Original Article

CEREAL GRAINS AND GLYCEROL IN Agaricus blazei *CRYOPRESERVATION*

GRÃOS DE CEREAIS E GLICEROL NA CRIOPRESERVAÇÃO DE *Agaricus blazei*

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ABSTRACT: *Agaricus blazei* is an important commercial basidiomycete studied for its biological activity. However, little has been studied about the preservation techniques of this basidiomycete, and cryopreservation is one alternative. The aim of this study was to evaluate the physical and chemical effects of different cultivation media and glycerol on *A. blazei* cryopreservation at -20 ºC and at -70 ºC. A solid cultivation medium consisting of agar with ground whole cereal grains (hard endosperm wheat, medium hard endosperm wheat or hard endosperm rye) or malt extract agar, or whole cereal grains (hard endosperm wheat, medium hard endosperm wheat or hard endosperm rye) without agar was used. Cultivation media had zero or 5% glycerol addition. Cultivation medium grains or disks containing mycelia were transferred to cryotubes with glycerol at 5% with or without water and then were cryopreserved at -20 ºC or at -70 ºC. After one-year or two-year cryopreservation, fungus viability was evaluated. The results showed that the physical structure of whole grains of hard and medium hard wheat is effective ($p \leq 0.01$) for two-year cryopreservation at -70 ºC as well as the use of glycerol in the cultivation medium or as a cryoprotectant ($p \leq 0.01$) at -70 ºC. Cryopreservation at -20 ºC was ineffective ($p \leq 0.01$) to preserve fungus viability.

KEYWORDS: *Agaricus brasiliensis*. *Agaricus subrufescens*. Substrate. Freezing. Osmotic strength.

INTRODUCTION

Agaricus blazei Murrill *ss* Heinemann (HEINEMANN, 1993), classified as *Agaricus brasiliensis* Wasser et al. (WASSER et al., 2002) and *Agaricus subrufescens* Peck (KERRIGAN, 2005), is a native fungus of Brazil (COLAUTO et al., 2010a, b, c). A doubt still remains if *A. blazei ss* Murrill, *A. blazei ss* Heinemann or *A. brasiliensis* Wasser et al. and *A. subrufescens* Peck are the same species or not. However, Colauto et al. (2011b) reported that the name *A. brasiliensis* and *A. subrufescens* has been used before by Fries in 1830 and Ellis e Everhart in 1893, respectively, and, therefore, are illegitimate names. Nonethless, Wisitrassameewong et al. (2012) reported that in a recent update of the "Index Fungorum" (February 2012), *A. subrufescens* Peck was published a few days before *A. subrufescens* Ellis & Everhart although this information is not clearly visible in the journal where they were published. Therefore, while the dispute continues, it is preferable to use the most used name (*A. blazei*) or use the name according to the origin of the biological material collected until a consensus for the correct name of this fungus is reached. In this study, the name *A. blazei* will be used.

It is known for its antitumoral (JUMES et al., 2010; MOURÃO et al., 2009), immunomodulator (WASSER; WEIS, 1999), antimutagenic (SOUZA-PACCOLA et al., 2004), antioxidant (MOURÃO et al., 2011b), and anti-inflammatory (MOURÃO et al., 2011a) activities. Previous studies demonstrated that its production of enzymes (like laccase) (D'AGOSTINI et al., 2011) and its gastronomic value (ESCOUTO et al., 2005) are of great interest for human intake. Therefore, the development of preservation methods is still needed.

Basidomycete preservation is difficult due to the predominance of the vegetative mycelia, which is sensitive to environmental variations, and the absence of asexual spores in many species (HOMOLKA et al., 2001). The usual preservation technique of *A. blazei*, subculture, has disadvantages such as contaminations (HOMOLKA et al., 2006; MATA; PÉREZ-MERLO, 2003) and loss of biological, genetic or physiological characteristics (HOMOLKA et al., 2006; VOYRON et al., 2009). Other techniques (mineral oil, distilled water and lyophilisation) have been utilized less successfully in long-term basidiomycete preservation (COLAUTO et al., 2012; HOMOLKA et al., 2001; VOYRON et al., 2009). Cryopreservation is considered a safe technique due to metabolism inactivation with lower contamination and genetic degeneration risk (ITO; NAKAGIRI, 1996). However, because of the diversity of responses to cryopreservation process of basidiomycetes

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(SMITH, 1983), the search for alternative cryopreservation techniques for each species is needed (RYAN et al., 2000).

Cryopreservation in liquid nitrogen (MATA; ESTRADA, 2005; MATA; PÉREZ-MERLO, 2003) or at -80 ºC (ITO; NAKAGIRI, 1996; KITAMOTO et al., 2002) has been effective for several basidiomycetes with results varying from 78% to 100% with or without glycerol as cryoprotectant, and for one week or 15 years. For *A. blazei* at -80 ºC using rice grains with glycerol, mycelial viability was effective from 0% to 100% for one year (COLAUTO et al., 2011a) and at -70 ºC with malt extract agar disks and several cryoprotectants, mycelial viability was effective from 20% to 100% after four year cryopreservation (COLAUTO et al., 2012). However, cryopreservation at -20 ºC has been little explored and may be a low cost alternative for *A. blazei*, even though there is a greater risk of cryoinjuries. Based on these considerations, we evaluate the physical and chemical effects of different cultivation media and glycerol for *A. blazei* cryopreservation at -20 ºC or -70 ºC.

MATERIAL AND METHODS

Fungus and experimental design

A. blazei U2/1 (formerly ABL97/11), from the fungus collection of the Molecular Biology Laboratory, Universidade Paranaense, Brazil, was grown on malt extract agar (MEA) at 2%, previously autoclaved at 121 ºC for 20 min, and kept at 25 ºC ± 1 ºC in the dark. The inoculum was selected from the mycelium growth border with uniform appearance and without sectioning. The experiment had a completely random design and consisted of 42 treatments and 15 replications.

Cultivation media

In order to evaluate the effects on the physical factors of the substrate, cultivation media with whole grains of hard endosperm wheat (*Triticum aestivum* L.) cultivar IPR 136, medium hard endosperm wheat (*T. aestivum* L.) cultivar IPR 130 or hard endosperm rye (*Secale cereale* L.) cultivar IPR 89, from the Instituto Agronômico do Paraná (IAPAR), Brazil, were used and classified as HW, MW and HR, respectively. To assess the effects of substrate chemical factors, cultivation media were prepared from ground whole grains, without removing the covering of the grains, and classified as HWG, MWG and HRG, respectively. To evaluate the effect of cultivation medium osmotic strength, 5% glycerol (GY) was added to cultivation media with whole or ground grains and classified as HW+GY, MW+GY and HR+GY or as HWG+GY, MWG+GY and HRG+GY, respectively.

Cultivation media were prepared by washing grains in tap water for 1 min and immersing them in ultra-purified water excess (HW, MW and HR) or in GY solution at 5% (v v^{-1}) (HW+GY, MW+GY and HR+GY) and kept at 90 ºC for 45 min. The liquid excess was removed; around 500 grains were transferred to 50 mL Falcon® tubes and autoclaved at 121 ºC for 180 min. For the ground cultivation media ground grains (40 g L^{-1}) with lower than 355 µm granulometry and agar (14 g L^{-1}) were mixed with ultra-purified water (HWG, MWG and HRG) or GY solution (HWG+GY, MWG+GY and HRG+GY). The mixture was autoclaved at 121 ºC for 30 min and transferred to Petri dishes. MEA was used as control cultivation medium.

After the preparation, the cultivation media were inoculated with 0.6 cm diameter of MEA disks containing inoculum. The mycelial growth was carried out at 25 ºC ± 1 ºC, in the dark, until complete colonization of whole grains or until 80% of the Petri dish area with agar ground grains or MEA.

Cryopreservation

The used cryoprotectants were GY solution, ultra-purified water (UW) or without cryoprotectant (WC). Cryotubes were made of polypropylene plastic tubes (0.6 × 7.0 cm cylindrical juice straws) with one end thermo sealed (CHALLEN; ELLIOT, 1986). The cryoprotective solutions and the cryotubes were autoclaved separately at 121 °C for 20 min. Each cryotube received 800 µL of cryoprotective solution (glycerol at 5%, v v^{-1}, or with or without water) and five disks or grains containing mycelia. Cryotubes had the other end thermo sealed and were frozen from room temperature (23 ºC) to mechanical ultrafreezer temperatures (-20 ºC or -70 ºC). Each disk or grain was considered a replication.

Mycelial growth viability after cryopreservation

Fifteen replications were recovered from three cryotubes from each treatment. The cryotubes were thawed by submersion in water at 30 ºC for 15 min (MANTOVANI et al., 2008) then immersed in alcohol at 70% and in alcohol at 90%. One of the ends was cut and the excessive cryoprotectant removed. The disks or grains were transferred to MEA and kept at 25 ºC ± 1 ºC, in the dark, for 30 days. Each disk or grain was considered a replication. The recovery percentage of mycelial

growth was calculated and the differences were determined by Friedman's test ($p \leq 0.01$). The statistical test was only applied to the results obtained after the two-year cryopreservation. The treatments in which there was mycelial growth without sectioning from 75% or more replications were considered viable (COLAUTO et al., 2011a).

RESULTS AND DISCUSSION

The fungus cryopreserved at -20 ºC after one or two years was not viable in any treatment. The reduced freezing rate at -20 ºC favors water migration, increasing the ion-intracellular concentration and the big ice crystal intracellular formation (PEGG, 2007; UENO et al., 2004). Besides, according to Wa (1991), the intramembrane particle aggregation (IPA) in the plasma membrane could be the primary cause of freezing injury on cryopreservation at -20 ºC. The IPA is caused by the close plasma membrane with itself as a result of mechanical stress from the extracellular ice crystal formation. For basidiomycetes, Wa (1991) described that IPA is formed when slow cooling occurs mainly between -5 ºC and -30 ºC. The results found by Wa (1991) for basidiomycetes and this study with *A. blazei* indicated that cryopreservation at -20 °C has been a challenge. Despite that, the increasing addition of glycerol in association with whole grains could improve the mycelial viability and may be explored at -20 ºC cryopreservation development.

A. blazei cryopreservation at -70 ºC for one or two years was effective and had results that varied from 87% to 100% for HW cultivation medium and 5% glycerol as cryoprotectant (Table 1). Besides, the mycelial growth was quickly visualized in an average of 5.5 ± 2.5 days after thawing. Colauto et al. (2012) reported that the mycelial growth visualization of *A. blazei* cryopreserved at -70 ºC after four year-cryopreservation was 13 ± 4 days, and for Colauto et al. (2011a), 9 ± 4 days. This suggested that wheat grains as substrate and glycerol as cryoprotectant were effective ($p \leq 0.01$) for the cryopreservation and recovery of the mycelial viability of this fungus.

Table 1. *Agaricus blazei* mycelial viability (%) grown on different cultivation media, with or without 5% glycerol, and cryopreserved at -70 ºC for one or two years with 5% glycerol, ultrapure water or without cryoprotectant in the cryotube.

Cultivation medium*	Cryopreservation after 1 year Cryoprotectant in the cryotube			Cryopreservation after 2 years Cryoprotectant in the cryotube		
	GY	UW	WC	GY	UW	WC
HW	100	33	87	87a	0	73ab
MW	93	27	87	80a	0	53b
HR	93	27	87	68ab	0	53b
HW+GY	93	47	87	87a	0	67ab
MW+GY	93	27	87	87a	0	73ab
HR+GY	87	40	87	73ab	0	53b
HWG	87	13	67	0	0	0
MWG	93	13	60	0	0	0
HRG	93	13	80	0	0	0
HWG+GY	93	33	93	0	0	0
MWG+GY	87	27	93	0	0	0
HRG+GY	93	27	93	0	0	0
MEA	87	7	73	0	0	0
MEA+GY	60	20	73	0	0	0

*HW: whole grain of hard endosperm wheat, MW: whole grain of medium hard endosperm wheat, HR: whole grain of hard endosperm rye, GY: 5% glycerol, HWG: agar + ground HW, MWG: agar + ground MW, HRG: agar + ground HR, MEA: malt extract agar, UW: ultrapure water and WC: without cryoprotectant. Means followed by different letters indicate significant differences among the groups according to Friedman's test ($p \leq 0.01$).

Whole grains of hard endosperm wheat had the highest ($p \leq 0.01$) values of mycelial growth recovery, 87%, after two-year cryopreservation at -70 ºC (Table 1). It was similar to MW, HW+GY and MW+GY. This could indicate the grain capillary physical structure is more important than the grain chemical composition in the cultivation medium for the mycelial viability after cryopreservation. The grain capillary net provides reduced physical space that limits the presence of water molecules (HOSENEY, 1991; LOBO; SILVA, 2003) and protects mycelia from ice formation. Although malt

extract agar and potato dextrose agar disks are broadly used for fungus cryopreservation (CHVOSTOVÁ et al., 1995), hard endosperm wheat grains were a better alternative ($p \leq 0.01$) for *A. blazei* cryopreservation at -70 °C.

Regarding the glycerol addition to the cultivation medium, the mycelium grown on substrates without glycerol, MW and HR, presented 68% and 80% viability after two-year cryopreservation at -70 ºC, respectively. However, glycerol addition to these cultivation media, MW+GY and HR+GY, increased ($p \leq 0.01$) the viability to 73% and 87%, respectively. This positive effect was more evident in substrates with ground grain in which MWG and HRG that had 60% and 80% viability, respectively, in treatments with WC after one-year cryopreservation. Glycerol addition to these cultivation media (MWG+GY and HRG+GY) increased mycelial viability to 93% (Table 1).

The use of glycerol cryoprotective solution increased ($p \leq 0.01$) mycelial viability, but the use of water drastically decreased mycelial viability for all treatments cryopreserved at -70 ºC for two years. The absence of a cryoprotective solution or water was appropriate for the preservation indices that varied from 53% to 73% after two-year preservation (Table 1). Based on our findings, glycerol addition to the cultivation medium as a protective solution increased the effect of the mycelial viability preservation. Glycerol increases water linked to the mycelia, causing a smaller formation of ice crystals and showing the capacity of penetrating in the cell wall and plasmatic membrane, altering cellular permeability (HUBÁLEK, 2003). This promotes the binding of water inside the cell, avoiding excessive dehydration, reducing salt concentration, inhibiting osmotic shock, and preventing the formation of ice crystals (DUMONT et al., 2004; HUBÁLEK, 2003).

Therefore, our current data suggest that the use of glycerol seems an important adjuvant for basidiomycete cryopreservation, added to substrate or cryotube, although grain capillary physical structure seems to be the most important factor in maintaining the mycelial viability on cryopreservation.

CONCLUSIONS

The physical structure of whole grains of medium hard or hard endosperm wheat is effective as substrate for fungus growth and cryopreservation at -70 ºC for two years. Whole grains or ground whole grains of rye, with or without glycerol in substrate or cryotube, are effective only after one-year cryopreservation.

The use of glycerol in the cultivation medium or as cryoprotective solution makes in long-term cryopreservation at -70 ºC viable for the fungus. Cryopreservation at -20 ºC is ineffective for *A. blazei* mycelial viability preservation.

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RESUMO: *Agaricus blazei* é um basidiomiceto de importância comercial amplamente estudado quanto à sua atividade biológica. Entretanto, pouco foi estudado sobre as técnicas de preservação deste basidiomiceto, sendo a criopreservação uma alternativa. O objetivo deste trabalho foi avaliar os efeitos físicos e químicos de diferentes meios de cultivo e do glicerol na criopreservação de *A. blazei* à -20 ºC e à -70 ºC. Foi usado meio de cultivo sólido composto por ágar com farinha de grãos inteiros de cereais (trigo textura dura ou semidura ou centeio textura dura) ou ágar extrato de malte, ou grãos de cereais inteiros (trigo textura dura ou semidura ou centeio textura dura) sem ágar. Os meios de cultivo tiveram adição de glicerol (zero ou 5%). Grãos ou discos de meio de cultivo contendo o micélio foram transferidos para criotubos com glicerol a 5%, ou com ou sem água, sendo, em seguida, criopreservados (-20 ºC ou -70 ºC). Após um ou dois anos de criopreservação foi avaliada a viabilidade do fungo. Os resultados mostraram que a estrutura física de grãos inteiros de trigo semiduro ou duro é efetiva ($p \leq 0,01$), quando em -70 ºC para criopreservação por dois anos, assim como o uso de glicerol tanto no meio de cultivo ou como solução crioprotetora ($p \leq 0,01$). A criopreservação a -20 ºC mostrou-se ineficaz ($p \leq 0,01$) na manutenção da viabilidade do fungo.

PALAVRAS-CHAVE: *Agaricus brasiliensis. Agaricus subrufescens.* Substrato. Congelamento. Força osmótica.

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