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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 *A. 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 (PDA) or wheat grain (WG). 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 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.

Introduction

Agaricus subrufescens Peck (=*Agaricus blazei* Murrill *sensu* Heinemann; *Agaricus brasiliensis* Wasser et al.) is a basidiomycete with culinary[1] and medicinal characteristics and one of the most important mushroom produced in Brazil.[2] Its consumption has been increasing due to its functional properties as antitumor,[3,4] antimutagenic,[5] anti-inflammatory[6] 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 species[16] 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. subrufescens*. Mycelial biomass of basidiomycetes is very sensitive to freezing conditions[19] 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 cryoinjures 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 °C ± 1 °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 μm; 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 ≥300 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 μm; 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 °C ± 1 °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 μm; 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 °C ± 1 °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

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