NOTAS CIENTÍFICAS

ENTOMOPATHOGENIC FUNGAL (HYPHOMYCETES) COLLECTION: ASSESSMENT OF CONIDIAL VIABILITY[1]

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ABSTRACT - Twenty four strains of the entomopathogenic fungi (Hyphomycetes) *Beauveria bassiana*, *Metarrhizium anisopliae*, *Nomuraea rileyi*, *Paecilomyces farinosus*, *P. fumosoroseus* and *P. lilacinus*, maintained in the culture collection of Embrapa-Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia (Cenargen) and preserved by lyophilization and in liquid nitrogen, had their conidial viability assessed. Germination rates of 16- to 84-month-old cultures stored in liquid nitrogen decreased, on average, less than 13.3%. For 29- to 49-month-old cultures preserved by lyophilization, the viability loss ranged, on average, from 28.6 to 94.5%. The results demonstrated the efficiency of the tested methods, especially liquid nitrogen, in preserving the viability of entomopathogenic fungi.

COLEÇÃO DE FUNGOS ENTOMOPATOGÊNICOS (HYPHOMYCETES): AVALIAÇÃO DA VIABILIDADE CONIDIAL

RESUMO - Vinte e quatro isolados dos fungos entomopatogênicos (Hyphomycetes) *Beauveria bassiana*, *Metarrhizium anisopliae*, *Nomuraea rileyi*, *Paecilomyces farinosus*, *P. fumosoroseus* e *P. lilacinus*, mantidos na coleção de culturas da Embrapa-Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia (Cenargen) e preservados em nitrogênio líquido e liofilização, tiveram a viabilidade conidial avaliada. As taxas de germinação de conídios preservados em nitrogênio líquido diminuíram, em média, menos de 13,3% após 16 a 84 meses de armazenamento. A perda de viabilidade de isolados liofilizados, após 29 a 49 meses de armazenamento, variou de 28,6 a 94,5%. Os resultados demonstraram a eficiência dos métodos testados, especialmente nitrogênio líquido, na preservação da viabilidade de fungos entomopatogênicos.

In Brazil, the occurrence of over 20 genera of entomopathogenic fungi has been reported (Alves, 1992). Embrapa-Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia (Cenargen), in Brasília, Brazil, is engaged in establishing an entomopathogenic fungal culture collection

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(Embrapa, 1996), to support biocontrol research involving these microorganisms. Activities such as acquisition, preservation, identification and distribution of strains have been carried out during the last eight years. Currently, this collection holds approximately 600 strains belonging to 16 species and eight genera with potential for use in biocontrol programs.

This study was carried out to quantitatively determine the viability of entomopathogenic fungi after long-term preservation, considering the lack of information regarding the storage of this group of natural enemies. Fungal strains belonging to species *Beauveria bassiana*, *Metarrhizium anisopliae*,

TABLE 1. Origin of entomopathogenic fungal isolates and storage method tested.

Fungus	Isolate	Host/Substrate	Origin	Year	Storage method[1]
Beauveria bassiana	CG001	*Deois flavopicta*	Brasília, DF	1984	L
	CG004	*Deois flavopicta*	Brasília, DF	1984	L
	CG010	*Nezara viridula*	Londrina, PR	1988	L
	CG011	*Sternechus subsignatus*	Londrina, PR	1988	L
	CG014	*Podisus* sp.	Londrina, PR	1988	N
	CG019	*Nezara viridula*	Londrina, PR	1981	N
	CG021	Pentatomidae	France	1973	N
	CG022	*Nezara viridula*	Londrina, PR	1983	N
Metarrhizium anisopliae	CG028	*Mahanarva posticata*	Alagoas	-	N
	CG033	*Deois flavopicta*	Mato Grosso	-	N
	CG043	*Zulia entreriana*	Minas Gerais	1988	L
	CG053	*Deois flavopicta*	Brasília, DF	1986	N,L
	CG055	*Deois flavopicta*	Brasília, DF	1988	L
	CG057	*Deois flavopicta*	Brasília, DF	1988	L
	CG097	Scarabaeidae	Brasília, DF	1988	N
Nomuraea rileyi	CG130	*Spodoptera frugiperda*	Brasília, DF	1988	L
	CG132	*Anticarsia gemmatalis*	Brasília, DF	1988	L
	CG379	*Spodoptera frugiperda*	Brasília, DF	1989	L
	CG382	*Spodoptera frugiperda*	Brasília, DF	1990	N
	CG385	*Anticarsia gemmatalis*	Brasília, DF	1991	N,L
	CG388	*Anticarsia gemmatalis*	Brasília, DF	1991	N
	CG391	*Anticarsia gemmatalis*	Brasília, DF	1991	N
Paecilomyces farinosus	CG189	Soil	Brasília, DF	1991	N
	CG195	*Chlosyne lacinia*	Londrina, PR	1983	N
	CG196	Tachinidae	Poland	1983	L
	CG197	*Pyrrhalta luteola*	France	1983	L
	CG198	*Aedes sierrensis*	California, USA	1985	N,L
	CG199	*Lymantria dispar*	Maryland, USA	1986	L
Paecilomyces fumosoroseus	CG202	*Musca domestica*	France	1985	L
	CG203	*Bemisia tabaci*	Florida, USA	1990	N,L
	CG204	*Bemisia* sp.	Mexico	1990	N
	CG205	*Bemisia tabaci*	Florida, USA	1992	N,L
	CG269	Soil	Minas Gerais	1991	N
	CG334	Soil	Campinas, SP	1990	L
Paecilomyces lilacinus	CG036	*Deois flavopicta*	Brasília, DF	1991	L
	CG175	*Meloidogyne* sp.	Castanhal, PA	1992	N
	CG262	Soil	Tocantínia, TO	1991	N,L
	CG263	Soil	Minas Gerais	1991	L
	CG264	Soil	Itacarambi, MG	1991	N
	CG266	Soil	Balsas, MA	1991	L
	CG267	Soil	Goiás	1991	N

[1] N: liquid nitrogen; L: lyophilization.

Nomuraea rileyi, *Paecilomyces farinosus*, *P. fumosoroseus* and *P. lilacinus* were used (Table 1). Conidial viability of tested strains was not determined months before, when the preservation methods were applied. Therefore, germination rates of both fresh conidia and 24 hours following preservation procedures were estimated to allow assess the loss of viability over time. In order to do that, cultures originally stored in liquid nitrogen using 10% glycerol as protectant were thawed, and a conidial suspension plated on complete medium ($FeSO_4$ 0.001 g; KCl 0.5 g; KH_2PO_4 1.5 g; $NaNO_3$ 6 g; $ZnSO_4$ 0.001 g; hydrolyzed casein 1.5 g; yeast extract 0.5 g; glucose 10 g; peptone 2 g; agar 20 g, per 1 liter), followed by incubation at 28°C in darkness. *N. rileyi* strains were cultivated on SMAY (Sabouraud Maltose Agar with yeast extract 10 g; maltose 40 g; neopeptone 10 g; agar 20 g; per 1 liter) at 25°C. For each strain, conidia produced on the surface of culture media after 10 days incubation were harvested with a spatula and added to an aqueous suspension (0.1% Tween 80).

To determine viability before applying the preservation procedure, 100 μL of the conidial suspension containing approximately $8x10^6$ conidia mL^{-1} were spread on solid culture medium. Viability of cultures was estimated by microscopically assessing the conidial germination percentage between 18 and 48 hours following incubation, depending on tested strain. One hundred conidia were counted in each of four microscopic fields.

For lyophilization of conidia, a mixture of 3% glucose (Merck, Rio de Janeiro, Brazil) and 3% gelatin (Sigma, St. Louis, MO, USA) was used as the suspending medium. Glass ampoules (100 x 6 mm) containing cultures were kept at -80°C for 30 minutes before transferring to a Lyph-Lock 18 lyophilization apparatus (Labconco, Missouri, USA). After overnight lyophilization, ampoules were sealed under vacuum and stored at 4°C. For cryopreservation, a conidial suspension was prepared in 10% glycerol and transferred to 1.0 mL Nunc cryotubes. The cryotubes were kept at -20°C for approximately 20 minutes, before transferring to liquid nitrogen (-196°C).

Following storage for 24 hours in liquid nitrogen and by lyophilization, cryotubes and ampoules, respectively, had their contents resuspended in a 0.1% Tween 80 solution. Samples kept in liquid nitrogen were directly removed to room temperature. The percentage of conidial germination was then assessed as indicated above. To estimate the efficiency of the two methods after long-term storage, the same procedure was used for cultures kept in liquid nitrogen for periods varying from 16 to 84 months, and lyophilized cultures stored for 29 to 49 months. There was only one experimental replicate per strain because a limited number of vials, usually two, were stored months earlier.

A small reduction in conidial viability was observed 24 hours following storage in liquid nitrogen. The average reduction of germination ranged from 0.1 to 10.3%, with best results being observed for *B. bassiana* and *Paecilomyces* spp. (Table 2). For long-term storage in liquid nitrogen, the average reduction of germination varied from 0.2 to 5.9%, with the exception of *N. rileyi*. Considerable intraspecific variation was recorded for *N. rileyi*, for which the estimated total viability reduction ranged from 0 to 29.9%. For *B. bassiana*,

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TABLE 2. Viability of entomopathogenic fungal isolates before and after preservation in liquid nitrogen and by lyophilization.

Fungus	Liquid nitrogen					Lyophilization				
	Isolate	Months stored[1]	Germination rate (%)			Isolate	Months stored[1]	Germination rate (%)		
			Before storage	Reduction after 24 hours[2]	Total reduction[3]			Before storage	Reduction after 24 hours[2]	Total reduction[3]
B. bassiana	CG014	84	99.5	0.2	4.2	CG001	49	44.0	16.4	80.7
	CG019	84	99.8	0.8	1.5	CG004	49	99.8	3.3	40.9
	CG021	84	100.0	0.0	8.0	CG010	49	100.0	3.5	93.7
	CG022	84	100.0	0.2	8.2	CG011	48	100.0	2.2	43.0
				(a=0.3)[4]	(a=5.5)				(a=6.4)	(a=64.6)
M. anisopliae	CG028	84	100.0	16.2	6.0	CG043	47	98.5	45.4	95.4
	CG033	84	100.0	8.2	5.0	CG053	48	63.8	22.7	96.9
	CG053	84	99.8	8.5	3.5	CG055	48	98.3	44.8	96.1
	CG097	84	100.0	8.2	9.2	CG057	48	95.5	58.6	89.5
				(a=10.3)	(a=5.9)				(a=42.9)	(a=94.5)
N. rileyi	CG382	18	76.3	18.1	29.9	CG130	39	49.0	37.1	13.3
	CG388	17	81.5	9.2	0.0	CG132	29	62.8	8.0	32.3
	CG385	16	93.8	1.1	23.5	CG379	29	53.5	0.0	46.7
	CG391	17	88.3	5.7	0.0	CG385	29	96.0	35.9	72.1
				(a=8.5)	(a=13.4)				(a=20.3)	(a=41.1)

Continuation...

TABLE 2. Continuation.

Fungus	Liquid nitrogen					Lyophilization				
	Isolate	Months stored[1]	Germination rate (%)			Isolate	Months stored[1]	Germination rate (%)		
			Before storage	Reduction after 24 hours[2]	Total reduction[3]			Before storage	Reduction after 24 hours[2]	Total reduction[3]
P. farinosus	CG189	40	100.0	1.5	3.7	CG196	46	100.0	3.0	21.7
	CG195	50	100.0	0.0	10.2	CG197	47	100.0	2.2	44.3
	CG198	48	98.5	0.0	0.5	CG198	47	98.5	18.3	40.8
				(a=0.5)	(a=4.8)	CG199	47	100.0	0.0	16.2
									(a=5.9)	(a=30.8)
P. fumosoroseus	CG203	50	99.5	0.0	5.5	CG202	47	100.0	9.5	53.5
	CG204	50	100.0	0.5	10.0	CG203	47	99.5	9.2	89.1
	CG205	48	100.0	0.0	0.0	CG205	47	100.0	15.5	84.7
	CG269	49	100.0	0.0	0.0	CG334	47	100.0	17.0	99.5
				(a=0.1)	(a=3.9)				(a=12.8)	(a=81.7)
P. lilacinus	CG175	47	100.0	0.2	0.5	CG036	48	100.0	6.7	37.5
	CG262	42	100.0	2.0	0.0	CG262	48	100.0	2.2	28.5
	CG264	49	100.0	0.0	0.2	CG263	48	100.0	0.7	30.5
	CG267	48	100.0	5.2	0.0	CG266	48	94.8	2.6	17.9
				(a=1.9)	(a=0.2)				(a=3.1)	(a=28.6)

[1] Months under storage.
[2] Viability reduction due to storage procedure, calculated by comparing the germination rate observed 24 hours after storage with the germination rate observed before storage.
[3] Estimated total viability reduction, calculated by comparing the long-term germination rate with the germination rate before storage; one replicate performed per isolate.
[4] Group average.

P. farinosus and *P. fumosoroseus* the reduction of viability was more severe during the storage period, when compared to *M. anisopliae* and most strains of *N. rileyi*, for which the loss of viability seemed to be greater during the storage procedure itself.

Following lyophilization, *B. bassiana* and *Paecilomyces* spp. showed low loss of viability after 24 hours. Reduction in germination rates ranged from 3.1% for *P. lilacinus* to 42.9% for *M. anisopliae*. After long-term storage, the estimated loss of viability varied from 28.6% for *P. lilacinus* to 94.5% for *M. anisopliae*. In the present study, total loss of viability for *P. lilacinus* varied from 17.9 to 37.5% after four years storage. Recently, Rybnikár (1995) reported losses in viability during storage ranging from 30.4 to 96.6% for 26 lyophilized cultures; for instance, the loss of viability for *P. lilacinus* was 56.1% after 11 years.

Our results demonstrated an evident reduction of viability of *M. anisopliae* and *N. rileyi* due to the storage procedure, especially when lyophilized. For both liquid nitrogen storage and lyophilization, it was observed that for some strains the viability reduction due to the storage procedure was slightly greater than the estimated total loss of viability. The use of one replicate for each strain and the utilization of viability loss based on conidial germination of the strains stored at different times may explain these results.

With the exception of a few reports (Bunse & Steigleder, 1991; Rybnikár, 1995), in which the reduction in viability of lyophilized cultures was quantified by recording the number of Colony Forming Units (CFUs), previous publications dealing with the efficiency of long-term preservation of fungi considered only the capability of growth on synthetic media. According to this criterion, all strains used in the present study were viable following lyophilization, although the germination rates were extremely low in certain cases. For example, the germination rate of strain CG334 (*P. fumosoroseus)* was 0.5% after 47 months storage.

For the six strains stored in both liquid nitrogen and lyophilization, viability 24 hours after storage and following long-term storage (16 to 84 months) was considerably higher when cultures were preserved in liquid nitrogen. For cultures maintained in liquid nitrogen, loss of conidial viability following preservation for 24 hours and long periods varied from 0 to 8.5% and from 0 to 23.5%, respectively. In contrast, losses for lyophilized cultures ranged from 2.2 to 35.9% and 28.5 to 96.9%, respectively. The satisfactory results obtained with cryopreservation in this study support the idea that liquid nitrogen is the most reliable method for long-term preservation of fungi (Goos et al., 1967; Stalpers et al., 1987).

Although attempts to improve conidial viability of lyophilized fungal cultures were not successful, such as the combinations of protectants [skim milk (10%), glutamic acid (5%) and raffinose (10%)] suggested by Berny & Hennebert (1991) and the use of another lyophilization apparatus (Supermodulyo 12K Freeze Dryer, Edwards, USA), all strains tested showed some degree of viability.

According to Kirsop & Doyle (1991), different taxonomic groups and even strains within a given species can exhibit considerable differences in response to stresses imposed by the storage procedure itself and/or recovery methods used. Rate of cooling, size of propagules, and thickness of cell wall are important parameters not considered in this study (Berny & Hennebert, 1991; Schoenlein-Crusius et al., 1993; Tan et al., 1995), and they may explain the observed intra and interspecific variability of fungal cultures as measured by conidial viability after different storage periods.

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