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Preservation of *Agaricus subrufescens* strains at low temperature by using cultures on sorghum grains

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ABSTRACT

Background: One of the main problems for the preservation of genetics resources of *Agaricus subrufescens* is to maintain the viability of the strains because the mycelium is very sensitive to cooling and therefore it ages rapidly.
Aims: Evaluate the viability of *A. subrufescens* strains stored as cultures on sorghum grain (spawn) at different temperatures.
Methods: Eighteen strains of *A. subrufescens* and three strains of *Agaricus bisporus* were studied. Spawn's viability was evaluated under the following conditions: (1) control at 25 °C (C), (2) cooling to 4 °C (R) and (3) freezing in liquid nitrogen at −196 °C (LN). Samples were recovered from week 4 every 2 weeks until week 12 and week 24 in C and R, whereas in LN samples were recovered at 4, 12 and 24 weeks. Viability was evaluated in 50 seeds, by strain and condition, recovering the mycelium in Petri dishes with potato dextrose agar medium (PDA). Mycelium growth was also evaluated on PDA after 14 days of recovery.
Results: Most strains showed 100% viability and they were recovered usually in 1 day. In LN the viability ranged between 84 and 100% depending on the strain, but in some cases recovery took more than 10 days. Mycelial growth decreased gradually over time and although the results show significant differences between treatments C and R, the decline is associated with ageing of the mycelium rather than the treatment itself.
Conclusions: Culture on sorghum grain and storage at low temperature is an interesting way to preserve genetic resources of *A. subrufescens*.

Preservación de cepas de *Agaricus subrufescens* a bajas temperaturas con cultivos en semillas de sorgo

Palabras clave:
Agaricus blazei
Agaricus bisporus
Hongo almendra
Seta del sol
Setas comestibles
Germoplasma
Himematsutake

RESUMEN

Antecedentes: Uno de los principales problemas para la preservación de los recursos genéticos de *Agaricus subrufescens* es mantener la viabilidad de sus cepas ya que el micelio es sensible al frío y, en consecuencia, envejece rápidamente.
Objetivos: Evaluar la viabilidad de cepas de *A. subrufescens* y cepas de *Agaricus bisporus* en cultivos en semillas de sorgo a diferentes temperaturas.
Métodos: Se estudiaron 18 cepas de *A. subrufescens* y 3 cepas de *A. bisporus*. Se evaluó la viabilidad de *A. subrufescens* en las condiciones siguientes: (1) control a 25 °C (C), (2) enfriamiento hasta 4 °C (E) y (3) congelación en nitrógeno líquido a −196 °C (NL). Las muestras se recuperaron a las 4, 6, 8, 10, 12 y 24 semanas en C y E, mientras que en NL se recuperaron a las 4, 12 y 24 semanas. La viabilidad se evaluó en 50 semillas, por cepa y condición, en placas de Petri con medio de agar patata dextrosa (APD). También se evaluó el crecimiento de los micelios en APD tras obtención a los 14 días.
Resultados: La mayoría de cepas mostraron un 100% de viabilidad, y en general, se obtuvieron en 24 h. En la condición NL la viabilidad varió entre el 84 y el 100%, pero en algunos casos su obtención requirió > 10 días. El crecimiento de los micelios se redujo gradualmente con el tiempo y, aunque los resultados indicaron

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diferencias significativas entre los tratamientos C y E, este declive se asocia al envejecimiento del micelio más que al propio tratamiento.
Conclusiones: El cultivo en semillas de sorgo y el almacenamiento a bajas temperaturas es un medio eficaz para preservar los recursos genéticos de *A. subrufescens*.

Agaricus subrufescens Peck, also named *Agaricus blazei* Murrill *sensu* Heinemann, *Agaricus rufotegulis* Nauta or *Agaricus brasiliensis* Wasser, M. Didukh, Amazonas & Stamets[11] is a commercially cultivated mushroom appreciated by its medicinal and therapeutic properties, of note its antitumor, immunomodulatory, antioxidant, antimutagenic, antiviral, and antibacterial capacities, and its effects on autoimmune diseases, such as lupus and arthritis.[1,2,4,6,7,13,15,17,19,28,33,34] This species is popularly known as cogumelo do sol, cogumelo piedade, cogumelo de deus, almond mushroom, almond Portobello, king *Agaricus*, Himematsutake or Kawariharatake, among others[8,32] Commercially is often found with the initials ABM, abbreviation of *Agaricus blazei* mushroom. For taxonomy and synonymy of this taxon we followed Kerrigan,[11] Arrilaga & Parra,[3] Ludwig[21] and Cappelli[5] and the fact that the homonyme *A. subrufescens* Ellis & Everh is posterior as this has been corrected in Index Fungorum (http://www.indexfungorum.org).

One of the main attractions of the cultivation of *A. subrufescens* is its ability to grow at relatively high temperatures, making this species an ideal candidate for cultivation in the tropics and subtropics. Brazilian and Japanese authors have investigated the genetic polymorphism among cultivated strains, mainly by using RAPD markers, and they showed a high genetic homogeneity.[16] In each country, the strains currently cultivated probably derived from a single or a very few sporophores, because the growers select the better strains. In addition to the cultivated strains with their low genetic diversity presented above, the collection of genetic resources for this organism is enriching with few North American,[11] European,[20] and more recently Asiatic isolates (Callac, personal communication). Due to the new interest for this mushroom with the growing industry of *A. subrufescens*, which has consolidated in recent years, works are in progress for increasing the number of specimens in collections and studying their diversity. Breeding programs leading to hybrid production are in progress.[12] However both mushroom growers, breeders and biologists studying the biodiversity of this species have problems linked to the management of the strains because *A. subrufescens* is the only species of the genus *Agaricus* known suffering damage when exposed for prolonged periods at temperatures of 4 °C or lower.[11] Growers have difficulties for maintaining spawn under refrigeration for long time periods without loss of mycelium viability and breeders and biologists have to find other preservation methods of the genetic resources than conventional storage of mycelium at low temperature. Mushroom spawn is made of mycelium grown under sterile conditions on cereal grains. Most commercial growers acquire spawn and they store it, usually under refrigeration, until it is used for compost inoculation. The use of spawn as an alternative support for preservation of *Agaricus* genetic resources has been proposed by several authors after San Antonio and Hwang[31] and Mata and Pérez-Merlo.[23]

The present work evaluated mycelium viability of both cultivars and wild strains from different origins of *A. subrufescens* using spawn on sorghum grain during storage at 4 °C and in liquid nitrogen (−196 °C). The aims were to check if the use of mycelium developed on cereal grains could be an efficient way for storage and preservation of *A. subrufescens* strains as previously shown for the white button mushroom, *A. bisporus*,[23] to enlarge the ways of preservation in addition of the recent proposition by Colauto et al.[7] of cryoconservation at −80 °C on rice grain and the low-cost preservation by immersion proposed by Maia et al.,[22] and to observe the phenotypic variability between strains representative of the diversity in the today germplasm of *A. subrufescens*.

Materials and methods

Studied strains and spawn preparation

Eighteen strains of *A. subrufescens* and 3 strains of *A. bisporus* from different origins were studied. Table 1 shows the strains with their registration numbers in the strain collection at INRA in Bordeaux, France (CGAB) and the Institute of Ecology AC in Mexico (IE). The strains were maintained in culture medium of potato dextrose agar (PDA).

The strains were cultured for 7 days in Petri dishes with PDA. Spawn was prepared according to the method of Guzman et al.[9] in sorghum seeds (*Sorghum vulgare* Pers.) 65% hydrated and sterilized at 121 °C for 1 h. The seeds, placed in Petri dishes were inoculated with a disc (±0.5 cm diameter) of agar with mycelium precultures of each strain and incubated in the dark for 2 weeks at 25 °C to allow the grains were completely covered by mycelium.

Preservation treatments

After the 2-week incubation of spawn, Petri dishes were placed with spawn of each strain under refrigeration (R) at 4 °C. The samples were recovered every 2 weeks from week 4 until week 12 and at week 24. Control samples (C) were maintained at 25 °C and recovered in the same way that the treatment R.

For freezing samples the method proposed by Mata and Perez Merlo[23] was used. Fully incubated sorghum seeds were placed in sterile polycarbonate (Nalgene) vials (25 seeds per vial) each vial containing 1.5 ml of sterile cryoprotectant solution prepared with 10% glycerol (v/v). The seeds remained in contact with the cryoprotective solution for 1 h and then samples were placed in polycarbonate boxes and transferred directly into the container of liquid nitrogen (−196 °C) (LN). Samples were thawed at 4, 12 and 24 weeks, in polycarbonate boxes dipping in water at 30 °C for 10 min.[26] Once thawed, the vials were cleaned for 1 min in an alcohol solution (70%, v/v), then seeds were removed from vials and placed in Petri dishes with PDA.

Viability and vitality of the samples

To evaluate the effect of cooling on mycelial viability, after treatments (C, R, LN) seeds were placed in Petri dishes containing PDA and incubated at 25 °C. After treatments the percentage of sample recovery was evaluated through daily observations of the seeds. A sample was considered recovered when mycelial growth was noted by observing the seeds with a stereoscopic microscope. The delay for recovering was also recorded. For each treatment and week of incubation recovery was evaluated with 50 spawn seeds.

Moreover, the mycelial growth was evaluated by placing a spawn seed in a Petri dish with PDA and recording the diameter of the mycelium on 2 perpendicular axes.[25] Ten samples were prepared per treatment and strain. Petri dishes with spawn seeds