

SPAWN CRYOPRESERVATION OF *AGARICUS BISPORUS* AND *A. SUBRUFESCENS* STRAINS

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ABSTRACT

One of the main problems for conservation of mushroom germplasm is that the traditional method of subculturing facilitates aging and contamination of cultures. The conservation of strains at ultra-low temperature in liquid nitrogen (-196 °C) is a widely used method, however, this method become expensive in collections with large number of strains and requires highly specialized personnel. The aim of this study was to evaluate the viability of spawn of *A. bisporus* and *A. subrufescens* strains frozen at different temperatures (-20 and -80 °C). Two strains of *A. subrufescens* and one strain of *A. bisporus* were studied using spawn prepared in sorghum seeds. Fully-incubated sorghum seeds were placed in polycarbonate vials with a sterile glycerol solution (10% v/v) (G) or without glycerol (WG) and frozen at -20 or -80 °C. After 3, 6 and 12 months the samples were thawed (30 °C, 10 min) and the percentage of sample recovery was evaluated. Mycelial growth was also evaluated in potato dextrose agar (PDA) or a medium with glucose and compost extract (C) recording mycelial diameter after 7 days of incubation at 25 °C. The results showed that in the samples frozen at -20 °C, the recovery was minimal (2 to 4%). However, the samples frozen at -80 °C showed a recovery of 100% from 1 to 6 days after thawing. The recovered samples had a significantly higher growth in the medium C. In all cases the recovered samples produced normal basidiomata when cultivated on compost substrate.

Keywords: white button mushroom, *Agaricus subrufescens*, ultra-low temperatures, edible mushrooms, germplasm conservation

INTRODUCTION

Edible mushrooms of the genus *Agaricus* are currently valued not only for their nutritional properties but also for their medicinal and organoleptic qualities. The white button mushroom, *A. bisporus* (JE Lange) Imbach, is the most cultivated edible species in the world, with an estimated production of over 4 million tonnes annually [1]. Its nutritional and culinary properties have given it an important place in the world market preferences. Moreover, *A. subrufescens* Peck (named also in some publications *A. blazei* Murrill or *A. brasiliensis*), popularly known as cogumelodo sol, almond mushroom, or Himematsutake is a well recognized species for its medicinal properties and for its delicate flavor [2]. One of main features of this latter species is its ability to grow at relatively high temperatures which makes it ideal candidate for growing in tropical and subtropical areas [3].

Growers face difficulties for maintaining spawn under refrigeration for long time periods without loss of mycelium viability and breeders and biologists have to find new preservation methods of the genetic resources other than conventional storage of mycelium at low temperature. Most of Basidiomycetes are usually preserved by successive mycelial subculturing but this method occasionally causes genetic degenerations, lost of commercial characteristics and contamination by other microorganisms (mainly moulds and bacteria) [4]. Furthermore this method become expensive to maintain collections with many strains and requires highly specialized staff for managing them. On another hand, cryopreservation with liquid nitrogen at -196 °C has been successfully used for preservation of fungal species [5]. Other methods have been also tested for mycelial preservation [6], however one of the cheapest method is to use spawn of the strains as an alternative support for preservation [3, 6-9]. 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 [10] and Mata and Pérez-Merlo [8]. Among *Agaricus* species, *A. subrufescens* is the only species of the genus known to suffer damage when exposed for prolonged periods at temperatures of 4 °C or lower [11]. It diminishes its ability to grow in relatively short periods of time [3]. With the objective to find

options of spawn preservation, the aim of this work was to evaluate two different temperatures (-20 and -80 °C) to preserve spawn of *A. bisporus* and *A. subrufescens*.

MATERIALS AND METHODS

Studied strains and spawn preparation

Three strains of *Agaricus* genus from different origins were studied: IE 623 strain of *A. bisporus* native from Mexico and strains of *A. subrufescens* IE 829 (= INRA Ca 454) originating from Brazil and IE 830 (= INRA Ca 487) originating from France, see Llarena *et al.* [12] for strain description. 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.* [13] 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 PDA with mycelium pre-cultures of each strain and incubated in the dark for 3 weeks at 25 °C to allow the grains to get completely covered by mycelium.

Freezing samples (-20 and -80°C)

For freezing samples the method proposed by Mata and Perez Merlo [8] 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 (G) and then samples were placed in polycarbonate boxes and transferred directly into the freezer at -20 or -80 °C. Moreover vials containing 25 seeds without glycerol were also prepared (WG) and immediately placed into the freezers. All samples were kept frozen at -20 or -80 °C for 3, 6 and 12 months (3M, 6M, 12M). After that time samples were thawed in polycarbonate boxes by dipping in water at 30 °C for 10 min [7]. Once thawed, the vials were cleaned for 1 min in an alcohol solution (70% v/v) and then seeds were removed from vials and placed in Petri dishes with PDA and incubated at 25 °C.

Viability and vitality of the samples

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 (maximum 15 days). For each treatment (G, WG) and time of freezing (3M, 6M, 12M) recovery was evaluated with 50 spawn seeds.

Moreover, mycelia recovered from freezing treatments were used to prepare new spawn in order to evaluate mycelial growth in two culture media: PDA and other culture medium prepared with agar, glucose (100 g/l) diluted in compost extract (C). Mycelial diameter was recorded by placing a spawn seed in a Petri dish with PDA and C on 2 perpendicular axes after 7 days of incubation at 25 °C [14]. Ten samples were prepared per treatment and strain.

Basidiomata production test

To assess basidiomata formation, samples of 4 kg of commercial compost were inoculated with spawn (5%) prepared as above from each of the strains recovered after freezing at -80 °C. After 15 days of incubation at 25 °C a casing layer of 5 cm was added to the samples and incubation continued for another 7 days. To favor appearance of primordia, samples were placed in a room with relative humidity of 90–95%, ventilation for 10 min 4 times a day, 12 h light and 12 h dark at 18 °C for IE 623 strain and at 25 °C for IE 829 and IE 830 strains was provided. Formation of basidiomata was registered.

Statistical analysis of data

Data recorded for mycelial diameter were analyzed using ANOVA followed by Tukey's multiple-range test ($p = 95\%$) to identify statistical differences in the average diameter of the mycelia obtained with different treatments.

RESULTS AND DISCUSSION

The results showed that in the samples frozen at -20 °C the recovery was minimal. In the 3M frozen samples, only one strain of *A. subrufescens* (IE 829) exhibits a recover percentage between 2 and 4%. No strain was recovered at 6M or 12M in samples frozen at -20 °C. However, the samples frozen at -80 °C with or without cryoprotectant (G, WG) during the freezing times tested (3M, 6M, 12M) showed a recovery of 100% from 1 to 6 days after thawing (Table 1). In all strains and conditions tested at -80 °C, the recovered mycelia showed normal appearance (in color and texture, with no apparent alteration (Fig. 1).

Table 1. Percentage of recovered samples after frozen at -20 or -80 °C with (G) and without glycerol (WG)

		-20 °C		-80 °C		
		3M		3M	6M	12M
G	IE 623	0		* (5)	* (5)	* (3)
	IE 829	2 (14)		* (2)	* (5)	* (3)
	IE 830	0		* (2)	* (6)	* (2)
WG	IE 623	0		* (2)	* (5)	* (3)
	IE 829	4 (14)		* (2)	* (5)	* (5)
	IE 830	0		* (1)	* (5)	* (1)

* Means total recovery of 50 samples = 100 % recovery.

Numbers in parentheses are days for recovery.

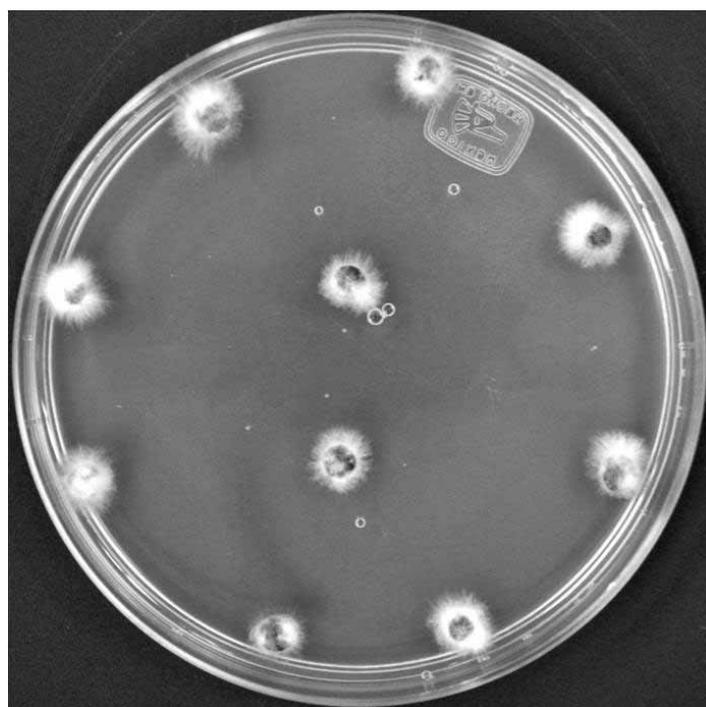


Figure 1. Mycelia recovered after 12 months of freezing at -80 °C with glycerol (G) as a cryoprotectant

In Table 2 the results of the mycelial growth of the strains frozen at -80 °C are shown. In general, all strains showed higher growth in the culture medium C with significant differences from the average obtained in PDA, the medium of compost extract and glucose seems to favor mycelial

growth after freezing. The average growth of the samples recovered from the WG treatment was 46.1 mm while in treatment G was 47.0 mm, so it was not possible to establish significant differences between treatments. These results confirm those obtained in other studies on the recovery of mycelia frozen without cryo protectant using seeds as vectors [3, 8, 14].

The strain IE 623 of *A. bisporus* showed no difference in their growth both 3M and 12M samples in treatments G and WG in both culture media (PDA and C). Further more the strains IE 829 and IE830 of *A. subrufescens* showed significantly lower growth after 12 months. Under similar conditions, using vectors of sorghum seed, but frozen in liquid nitrogen, these *A. subrufescens* shown greater recovery than strain IE 623 of *A. bisporus* [3], however, the results obtained in this work confirm that the strains of *A. subrufescens* are sensitive to low temperatures [3, 6], and therefore, protocols for preservation must be carefully selected and tested. The average mycelial growth in samples recovered from the 3M (49.9 mm) and 12M (43.2 mm) showed significant differences, suggesting as lightly negative effect due to freezing.

Spawn prepared from mycelia recovered after freezing at -80 °C (3M and 12 M) showed a normal growth in compost, completely covering the substrate at 15 days and having

Table 2. Micelial diameter in mm after 7 days of incubation in samples recovered from -80 °C

	IE 623				IE 829				IE 830			
	PDA		C		PDA		C		PDA		C	
	G	WG	G	WG	G	WG	G	WG	G	WG	G	WG
3M	20.6a	23.7a	50.8b	52.8b	44.6b	48.6b	72.5d	68.7cd	39.9b	33.0ab	75.3d	66.5c
12 M	22.1a	24.3a	54.1b	55.3b	24.6a	31.7a	61.8c	61.2c	30.1a	28.9a	66.7c	58.3c

Different in the columns by species indicate significant differences in mycelial diameters using Tukey's multiple range test ($p = 0.05$).

primordial from day 21, which means 6 days after adding the casing. The basidiomes obtained in all cases showed normal morphological (color, size, shape) and organoleptic (odor) characteristics (Fig. 2).

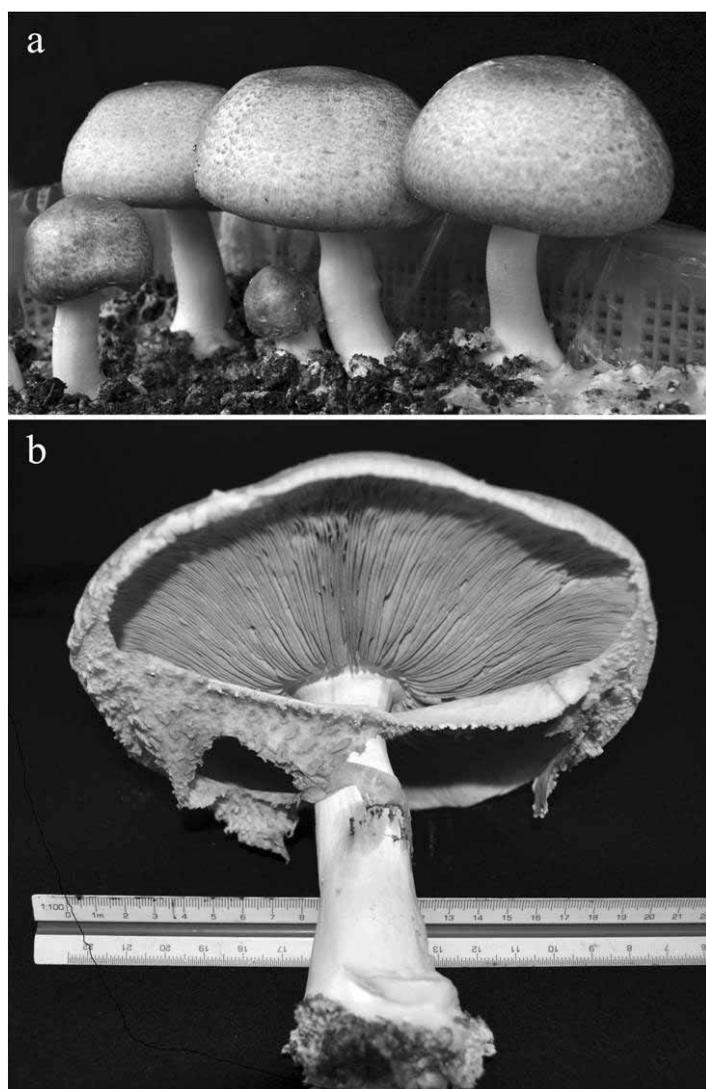


Figure 2. Basidiomata of *Agaricus subrufescens* (strain IE 830) obtained after 12 months of freezing at -80 °C

CONCLUSION

Samples frozen at -20 °C showed a minimal recovery (2 to 4%), however, samples frozen at -80 °C showed a recovery of 100% with or without cryoprotectant (G, WG) in all freezing times tested (3M, 6M, 12M). The recovered samples had a significantly higher growth in the medium prepared with compost extract and glucose (C), so this culture medium could be recommended to favor recovery samples. The results showed a slight decrease in growth in the samples of *A. subrufescens* strains recovered if 12 M samples are compared with 3 M samples. In spite of good results obtained in the recovery of samples, strains of *A. subrufescens* appear to be more sensitive than *A. bisporus* to freezing at -80 °C. Although in all recovered strains normal basidiomata were obtained when they were grown in compost, it would be advisable to test freezing during longer periods of time.

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