Semisolid culture medium improves mycelial recovery of *Agaricus subrufescens* cryopreserved in cereal grains

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Received: 11 June 2018 / Accepted: 7 December 2018 / Published online: 8 March 2019
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Abstract

This study aimed to evaluate the effects of the solid and semisolid culture medium on the mycelial viability of *A. subrufescens* after 5-year cryopreservation at -70°C . Mycelia were grown in three types of whole or ground grains, with or without 5% glycerol addition in the substrate and/or in a cryotube. After 5 years of cryopreservation at -70°C , every treatment was thawed and recovered in malt extract culture medium with 15 (solid culture medium) or 5 g L^{-1} (semisolid culture medium) of agar. The semisolid recovery culture medium increased the mycelial viability recovery capacity of *A. subrufescens* cryopreserved for 5 years in grains with glycerol only in the cryotube, and specifically with medium-hard wheat grain without glycerol addition at all. Agar-based substrates such as malt extract agar, agar-ground grain, or the one with glycerol addition to the substrate were not effective to keep the mycelial viability, regardless of the recovery culture medium consistency. Hard and medium-hard endosperm wheat grains or hard endosperm rye grains with addition of glycerol as cryoprotectant only to the cryotube were effective to cryopreserve the fungus for 5 years without cryoprotectant addition in the substrate.

Keywords *Agaricus blazei* · Semisolid culture medium · Water activity · Mycelial cryopreservation · Thawing

Introduction

Agaricus subrufescens Peck (*Agaricus blazei* Murrill ss. Heinemann; *Agaricus brasiliensis* Wasser et al.) has received attention due to its antitumor [1–3], immune modulator [4], antimutagenic, photoprotective [5], antioxidant [6, 7], and anti-inflammatory [8] properties. Also, it is a laccase producer [9, 10] and is able to bioaccumulate zinc in mycelia [11].

Most basidiomycete cryopreservation studies evaluate the cryopreservation effect of temperature [12–14], substrates for fungal growth before cryopreservation [15–17], class and concentration of cryoprotectants [18, 19], and maintenance period [18, 20–22]. However, few studies assess the effect of cereal grains for cryopreservation and the consistency of recovery culture medium on the mycelial viability after cryopreservation.

Tanaka et al. [17] described the protective effect of grains due to the presence of a capillary net within the grain. Thus, the utilization of whole grains as substrate can increase even more the efficiency of the cryopreservation process for this fungus.

High osmotic pressure in a culture medium induces the fungal accumulation of polyols such as glycerol, mannitol, erythritol, and arabinol. Fungal polyol production is a chemical response to keep cell turgor pressure and growth in low moisture content environment [23]. Our hypothesis is that the increase of the osmotic pressure in the culture medium could affect cell turgor pressure and possibly reduce mycelial free water. It could be an advantage to cryopreservation due to less mycelial free water with consequent decrease of the ice crystal size and osmotic stress caused by the freezing process.

There are many studies about the initial and intermediate stages of the cryopreservation process but few studies on the recovery culture media after thawing [22]. Recovery cultivation media are usually the same ones used for fungal growth before freezing. The most used ones are potato dextrose agar (PDA) and malt extract agar (MEA) [22], but they are not always the best choice. Fungal cryopreservation is a very stressful freezing process and our hypothesis is that the increase in free water using semisolid media after thawing could improve mycelial recovery viability. Therefore, this study

Responsible Editor: Derlene Attili de Angelis

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aimed to evaluate the mycelial viability of *A. subrufescens* cryopreserved at $-70\text{ }^{\circ}\text{C}$ for 5 years on hard and medium-hard endosperm wheat grains, hard endosperm rye grains, with or without addition of glycerol to the substrate and/or cryotube, and the fungal recovery in solid and semisolid culture medium.

Material and methods

Fungus and experimental design

A. subrufescens U2-1 (ABL97/11) from the basidiomycete collection of the Laboratory of Molecular Biology, Paranaense University, was grown on MEA (20 g L^{-1} malt extract and 15 g L^{-1} agar; Himedia®), previously autoclaved at $121\text{ }^{\circ}\text{C}$ for 30 min, and kept at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ in the dark. The inoculum was selected when the mycelial growth edge had uniform appearance without sectoring.

Substrates

Mycelia were grown in three types of whole or ground grains, with or without glycerol in the substrate and/or the cryotube according to Tanaka et al. [17] Whole grains were hard endosperm wheat (*Triticum aestivum* L.) cultivar IPR 136, medium hard endosperm wheat (*T. aestivum* L.) cultivar IPR 130, and hard endosperm rye (*Secale cereale* L.) cultivar IPR 89, from the Instituto Agronômico do Paraná, Brazil. The same whole grains (40 g L^{-1}) were ground ($355\text{ }\mu\text{m}$ granulometry) and agar was added (15 g L^{-1}) to make agar-ground grains [17]. Whole or agar-ground grains with or without 5% glycerol (volume/volume) and/or the cryotube was used. MEA with or without 5% glycerol in the substrate and/or cryotube was used as controls. The treatments regarding substrates, with or without the cryoprotectant in the substrate and/or the cryotube were codified and shown in Table 1.

Whole grains were prepared by washing grains in tap water for 1 min and immersing them in ultrapurified water or in 5% glycerol, and kept at $90\text{ }^{\circ}\text{C}$ for 45 min. The liquid excess was removed; around 500 grains were transferred to 50 mL Falcon® tubes and autoclaved at $121\text{ }^{\circ}\text{C}$ for 180 min. Another grain sample was prepared by grinding whole grains at $355\text{ }\mu\text{m}$ and 40 g L^{-1} was added to agar (15 g L^{-1}) with ultrapurified water or 5% glycerol. MEA with or without 5% glycerol in the culture medium and/or cryotube were the controls. The agar-based media were autoclaved at $121\text{ }^{\circ}\text{C}$ for 30 min and transferred to Petri dishes (90 mm diameter). Each substrate was inoculated with 0.6-cm diameter MEA disks containing inoculum. The mycelial growth was carried out at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, in the dark, until complete colonization.

Cryopreservation

Cryoprotectants and cryotubes were autoclaved separately at $121\text{ }^{\circ}\text{C}$ for 20 min. Each cryotube received $800\text{ }\mu\text{L}$ of 5% glycerol or without cryoprotectant and five disks or grains containing mycelia were added. Cryotubes were frozen in a mechanical ultrafreezer at $-70\text{ }^{\circ}\text{C}$. The cryotubes and the details of the cryopreservation process were the same as the ones described by Tanaka et al. [17]

Mycelial recover viability after cryopreservation

The culture media for recovery of cryopreserved fungus were of 20 g L^{-1} malt extract (Himedia®) added of 15 g L^{-1} (solid culture medium) or 5 g L^{-1} (semisolid culture medium) agar (Himedia®). The water activity in the culture media was evaluated in a water activity analyzer (Labswift; Novasina®).

Six cryotubes of each treatment were recovered from an ultrafreezer after 5-year cryopreservation. The cryotubes were thawed by immersion in previously autoclaved-ultrapure water at $30\text{ }^{\circ}\text{C}$ for 15 min, and immersed in alcohol at 70% and at 90% for 30 s [24]. The cryotube ends were cut and the excessive cryoprotectant removed. The disks or grains of the cryotubes were transferred to solid and semisolid culture media and kept at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, in the dark, for at least 30 days. Each treatment had 15 replications, that is, three cryotubes for each culture medium. Each disk or grain was considered a replication. Treatments with mycelial growth without sectoring in 75% or more replications were considered viable [12].

Results

The solid culture medium, conventionally utilized in the recovery of cryopreserved *A. subrufescens* mycelial viability [13, 16–19, 25], was not effective to recover the mycelial viability ($<75\%$) of all treatments after 5-year cryopreservation of the fungus (Fig. 1). However, the semisolid culture medium resulted in mycelial viability ($\geq 75\%$) for the treatments HWW + WC/GY, MWW + WC/GY, MWW + WC/WC, and HRW + WC/GY after 5-year cryopreservation (Fig. 1). The semisolid consistency of the recovery culture medium provided mycelial viability increase from 55 to 75% for HWW + WC/GY, 24 to 80% for MWW + WC/GY, 16 to 77% for MWW + WC/WC, and 36 to 86% for HRW + WC/GY when compared to the solid culture medium (Fig. 1). The solid or semisolid malt extract agar culture medium showed the same water activity values of 0.965 ± 0.001 .

In treatments in which the cultivation substrate was MEA, agar-ground grain or when glycerol was added to the substrate, the mycelial viability was not effective, regardless of the consistency of the recovery culture medium (Fig. 1). This suggests that agar-based culture media are less effective than

Table 1 Codification of treatments and controls: substrates with or without 5% glycerol for *Agaricus subrufescens* growth, with or without 5% glycerol in the cryotube as a cryoprotectant for 5-year cryopreservation

Substrate	Whole grain (W)	Agar-ground grain (G)	Cryoprotectant added to substrate		Cryoprotectant added to cryotube		Code
			With glycerol (+GY)	Without cryoprotectant (+WC)	With glycerol (/GY)	Without cryoprotectant (/WC)	
Hard endosperm wheat (HW)	X	-	X	-	X	-	HWW + GY/GY
	X	-	X	-	-	X	HWW + GY/WC
	X	-	-	X	X	-	HWW + WC/GY
	X	-	-	X	-	X	HWW + WC/WC
	-	X	X	-	X	-	HWG + GY/GY
	-	X	X	-	-	X	HWG + GY/WC
	-	X	-	X	X	-	HWG + WC/GY
	-	X	-	X	-	X	HWG + WC/WC
Medium hard endosperm wheat (MW)	X	-	X	-	X	-	MWW + GY/GY
	X	-	X	-	-	X	MWW + GY/WC
	X	-	-	X	X	-	MWW + WC/GY
	X	-	-	X	-	X	MWW + WC/WC
	-	X	X	-	X	-	MWG + GY/GY
	-	X	X	-	-	X	MWG + GY/WC
	-	X	-	X	X	-	MWG + WC/GY
	-	X	-	X	-	X	MWG + WC/WC
Hard endosperm rye (HR)	X	-	X	-	X	-	HRW + GY/GY
	X	-	X	-	-	X	HRW + GY/WC
	X	-	-	X	X	-	HRW + WC/GY
	X	-	-	X	-	X	HRW + WC/WC
	-	X	X	-	X	-	HRG + GY/GY
	-	X	X	-	-	X	HRG + GY/WC
	-	X	-	X	X	-	HRG + WC/GY
	-	X	-	X	-	X	HRG + WC/WC
Malt extract agar (MEA)	-	-	X	-	X	-	MEA + GY/GY
	-	-	X	-	-	X	MEA + GY/WC
	-	-	-	X	X	-	MEA + WC/GY
	-	-	-	X	-	X	MEA + WC/WC

X, presence; -, absence

Fig. 1 Mycelial viability of *Agaricus subrufescens* cryopreserved for 5 years in wheat or rye grains, with or without 5% glycerol added to the substrate and/or cryotube, and recovered in solid (15 g/L agar) or semisolid (5 g/L agar) malt extract (20 g/L) agar culture medium. Treatments without mycelial viability were not displayed (Table 1)

cereal grains in mycelial viability cryopreservation, probably because grains provide an internal capillary net, besides protectant substances, which works as a physical protection to mycelia, confirming the reports of Tanaka et al. [17] and Mata and Pérez-Merlo [26]. Another aspect is that glycerol addition to the substrate in order to obtain a “preservation substrate” [22] was not effective to keep the mycelial viability in all treatments after 5-year cryopreservation.

The association of the semisolid culture medium was effective to recover the mycelial viability only when wheat or rye grains were used for mycelial growth, without glycerol addition to the substrate, and with glycerol addition to the cryotube. Except for the medium hard endosperm wheat, without cryoprotectant addition (or any solution) in the substrate and in the cryotube, which was effective to keep the mycelial viability of 5-year cryopreservation, the little explored potential of grains as natural preservation substrate for fungi is evident. For hard- or medium-hard endosperm wheat and hard endosperm rye only the treatment with cryoprotectant addition to the cryotube has mycelial viability after 5-year cryopreservation. This showed that glycerol can be utilized in association with preservation substrates such as wheat or rye if the semisolid culture medium is used to recover the mycelial viability after thawing.

Discussion

Glycerol in the culture medium for fungal growth before cryopreservation reduced the mycelial viability after cryopreservation, although glycerol used as a protectant solution for cryopreservation increased the mycelial viability recovery. Glycerol can bind water causing smaller formation of ice crystals and it can also penetrate the cell wall altering cellular permeability [27]. Cellular permeability alteration avoids excessive dehydration, reducing salt concentration, inhibiting osmotic shock, and preventing ice crystal formation [27, 28]. The same positive effect of glycerol as cryoprotectant was observed by Tanaka et al. [17] for *A. subrufescens* 2-year cryopreservation at $-70\text{ }^{\circ}\text{C}$.

For the 5-year cryopreservation at $-70\text{ }^{\circ}\text{C}$ of *A. subrufescens*, the fungal growth in whole grains of hard endosperm wheat or rye is recommended, even though MEA and potato dextrose agar disks were broadly used in the basidiomycete cryopreservation [22]. Colauto et al. [12] reported effective mycelial viability recovery of *A. subrufescens* grown in whole grains of rice with hulls and cryopreserved at $-86\text{ }^{\circ}\text{C}$ for 1 year with 10% glycerol. In addition, Tanaka et al. [17] reported effective mycelial viability recovery of *A. subrufescens* grown in whole grains of hard endosperm wheat and cryopreserved at $-70\text{ }^{\circ}\text{C}$ for 2 years with 5% glycerol. For these authors, there was mycelial viability of *A. subrufescens* recovered in MEA. In addition,

Colauto et al. [18] reported effective mycelial viability recovery of *A. subrufescens* grown in MEA and cryopreserved at $-70\text{ }^{\circ}\text{C}$ for 1 year with 6% glycerol; however, after a 4-year cryopreservation, glycerol was not effective anymore and was supplanted by 15% sucrose or 4% glucose. In our study, after a 5-year cryopreservation, no agar-based culture medium, mainly MEA, was effective to keep *A. subrufescens* mycelial viability, except for cereal grains which were effective. It indicates the limit of mycelial viability preservation for agar-based culture medium for 2 years (maybe 3 years) and the advantage of utilizing cereal grains in long-term cryopreservation for this species.

No reports were found for studies evaluating the effect of agar concentration in the culture medium to recover the mycelial viability after cryopreservation. The most utilized culture media to recover basidiomycetes contain from 8 to 20 g L^{-1} agar such as PDA [13, 19, 29], MEA [16–18], wort agar [14, 20], and MGL1 (barley flour, yeast extract, sucrose, and agar) [30]. This makes the comparison between studies difficult, besides the number of different variables involved. However, in our study, the lowest agar concentration (5 g L^{-1}) in the culture medium favored the increase of *A. subrufescens* mycelial variability. Therefore, this variable must be better explored in the recovery of cryopreserved basidiomycetes. In addition, preliminary tests with the utilization of liquid culture medium to recover the mycelial viability after cryopreservation were not effective (data not shown). The submerge culture has lower oxygen pressure with anaerobic areas, increasing the metabolic demand and reducing the chances of fungal recovery after cryopreservation.

The effect of agar concentration in the culture medium utilized after basidiomycete thawing has been little evaluated mainly in long-term cryopreservation studies. Our study makes evident that the water activity did not explain the alterations in the mycelial viability recovery capacity after cryopreservation. Since the concept of water activity was introduced by Scott [31], it has provided a basis to understand the effect of water on microbial growth in solutions and solid matrices. However, Huang et al. [32] demonstrated that water activity cannot explain the effect on agar concentration on the fungal germination and growth. For these authors, filamentous fungi grow by formation and extension of hyphae, and produce lateral or apical branches [33]. Hyphal extension provides a means of exploring new regions for fresh nutrients, while branch formation enables full utilization of the culture medium when already colonized. Nevertheless, under unfavorable conditions, fungi can initially reduce spatial branching growth to maintain their extension rate [33].

Thawing is a general procedure of cell reactivation after metabolic suspension of cryopreservation when the cell is in a stressful and fragile situation before starting growing again. Our results improved the cryopreservation process of *A. subrufescens* due to a modification of the agar

concentration in the recovery culture medium, allowing us to verify which technique is actually preserving the mycelial viability during cryopreservation. This important result has improved the knowledge on basidiomycete cryopreservation conditions, focusing mainly on *A. subrufescens*.

Conclusion

The semisolid consistency of the recovery culture medium increases the mycelial viability recovery capacity of *A. subrufescens* cryopreserved for 5 years with grains and glycerol in the cryotube, and with medium-hard endosperm wheat grains without addition of any cryoprotectant or solution. Agar-based substrates such as MEA and agar-ground grain, with or without addition of glycerol to the substrate, are not effective to preserve the mycelial viability regardless of the recovery culture medium consistency. Hard- and medium-hard endosperm wheat grains or hard endosperm rye with glycerol added to the cryotube are effective to cryopreserve the fungus for 5 years. However, medium-hard endosperm wheat is effective to cryopreserve fungus for 5 years without the utilization of cryoprotectant in the substrate and the cryotube.

Funding information The authors thank Universidade Paranaense, Programa de Pós-graduação em Biotecnologia Aplicada à Agricultura, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—finance code 001, Fundação Araucária, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support and fellowship.

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