of 53 *S. schenckii* strains maintained under mineral oil, 37 were viable (69.8%) for a period ranging from 49 to eight years.

Tables 1 and 2 show the data concerning the viable strains preserved over different periods of time.

None of the strains maintained in soil were viable.

Table 1. Viability of *B. dermatitidis* strains preserved under mineral oil for different periods of time.

No of strain	Entry date	Preservation year	Oil depth (cm)	Viability
1183	1928	1981	d	-
		1981	d	-
		1978	0.3	-
		1978	0.2	-
		1978	0.2	+
		1978	0.3	+
		u	0.3	-
1297	1929	1962	0.3	+
		u	d	-
		u	d	-
		u	d	-
1298	1929	1962	0.7	+
		1966	d	-
		u	0.5	+
1300	1929	1966	d	-
		u	0.4	+
		u	d	-

u = unknown; d = dehydration of preservation medium

Table 2. Viability of *S. schenckii* strains preserved under mineral oil for different periods of time.

No of strain	Entry date	Preservation year	Oil depth (cm)	Viability
1113	1927	1983	2.2	+
		1983	3.7	+
		1966	0.8	-
		1962	1.2	-
		1957	1.9	-
1146	1927	1953	0.3	+
		u	1.2	+
1186	1928	u	0.7	+
1226	1928	1953	0.7	-
		u	1.1	+
		u	0.6	+
1232	1927	1983	2.4	+
		1983	2.6	-
		1957	0.3	-
		1958	0.6	-
		u	1.2	-
		u	0.6	+
1275	1929	1982	2.3	+
		1982	1.4	+
		1966	1.3	-
		1962	0.9	-
		u	1.3	-
		u	1.1	-
1697	1934	1991	0.7	+
		1991	0.8	+
		u	1.5	-
		u	1.9	+
		u	0.6	-
1791	1935	1983	2.9	-
		1983	2.1	+
		u	1.4	+
1799	1935	u	1.7	+
		u	2.2	+
		u	0.6	+
1806	1935	u	2.1	+
		u	1.9	+
		u	0.3	-
		u	1.3	+
		u	0.9	+
		u	0.4	+
1824	u	1966	1,3	-
		1961	0,8	+
		u	0,5	+
1835	1936	1983	2,9	+
		1983	2,0	+
		u	0,3	+
		u	0,3	-
1846	u	1957	0.9	-
		u	1.9	+
		u	1.4	+
1904	1945	1983	2.7	+
		1983	2.4	+
		1960	1.0	-
		u	0.7	+
		u	0.6	+
1912	1945	1983	2.4	+
		1983	2.1	+
		1961	0.3	-
		u	1.4	+
		u	1.3	-
2047	u	u	1.3	+
2314	1947	1981	0.6	+
		1978	0.6	+
		1978	1.0	+
		1978	0.6	+
		1978	0.6	-
2547	1948	1983	2.4	+
		1983	3.4	-
		1953	0.4	+
		u	0.4	-
		u	0.4	-
		u	0.5	+
2562	1948	1983	2.5	+
		1983	2.6	+
		1957	0.4	-
		1966	0.6	-
2832	1950	1959	0.8	+
		1950	1.0	+
2835	1950	1983	2.4	+
		1983	2.5	+
		1957	1.5	+
		u	1.5	-
2843	1950	1959	0.6	+
		u	1.8	+
		u	0.6	-
2844	1950	1983	2,0	+
		1983	2,4	+
		1944	0,5	-
			0,3	-
2845	1950	1958	0,3	+
2847	1950	1983	1.9	+
		1983	2.9	+
		1944	0.4	-
		u	1.9	-
		u	1.7	-
2848	1950	1983	2.5	+
		1983	2.6	+
		u	0.3	+
		u	1.6	-
		u	1.4	-
		u	0.4	-
2849	1950	u	2.6	+
2852	1950	u	1.1	-
		u	2.1	+
		u	0.9	-
2855	1950	u	2.2	+
		u	2.5	-
2993	1951	1980	1.1	+
		1980	0.8	+
		1980	1.5	-
		1980	1.0	-
		1977	0.9	+
		1977	1.1	-
		1977	1.5	-
3108	1953	1966	0.7	+
		u	1.8	-
3362	1956	1983	2.4	+
		1983	2.6	-
		1966	0.9	+
		1957	1.9	+
		u	0.7	-
		u	0.3	-
3411	1958	u	1.8	+
3429	1959	1982	2.4	+
		1982	2.1	+
		1960	1.8	-
		u	0.3	-
3606	1965	1980	1.1	+
		1980	0.5	+
		u	0.2	-
		u	0.6	-
3645	1971	1986	2.2	-
		1986	2.1	+
		1983	1.9	+
		1983	2.3	+
		u	0.6	-
		u	1.2	-
3735	1983	1984	1.3	+
		1984	1.3	+
		1984	2.2	-

u = unknown

The four viable *B. dermatitidis* strains, maintained under oil without a change of medium, presented microscopic morphology with the following characteristics: thin and thick filaments with interseptal and terminal dilatations. Some of the dilatations, similar to yeast (Y) cells, presented a cell wall thicker than the mycelial (M) cell wall (Figure 2).When the strains were subcultured on PYG and Sabouraud agar at room temperature, their micromorphological characteristics did not differ significantly from those of strains preserved under oil. However, when the strains were subcultured on CMA at room temperature, large numbers of conidia were produced, showing that the dimorphic process had been completed to the M phase (Figure 3).

The only viable *H. capsulatum* var. *capsulatum* strain grew on PYG and SABHI agar at room temperature as membranous cream colonies with some aerial mycelium. At 36°C the colonies were membranous with a wrinkled surface cream to brown in color. Microscopic morphology showed thin and thick filaments with dilatations at room temperature and at 36 °C. On the other hand, on Sabouraud agar the colonies grew with short aerial mycelium with floccose texture, white in color. The same transitional morphology was seen. However it was impossible to determine the fungal identity in the different media used because of their inability to complete the whole dimorphic process (M $\Leftrightarrow$ Y).

The 37 viable *S. schenckii* strains grew as dirty white membranous colonies with or without dark brown pigment. Micromorphological study revealed septate hyaline hyphae and oval, pyriform or elongated hyaline conidia. Conidia emerged singly or in clusters or bouquets at the top of fertile erect branches in 28 strains (Figure 4). Pigmented spherical to conical conidia were also observed (data not shown) [9]. Our group observed the same characteristics in a previous study on some of these strains [10], in which, however, we did not evaluate their ability to complete the dimorphic process.

The experiment carried out here to evaluate the ability to complete the dimorphic process (M $\Rightarrow$ Y) showed that 12 *S. schenckii* strains were able to produce typical Y cells in BHI broth at 36 °C (Figure 5) while the other strains remained in the transitional phase (data not shown). *B. dermatitidis* strains grew in SABHI broth in the M phase at 36 °C. However, when they were subcultured on SABHI agar at the same temperature they produced transitional forms with dilatations similar to Y cells (Figures 6 and 7).

The longevity (years) of the strain subcultures with known dates of storage under mineral oil is presented in table 3.

Table 3. Longevity of subcultures of *B. dermatitidis*, *H. capsulatum* var. *capsulatum* and *S. schenckii* strains preserved under mineral oil.

No of strain	Longevity*	No of strain	Longevity*
Bd 1183	21	Ss 2562	17
Bd 1297	37	Ss 2832	40/49
Bd 1298	37	Ss 2835	17/43
Hc 3734	15	Ss 2843	40
Ss 1113	16	Ss 2844	17
Ss 1146	46	Ss 2845	41
Ss 1232	16	Ss 2847	17
Ss 1275	17	Ss 2848	17
Ss 1697	8	Ss 2993	20/23
Ss 1791	16	Ss 3108	33
Ss 1824	38	Ss 3362	17/34/43
Ss 1835	16	Ss 3429	18
Ss 1904	16	Ss 3606	20
Ss 1912	16	Ss 3645	14/17
Ss 2314	18/21	Ss 3735	16
Ss 2547	17/47		

* Number of years of survival without a change in medium.
Bd = *B. dermatitidis*; Hc = *H. capsulatum* var. *capsulatum*; Ss = *S. schenckii*

DISCUSSION

The dimorphic fungi studied here were kept for long periods of time under mineral oil. The microaerobiosis, the loss of nutrients and the accumulation of toxic metabolites during the process of reduction of metabolic rates on which this preservation method is based [11] affected the development, the formation of conidia and the dimorphic process of the majority of strains evaluated. However, there were differences among species as to their ability to survive the preservation methods. Barnes [2] reported that the longevity of microorganisms, mainly under mineral oil, varies with species, temperature, and probably with culture medium. Other important factors are the exact time to reduce the metabolic activity, the inoculum age, the depth of the layer and the quality of the mineral oil used [12-14].

According to Pumpyanskaya [14], an oil layer of 0.2 to 0.5 cm prevents dehydration of the medium and allows sufficient oxygenation, while a layer of 1 cm or more creates relatively anaerobic conditions that can lead to cellular damage. Most of our strains were preserved under an oil layer of more than 0.6 cm, a fact that probably contributed to high cell death rates, except for *S. schenckii* strains that remained viable for 49 years (Ss 2832) and 16 years (Ss 1113) under an oil layer of 1 cm and 3.7 cm, respectively.

Phenotypic study of the viable strains harvested from oil showed that in *B. dermatitidis* there was abundance of intermediate forms between mycelial and yeast phases at room temperature and at 36 °C, similar to the forms studied by Mendes da Silva *et al.* [5]. *B. dermatitidis* strains were isolated and preserved under mineral oil in yeast forms and remained for long periods of time at room temperature varying from 22 to 35 °C. So it is possible that the dimorphic process started under oil, forming the transitional configurations *in situ*, which were probably more sensitive to low oxygen concentration and to the toxic metabolites that are accumulated under oil, leading to the loss of viability for most strains and to irreversible M $\Rightarrow$ Y transition for the viable strains in the media tested. But when they were cultured in poor medium at room temperature all of them were able to sporulate, suggesting that nutritional factors may be involved in the dimorphic process after a long period of latency. Spontaneous mutation after many passages *in vitro* at 35-37 °C was observed in one *B. dermatitidis* strain [15]. The authors reported that the mutant strain was attenuated in virulence to mice and showed a slower conversion to the mycelial phase at 22 °C compared to the virulent strain.

In the present study, the agar added to the SABHI medium for *B. dermatitidis* strains somehow permitted the formation of transitional forms with dilatations similar to Y cells at 36 °C, a fact that did not occur when no agar was added to the same medium. Physical and electrochemical factors of the macromolecular pattern of the agar used here, when in contact with cells, may play an important role in the dimorphic process of these strains. Furthermore, according to Lilly and Barnett [16], agar introduces physiologically active elements into the medium, possibly containing significant amounts of zinc and other essential trace elements.

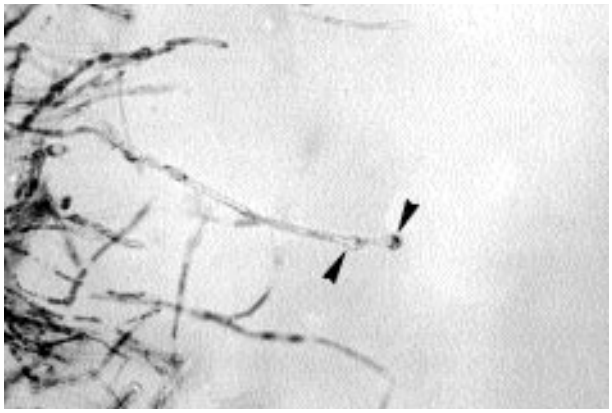

Figure 2. Morphological characteristics of *B. dermatitidis* under mineral oil. Presence of thin and thick filaments with interseptal and terminal dilatations similar to yeast cell (arrowheads). Original magnification 400x.

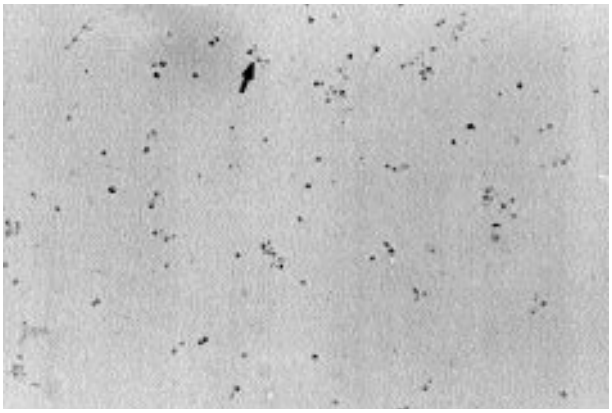

Figure 5. Micromorphological characteristics of *S. schenckii* in BHI broth at 36 °C. Production of typical yeast cells (arrow). Original magnification 400x.

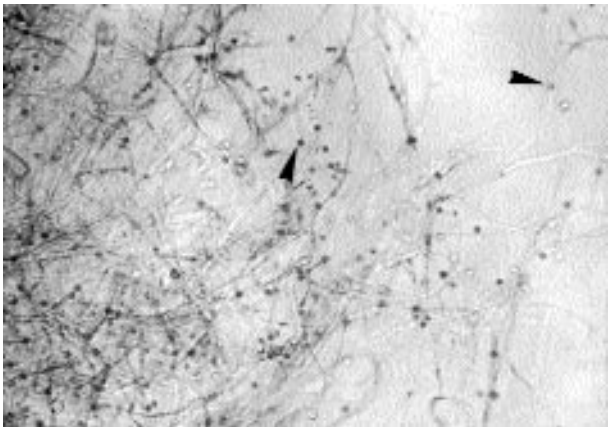

Figure 3. *B. dermatitidis* growing on CMA at room temperature. Presence of conidia (arrowheads). Original magnification 400x.

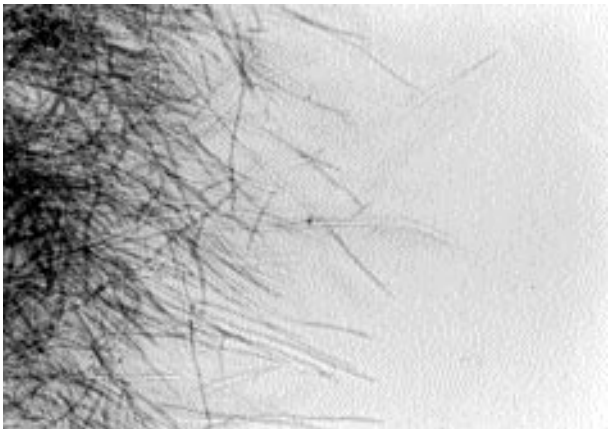

Figure 6. *B. dermatitidis* growing in SABHI broth at 36 °C. Only filaments were produced. Original magnification 400x.

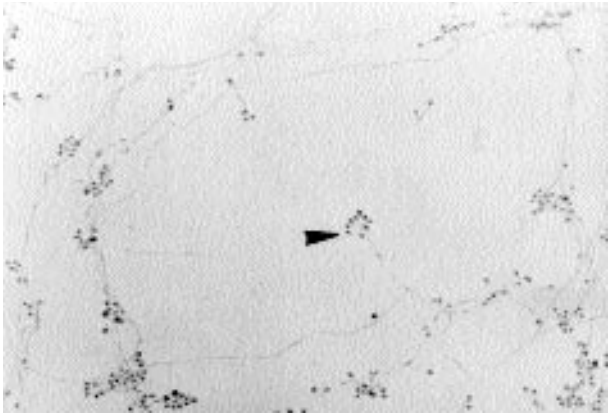

Figure 4. Microculture of *S. schenckii* on MEA. Conidia emerged singly or in bouquets at the top of fertile erect branches (arrowhead). Original magnification 400x.

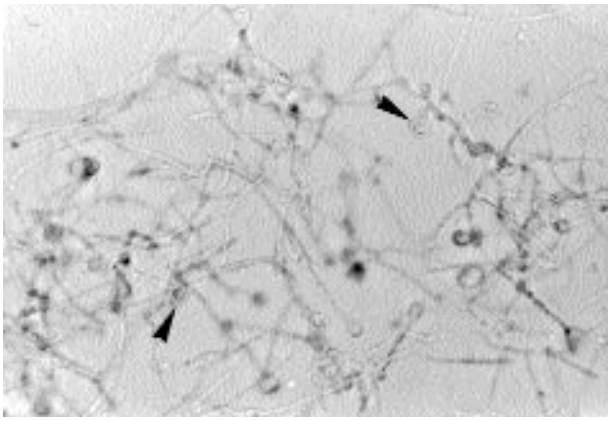

Figure 7. *B. dermatitidis* growing on SABHI agar at 36 °C. Formation of transitional forms with dilatations similar to Y cells (arrowheads). Original magnification 400x.

H. capsulatum var. capsulatum strains were kept under oil in the mycelial phase and maintained in this phase at room temperature and at 36 °C. The alterations suffered during preservation were the loss of the ability to sporulate and to complete the dimorphic process under the conditions used here. Klimpel and Goldman [17,18] by a simple selection strategy based on successive subculturing of nonclumping yeast cells, produced a series of stable avirulent clones from virulent strains. These clones did not have a-1.3 glucan in the cell wall, grew in a disperse way in broth medium and produced colonies with smooth texture when compared to colonies of the virulent parental cells. On this basis, we concluded that *H. capsulatum var. capsulatum* strains easily lose their original characteristics when kept *in vitro*. Thus, the low viability and the loss of the ability to sporulate and of dimorphic competence of the strains studied clearly show the high requirements of this fungus for the factors related to good development.

S. schenckii strains survived better than other species studied here since they presented typical macro- and microscopic morphology and good sporulation and some of them were able to complete the dimorphic process (32.4%). The data obtained agree with those reported by our group in a previous study [10] and confirm the fact that mineral oil is efficient in preserving *S. schenckii* strains.

None of the strains studied survived when preserved in soil, in agreement with Windels [4], who reported that the use of soil culture for long-term storage may cause mutations with lost morpho- and physiological characteristics or cellular death.

The results reported here demonstrate that preservation of fungi in culture collections requires appropriate environmental conditions and culture, less widely spaced transplants and the choice of the exact time of intervention to induce growth and development restriction in each strain, an essential factor for successful preservation, in addition to periodic monitoring.

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