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Cryopreservation at -75°C of *Agaricus subrufescens* on wheat grains with sucrose



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ABSTRACT

Agaricus subrufescens is a basidiomycete which is studied because of its medicinal and gastronomic importance; however, less attention has been paid to its preservation. This study aimed to evaluate the effect of sucrose addition to substrate and cryotube on the viability of *Agaricus subrufescens* cryopreserved at -20°C and at -75°C for one and two years. Zero, 10% or 20% sucrose was added to potato dextrose agar or wheat grain. The mycelia were cryopreserved in the absence of cryoprotectant or with sucrose solutions at 15%, 30% or 45%. After one or two years at -75°C or at -20°C , mycelia were thawed and evaluated about viability, initial time of growth, colony diameter and genomic stability. Cryopreservation at -20°C is not effective to keep mycelial viability of this fungus. Cryopreservation at -75°C is effective when sucrose is used in substrates and/or cryotubes. Without sucrose, cryopreservation at -75°C is effective only when wheat grains are used. Physiological characteristic as mycelial colony diameter is negatively affected when potato dextrose agar is used and unaffected when wheat grain is used after two-year cryopreservation at -75°C . The fungus genome does not show alteration after two-year cryopreservation at -75°C .

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Introduction

Agaricus subrufescens Peck (= *Agaricus blazei* Murrill sensu Heinemann; *Agaricus brasiliensis* Wasser et al.) is a basidiomycete with culinary¹ and medicinal characteristics and one of the most important mushroom produced in Brazil.² Its consumption has been increasing due to its functional proper-

ties as antitumor,^{3,4} antimutagenic,⁵ anti-inflammatory⁶ and antioxidant.⁷

This species is among the most produced and commercialized ones in Brazil and in the world along with *Pleurotus ostreatus* and *Lentinula edodes*. However, the traditional preservation method of this fungus is still the periodic subculture of mycelia. This method is inexpensive but requires periodic maintenance, time, and physical space, making it impractical to preserve large collections.⁸ In addition, it has several disadvantages such as loss of biological, genetic and/or physiological characteristics and/or loss by contamination.^{9,10}

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Therefore, other techniques such as cryopreservation could be an alternative to the preservation of this fungus.¹¹

Cryopreservation has been used to keep biological material viable for long periods at temperatures ranging from -20°C to -196°C .^{12,13} It is considered a safe technique due to metabolism inactivation with lower contamination and reduced risk of genetic degeneration.¹⁴ Cryopreservation at -20°C has been little explored and is a low-cost alternative for *A. subrufescens* even though there is a great risk of cryoinjuries. Cryopreservation at -80°C is a safe technique and avoids the dependence on constant refilling with liquid nitrogen.¹⁵ However, the use of low temperatures for *A. subrufescens* preservation is still a challenge due to the mycelium sensitivity of this species¹⁶ to cooling temperatures and to other more conventional techniques of culture preservation such as mineral oil overlay (paraffin method) and distilled water (Castellani's method).^{11,15}

Colauto et al.,¹⁵ Mantovani et al.¹⁷ and Tanaka et al.¹⁸ have used cereal grains in cryopreservation to increase mycelial viability and cryopreservation period. Tanaka et al.¹⁸ verified that the whole grains of hard endosperm wheat had the highest mycelial recovery after two-year cryopreservation at -70°C of *A. blazei*. Mycelial biomass of basidiomycetes is very sensitive to freezing conditions¹⁹ and, therefore, is immersed in cryoprotective solutions to reduce cryoinjuries.²⁰ Colauto et al.¹¹ reported that sucrose was more effective than glycerol for *A. blazei* cryopreserved at -70°C after four years. The combination of sucrose in the cultivation medium to reduce free water and improve mycelium resistance to cryoinjuries has never been described before for basidiomycetes. Therefore, this study aimed to evaluate the effect of sucrose addition to substrate and cryotube on the viability of *A. subrufescens* cryopreserved at -20°C and at -75°C for one and two years.

Materials and methods

Inoculum

Strain U2-1 (ABL 97/11) of *Agaricus subrufescens* from the Culture Collection of the Laboratório de Biologia Molecular of Universidade Paranaense was used to carry out all experimental phases. The inoculum was produced on potato dextrose agar (PDA, 39 g L^{-1} , ACUMEDIA[®]), previously autoclaved at 121°C for 20 min, and kept at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark. The inoculum was obtained from the mycelial growth edge – without reaching the edge of Petri dish – with uniform appearance and without sectoring after six days.

Substrates for cryopreservation

The moisture content of PDA or wheat grains was determined after drying it at 105°C until constant mass. The moisture was determined in order to calculate the amount of sucrose to be added to the substrates (PDA or wheat) to reach 10% or 20% sucrose (dry basis).

The sucrose solution at 50% (w v^{-1}), previously heated at 50°C for 1 h and filtered ($0.22\ \mu\text{m}$; Millipore[®]), was added to autoclaved (121°C for 20 min) PDA to obtain 10% or 20% sucrose (dry basis) and poured into Petri dishes (90 mm). The

absence of electrolytes in sucrose or ultrapure water solutions was verified by a conductivity meter (Quimis[™] Q795A). Hard endosperm wheat (*Triticum aestivum* L.) grains (cultivar IPR Catuara[™]; breeding or improver type with flour strength $\geq 300\text{ W}$) from Paraná Agronomical Institute were washed and cooked in abundant water (300 g grain to 1 L water) for 45 min at 90°C . The water excess was removed, the grains were transferred to Falcon[®] tubes (50 mL) and autoclaved at 121°C for 90 min.¹⁶ Filtered sucrose solution ($0.22\ \mu\text{m}$; Millipore[®]) was added to each tube containing autoclaved grains to obtain 10% or 20% sucrose (dry basis) and kept grain covered for 36 h for equalization of sucrose concentration before use. The sucrose solution was completely adhered to the grains.

The substrates, PDA in Petri dishes or wheat grains in Falcon tubes, were inoculated with PDA disks (6 mm diameter) containing mycelium and kept at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark until colonization of each substrate. Mycelial colonization was stopped when it reached 80% of the substrate (Petri dish or Falcon tubes) to avoid eventual mycelial physiological changes but only fully colonized substrate (PDA or grain) was transferred to cryopreservation tubes.

Cryopreservation

Mycelia were cryopreserved in cryotubes according to Challen and Elliot.²¹ Six 5-mm-diameter PDA disks and six wheat grains containing mycelium were transferred to a cryotube (6-mm diameter by 6-cm length; Strawplast[®]; model CS304). The end of each cryotube was thermosealed and each cryotube received 600 μL sucrose aqueous solution with 15%, 30% or 45% (w v^{-1}), previously filtered ($0.22\ \mu\text{m}$; Millipore[®]). The control was without any aqueous solution in the cryotube. After thermosealing the other end, the cryotubes were kept in an ultrafreezer at -75°C or -20°C in a vertical position.

Mycelial viability after cryopreservation

After one- and two-year cryopreservation, three cryotubes were removed from the ultrafreezer at -75°C and at -20°C and kept submersed in ultrapure water at 30°C for 15 min. After that, the cryotubes were immersed for 30 s in 70% alcohol and 96% alcohol, and dried in a laminar flow chamber for 2 min. Both ends of the cryotube were cut and the content was transferred to a sterile empty Petri dish, except the disk or grain on the top of the cryotube that was discarded. Five disks or grains were moved to PDA and incubated at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark. The total of replications was 15 (three cryotubes with five disks or grains each). For each treatment, the time for the beginning of the mycelial growth after thawing (TBMG) was recorded after two years of cryopreservation. The treatments with mycelial growth of 80% or greater, until 20 days, were considered viable after one and two years of cryopreservation. Variations of mycelial branching, color and vigor were verified in a stereoscopic and optical microscope.

Physiological characteristic of mycelia after cryopreservation

The treatments considered viable after one or two years of cryopreservation were analyzed regarding mycelial colony

diameter (MCD). For each treatment, one 4-mm-diameter disk of PDA with mycelia was transferred to the center of a Petri dish (90 mm) containing PDA. Mycelium kept by periodic subculture in PDA for one or two years at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark was used as control. The experiment was done in triplicate. The dishes were randomly arranged at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in the dark. The MCD was measured to all treatments when mycelia of one of the treatments reached the Petri dish edge closely.²²

Genetic stability after cryopreservation

For DNA extraction, the mycelium was kept in liquid medium of 3% malt extract (w v^{-1}) and 0.002% yeast extract (w v^{-1}) for 15 days and agitated at 90 rpm, at 28°C , in the dark. The obtained mycelium was vacuum-filtrated using a cellulose membrane (14 μm pore), previously autoclaved (121°C for 20 min), in a Buchner filter and washed with autoclaved (121°C for 20 min) ultrapure water. After that it was dehydrated in a stove at 40°C for 30 min, and then ground in liquid nitrogen. For each gram of mycelium, 2.1 mL of extraction buffer (40 mL 1 M Tris-HCl, pH 8, 10 mL 5 M NaCl, 10 mL 0.5 M EDTA, pH 8, 20 mL 10% SDS and 120 mL ultrapure water) was added and kept immerse in water at 65°C for 15 min. Next, 2.1 mL of phenol was added and the mixture was homogenized and centrifuged at 15,200 g for 10 min at 4°C . The organic phase was discarded and the same volume of chlorophane (phenol:chloroform, 1:1, v v^{-1}) was added to the aqueous phase, homogenized and centrifuged at 15,200 g for 10 min at 4°C . The previous step was repeated with chlorophyll addition (chloroform:isoamyl alcohol, 24:1, v v^{-1}) substituting chlorophane. The aqueous phase was discarded and 30 μL 3 M NaCl was added to the pellet to obtain final concentration of 0.3 M with 600 μL of cooled absolute ethanol. The samples were homogenized, kept at -20°C for 30 min, and centrifuged at 15,200 g for 15 min. The supernatant was discarded, the pellet was dried at 22°C on the bench, and then re-suspended in 50 μL TE buffer (1 mL 1 M Tris-HCl, pH 7.6, 0.2 mL 0.5 M EDTA, pH 8.0 and 100 mL ultrapure water).²³

For amplification by PCR-RAPD of genomic DNA of each treatment, three primers were utilized: A09 (5' GGG TAA CGC C 3'), A10 (5' GTG ATC GCA G 3') and A13 (5' CAG CAC CCA C 3') (Life Technologies™). The final volume of each amplification reaction was 30 μL , containing buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), a mixture of nucleotides (0.25 mM), primer (0.4 μM), 3.4 mM MgCl_2 , 2 U of Taq DNA polymerase and 15 ng genomic fungus DNA. The control reaction had all reaction components, except for genomic DNA. The amplification occurred in a thermocycler with initial denaturation at 94°C for 5 min, followed by 40 cycles of 1 min at 92°C , 1 min at 35°C , 2 min at 72°C and final extension of 5 min at 72°C . The reaction was done in triplicate using three different thermocyclers (Eppendorf®) in the same laboratory.

Amplification product samples (7 μL) were mixed with 3 μL loading buffer (0.25 g bromophenol blue, 15 g Ficoll™; 100 mL ultrapure water), submitted to electrophoresis at 3 V cm^{-1} in 1.4% agarose gel and stained with 1% ethidium bromide. DNA ladder (1 kb Plus Life Technologies™) was used as molecular mass standard. The similarity and integrity of amplification products were verified based on profile of bands by visual-

ization in ultraviolet light transilluminator. The result was digitally recorded.

Statistical analysis

Treatments were composed of two substrates, PDA or wheat grain (WG), with addition of sucrose (00%, 10% and 20%) in substrates and/or cryotubes (00%, 15%, 30%, 45%). Each experiment had a completely randomized design. Data were evaluated as arithmetic mean and standard deviation. A comparison among treatments was performed according to the Scott-Knott's test ($p \leq 0.05$) using analysis of variance (Assistat 7.7, Universidade Federal de Campina Grande, Campina Grande, Paraíba, Brazil).

Results

After one-year cryopreservation at -20°C , there was viability only in some treatments with wheat grains such as WG-00-15, WG-10-30, WG-10-45 and WG-20-00 (data not presented). For PDA there was not mycelial viability after cryopreservation at -20°C . Therefore, the use of wheat grains as substrate was determinant to recover mycelial growth after cryopreservation at -20°C . However, the high variability of the results with reduced mycelial recovery, cryopreservation at -20°C could not be considered effective for *A. subrufescens*.

For cryopreservation at -75°C , there was not mycelial viability for PDA without sucrose added to the substrate and cryotube (PDA-00-00) after one and two years of cryopreservation (Table 1). However, for wheat grains under the same conditions (WG-00-00), the mycelium presented viability after one and two years of cryopreservation (Table 1). Therefore, the wheat grain substrate without cryoprotectant was effective after one-year and two-year cryopreservation at -75°C . Treatments with mycelial viability showed no change in the mycelial branching, color and vigor.

The TBMG cryopreserved at -75°C for two years in PDA varied from 2.29 (PDA-20-45) to 5.88 (PDA-00-15) days, with an arithmetic mean of 3.65 days; however, in wheat grain it varied from 1.08 (WG-20-00) to 5.88 (WG-00-30) days, with a mean of 3.02 days (Table 1). TBMG shows how long it took mycelia to grow after thawing. The shorter the time, the more efficient the cryopreservation process was. TBMG was smaller for wheat grains (Table 1) indicating that this substrate is the best for cryopreservation. For PDA, the greatest addition of sucrose in the substrate and/or cryotube resulted in the smallest ($p \leq 0.05$) TBMG value. This suggests that sucrose improve the recovery capacity of the mycelium after cryopreservation. For wheat grains, the greatest sucrose addition to the substrate and/or cryotube resulted in greater ($p \leq 0.05$) TBMG values. TBMG increases with increasing sucrose addition to substrate is possibly related to water activity reduction, making mycelial growth difficult.

The addition of 10% or 20% sucrose in PDA reduced TBMG value from 5.88 (PDA-00-15) to 2.96 (PDA-10-15) and 2.46 (PDA-20-15) days, respectively (Table 1). The same result occurred for PDA-00, PDA-10 and PDA-20 (10% and 20% sucrose in the substrate) considering sucrose in the cryotube (30% and 45%).

Table 1 – Mycelial viability and time for beginning of mycelial growth after thawing (TBMG) of *Agaricus subrufescens* after one- and two-year cryopreservation at -75°C in several sucrose concentrations in substrate and/or cryotube.

Code		Mycelial viability (%) after 1 year	Mycelial viability (%) after 2 years	TBMG (day) after 2 years			
Substrate ^d	Sucrose in substrate (%)	Sucrose in cryotube (%)					
PDA	00	00	40	0	–		
		15	93	100	5.88 ± 0.05^c		
		30	100	100	4.33 ± 2.08^b		
		45	100	100	4.88 ± 1.42^c		
		00	100	100	5.29 ± 1.29^c		
	10	15	100	100	2.96 ± 0.17^a		
		30	100	100	3.13 ± 0.17^a		
		45	100	100	2.29 ± 0.38^a		
		00	100	100	3.75 ± 1.38^b		
		15	100	100	2.46 ± 0.54^a		
	20	30	100	100	2.88 ± 0.58^a		
		45	100	100	2.29 ± 0.54^a		
		Mean	94.4	91.7	3.34 ± 0.72		
		WG	00	00	93	100	2.46 ± 0.50^a
				15	100	100	2.62 ± 0.50^a
30	100			100	5.88 ± 0.04^c		
45	100			80	4.38 ± 1.75^b		
00	100			100	2.21 ± 1.00^a		
10	15		100	100	1.54 ± 0.46^a		
	30		100	100	2.00 ± 0.17^a		
	45		100	100	3.88 ± 1.88^b		
	00		100	100	1.08 ± 0.17^a		
	15		100	100	3.46 ± 1.50^a		
20	30		100	100	1.88 ± 0.46^a		
	45		100	100	4.88 ± 1.42^c		
	Mean		99.4	98.3	3.02 ± 0.82		

^d PDA, potato dextrose agar; WG, hard endosperm wheat grain. Arithmetic mean of 15 replications (five grains or disks per cryotube; three cryotubes). The arithmetic means followed by different letters indicate significant differences among the treatments according to Scott–Knott's test ($p \leq 0.05$).

This indicates an efficiency threshold of utilization of 10% sucrose in the substrate, and 15% sucrose in the cryotube for PDA (Table 1). Similarly, in general, the addition analysis of 00%, 15%, 30% or 45% sucrose in the cryotube for PDA-00, PDA-10 and PDA-20 treatments reduced TBMG values, except for PDA-00-45 (Table 1). TBMG values also indicate an efficiency threshold of utilization of 15% sucrose in the cryotube (Table 1).

The MCD after one-year cryopreservation at -75°C in PDA varied from 5.2 (PDA-20-00) to 7.8 (PDA-00-45) cm, with a mean of 6.1 cm; however, in wheat grain it varied from 5.5 (WG-20-15) to 7.2 (WG-00-30, WG-10-00, WG-20-45) cm with a mean of 6.4 cm (Table 2). The MCD for wheat grain was, in general, better and closer to control than PDA, but there is not significant difference among them (Table 2). Overall, the greatest sucrose addition to substrate and/or cryotube did not result in significant biological alterations when compared to control (Table 2). However, the MCD after two-year cryopreservation at -75°C in PDA varied from 3.7 (PDA-00-15, PDA-10-30) to 4.7 (PDA-20-00) cm, with a mean of 4.3 cm whereas wheat grains varied from 4.3 (WG-00-00) to 7.0 (WG-20-00) cm, with a mean of 6.1 cm (Table 2). In general, PDA and control had lower MCD values ($p \geq 0.05$) than wheat grains after two years of cryopreservation (Table 2). Any PDA treatment or wheat grain without sucrose (WG-00-00) had MCD values reduced and equal as control (Table 2).

The addition of 10% or 20% sucrose in PDA or wheat grains did not significantly affected MCD value after one- or two-year cryopreservation (Table 2). In general, the increasing addition of sucrose to the cryotube for PDA or wheat grains resulted in no additional benefit after one- or two-year cryopreservation (Table 2). However, sucrose addition in wheat grain better preserved primordial MCD values than PDA and control after two-year cryopreservation (Table 2).

The amplification products obtained by PCR-RAPD did not present alterations in the band pattern for all viable treatments after two-year cryopreservation (Fig. 1).

Discussion

The use of wheat grains as substrate for mycelial growth was effective for fungus cryopreservation. Alternative substrate for mycelial growth, such as perlite,²⁴ sawdust,²⁵ and other grains^{26–28} have been found to be more effective for cryopreservation of basidiomycetes than agar based media, mainly PDA or malt extract agar. Colauto et al.¹⁵ reported that *A. blazei* cryopreserved at -80°C during one year had higher mycelial viability when rice grains with husk were used as substrate. On the other hand, mycelia of *A. blazei* preserved by overlaying cultures with mineral oil was reported as ineffective after four

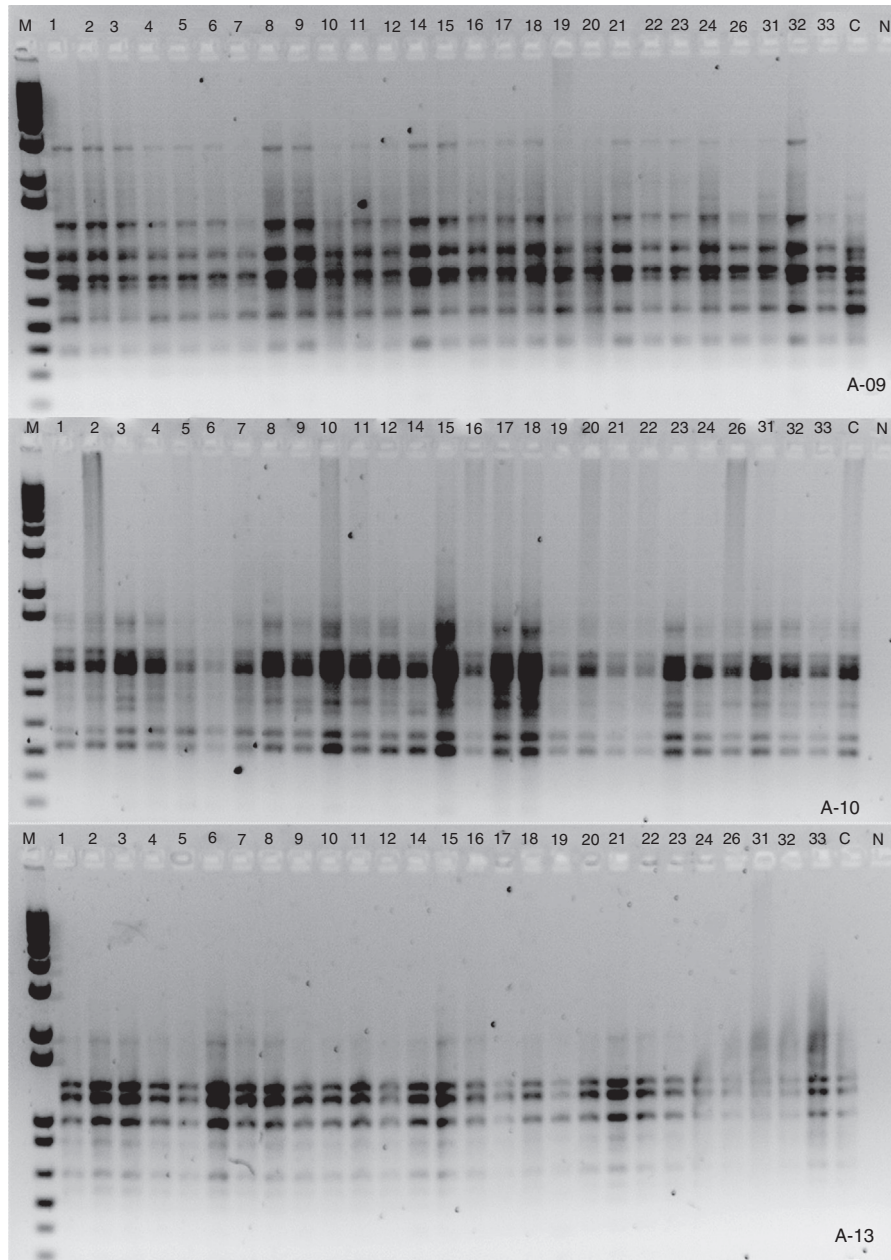


Fig. 1 – Agarose gel (1.4%) of PCR products referring to primers A09, A10 and A13 of viable treatments of *Agaricus subrufescens* cryopreserved at -75°C during two years and at -20°C for one year. M = 1 kb molecular mass marker; 1–12 = mycelium grown in wheat grain and cryopreserved at -75°C ; 14–24 = mycelium grown in potato dextrose agar (PDA) and cryopreserved at -75°C ; 26, 31–33 = mycelium grown in wheat grains and cryopreserved at -20°C ; C = PCR product of the same preserved fungus by periodic subculture (positive control); N = PCR reaction without DNA (negative control).

years at 4°C or 20°C but it could be effective for periods of one year at 20°C .¹¹

Tanaka et al.¹⁸ evaluated the effect of chemical composition and physical aspects of wheat grain in *A. blazei* cryopreservation. They verified that the whole grains of hard endosperm wheat had the highest ($p \leq 0.01$) values of mycelial recovery after two-year cryopreservation at -70°C . For Tanaka et al.,¹⁸ the grain capillary physical structure is more important than the grain chemical composition for the mycelial viability on cryopreservation. The grain capillary net provides reduced physical space that limits the presence of

water molecules²⁹ and protects mycelia from ice formation. Moreover, Griffith et al.³⁰ reported that wheat grain of winter varieties produces anti-freezing proteins, found mainly in intercellular spaces such as the apoplast. This last piece of information corroborates the study by Tanaka et al.¹⁸ in which hard endosperm wheat grain, a winter culture, is recommended as the most effective to cryopreserve *A. blazei*. Therefore, future studies related to substrate capillarity, water activity in the cultivation medium and anti-freezing proteins are relevant on the cryopreservation process of basidiomycetes.

Table 2 – Mycelial colony diameter (MCD) of *Agaricus subrufescens* after one- and two-year cryopreservation at -75°C with several sucrose concentrations in substrate and/or cryotube (treatments), and preserved in PDA by subculture (control) for one and two years at 25°C .

Code			MCD (cm) cryopreserved for 1 year	MCD (cm) cryopreserved for 2 years
Substrate ^d	Sucrose in substrate (%)	Sucrose in cryotube (%)		
Control	PDA subculture		6.4 ± 0.6^a	4.3 ± 0.1^b
			–	–
PDA	00	00	–	–
		15	6.3 ± 0.6^a	3.7 ± 0.4^b
		30	5.7 ± 0.3^b	4.2 ± 0.3^b
		45	7.8 ± 1.3^a	4.4 ± 0.5^b
	10	00	5.6 ± 0.6^b	5.5 ± 0.8^a
		15	6.4 ± 0.5^a	4.6 ± 0.1^b
		30	6.8 ± 0.4^a	3.7 ± 0.3^b
		45	5.5 ± 0.4^b	4.0 ± 0.1^b
	20	00	5.2 ± 0.3^b	4.7 ± 0.2^b
		15	6.0 ± 0.3^b	3.9 ± 0.2^b
		30	6.6 ± 1.7^a	3.9 ± 0.4^b
		45	5.3 ± 0.3^b	4.1 ± 0.8^b
	Mean	6.1 ± 0.8	4.2 ± 0.5	
WG	00	00	5.8 ± 0.7^b	4.3 ± 0.1^b
		15	5.9 ± 0.2^b	6.6 ± 0.1^a
		30	7.2 ± 1.2^a	6.4 ± 0.9^a
		45	6.3 ± 0.8^a	5.7 ± 0.2^a
	10	00	7.2 ± 0.9^a	6.1 ± 0.1^a
		15	6.8 ± 0.9^a	6.3 ± 0.5^a
		30	6.9 ± 0.8^a	5.9 ± 0.1^a
		45	6.5 ± 1.2^a	6.6 ± 0.6^a
	20	00	6.0 ± 0.7^b	7.0 ± 1.0^a
		15	5.5 ± 0.1^b	6.1 ± 0.6^a
		30	6.0 ± 0.3^b	6.2 ± 0.1^a
		45	7.2 ± 0.5^a	6.5 ± 0.6^a
	Mean	6.4 ± 0.6	6.1 ± 0.7	

^d Control PDA, preserved by periodic subculture (25°C); WG, hard endosperm wheat grain; PDA, potato dextrose agar (cryopreserved); –, treatment without mycelial viability. The arithmetic means followed by different letters indicate significant differences among the treatments according to Scott-Knott's test ($p \leq 0.05$).

The addition of sucrose to substrate and cryotube maintained primordial MCD values ($p \leq 0.05$) compared with treatments without sucrose and control after two-year cryopreservation at -75°C (Table 2). In general, PDA and control had lower MCD values ($p \geq 0.05$) than wheat grains after two years of cryopreservation (Table 2). This indicates a significant biological alteration in PDA and control (periodic subculture) compared with wheat grains. Mycelial degeneration due to periodic subculture was already expected, but it is unforeseen that protection against mycelial degeneration occurred only when wheat grains with sucrose was used in cryopreservation. It is also important to determine the highest cryoprotective concentration that is not toxic to the fungus and does not remain as a barrier to be overcome. Colauto et al.¹¹ reported 100% mycelial viability when 15% sucrose was used as cryoprotectant after one-year and four-year cryopreservation at -70°C of *A. blazei*. Although it was high, addition of 20% sucrose to the substrate and 45% sucrose to cryotube in our study was still effective to keep mycelial viability and did not affect MCD after two-year cryopreservation, especially for WG group (wheat grains). This indicates that sucrose concentration limit still has not been reached even though it is in the

operational viability limit of the solution. Also, this suggests that sucrose associated with wheat grain is fundamental to a better preservation of mycelial characteristics of this fungus. Mantovani et al.¹⁷ reported that only after two-year cryopreservation it is possible to verify the effect of long-term preservation on mycelial viability maintenance. Moreover, the long-term cryopreservation could affect not only the mycelial cell survival but also other microorganisms inside the mycelia such as endosymbionts, similar to endophytes,³¹ which could interfere with mycelial growth.

After two years of cryopreservation at -75°C in wheat grain, TBMG average was 3.32 days (Table 1). The mycelial visualization started at nine days for *A. blazei* cryopreserved at -80°C for one year in rice grains¹⁵; four days for *Agaricus bisporus* cryopreserved in liquid nitrogen²⁶; 5.5 days for *A. blazei* cryopreserved at -80°C in different wheat grains¹⁸; from 9 to 17 days for *A. blazei* cryopreserved at -70°C during four years using different cryoprotectants.¹¹ There is no consensus for TBMG after cryopreservation but the faster the mycelial growth starts, the more it indicates that there were less cryoinjuries in the cryopreservation process. On the other hand, greater TBMG values could be related to the reduction of

mycelial growth due to increase in the osmotic. Nevertheless, TBMG values found in our study were in general smaller than the ones cited in the studies reported above, on the same fungus, suggesting a greater efficiency or at least an improvement of the cryopreservation process of our study.

Basidiomycete cryopreservation at -20°C has not been considered appropriate because it caused cell damages, probably due to low freezing rate and formation of ice crystals, resulting in low fungus survival.³² In our study, the mycelial viability was not effective after one and two years of cryopreservation at -20°C . These results are similar to the ones in other studies of our group¹⁸ which did not report mycelial viability for *A. blazei* cryopreserved at -20°C . According to Pegg³² and Ueno et al.,³³ the reduced freezing rate at -20°C favors water migration, increasing the ion-intracellular concentration and the intracellular formation of big ice crystals. Moreover, Fujikawa³⁴ reported that the intramembrane particle aggregation could be the cause of cryoinjuries as a result of mechanical stress from the extracellular ice crystal formation in cryopreservation at -20°C in basidiomycetes. However, Mantovani et al.¹⁸ obtained mycelial viability of 67% for *Pleurotus ostreatus* cryopreserved at -20°C for one and three years in wheat grains. This shows that it is possible to cryopreserve other basidiomycetes in this temperature range.

As verified by our group, cryopreservation of *Agaricus* genus at -20°C rarely presents satisfactory results of mycelial viability and growth. However, studies utilizing ultralow temperatures for preservation in liquid nitrogen have also shown reduced mycelial recovery rates. Colauto et al.¹¹ reported little satisfactory results of cryopreservation in liquid nitrogen during 1.5 year for *A. blazei* in agar-based cultivation medium using DMSO as cryoprotectant. This shows that *A. blazei* is sensitive to the formation of intracellular ice crystals or even to the quick expansion of the cellular volume. Probably the determining factor for the reduced *A. blazei* viability under cryopreservation at -196°C is the moisture content over 90%.¹¹ Cells with high moisture content generally need slow freezing to dehydrate and reduce the formation of ice crystals and volume expansion, the main responsible factors for the rupture and loss of cellular viability.²⁰ Similar results were reported by Chen³⁵ who observed more cellular damages with other basidiomycete cells cryopreserved by fast freezing.

Colauto et al.¹⁵ checked that fast water freezing (-196°C) caused cryoinjuries for *A. blazei* probably due the low elasticity of its plasmatic membrane or even great rigidity of its cell wall. On the other hand, the slow freezing process (-80°C) was more efficient for *A. blazei* cryopreservation.¹¹ Thus, the freezing and maintenance temperature interval between -70 and -80°C seems to be the most indicated for the preservation of this fungus. Another option is to use control of temperature decrease in which ice formation is primarily extracellular and there is time for the cell to adapt to its new volume, causing less cellular damage by rupture, despite of its high cost.³²

Our results show that cryopreservation did not change the tested genome regions. This suggests that cryopreservation at -75°C , after two years did not affect the genetic stability (Fig. 1). Also, it indicates that the reduction of MCD value in PDA, after two-year cryopreservation (Table 2) could be temporary or reversible considering that PCR-RAPD showed no alteration (Fig. 1). For Odani et al.³⁶ freezing and thawing

can cause functional re-arrangement of genome. Ryan et al.³⁷ detected DNA polymorphism for *Metarhizium anisopliae* after freezing in liquid nitrogen. Basidiomycetes withstand freezing in liquid nitrogen well^{38,39} with no changes in morphology and metabolism for *Armillaria*, *Pleurotus*, *Pluteus* and *Polyporus* genera.^{19,40} Phenotypic stability after cryopreservation in liquid nitrogen has been reported for 11 species of edible fungi. In *Agaricus* and *Pleurotus* genera, no changes were found in 5.8S rDNA isolated from mycelia.²⁸ For Camelini et al.,⁴¹ *A. subrufescens* showed by PCR-RAPD a genetic variability reduction after 4, 8 and 12 months of cryopreservation, indicating the occurrence of cryoinjuries in the genomic material. Singh et al.²⁸ did not detect genotypic variations of several basidiomycetes preserved in wheat grains in liquid nitrogen for 3.5 years, but they analyzed a more restrict area of the genome, the ITS region. The results from our study contribute to the adjustment of cryopreservation techniques in basidiomycetes and help studies on long-term stability variation.

Conclusion

Cryopreservation at -20°C is not effective to keep mycelial viability of this fungus. Cryopreservation at -75°C is effective when sucrose is used in substrates and/or cryotubes. Without sucrose, cryopreservation at -75°C is effective only when wheat grains are used. Physiological characteristic as mycelial colony diameter is negatively affected when PDA is used and unaffected when wheat grain is used after two-year cryopreservation at -75°C . The fungus genome does not show alteration after two-year cryopreservation at -75°C . Although all treatments that use sucrose as cryoprotectant and only grains without the cryoprotectant are effective for the preservation of this fungus, we recommend the use of wheat grains with 10% and 15% sucrose solution in substrate and cryotube, respectively, for cryopreservation at -75°C at this time. It is always cautious to use more than one technique to preserve important strains. Cryopreservation at -75°C is an alternative method to maintain *A. subrufescens* for 2 years and avoid mycelial degeneration on periodic subculture.

Conflicts of interest

The authors declare no conflicts of interest.

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