Viability in spawn stocks of the white button mushroom, *Agaricus bisporus*, after freezing in liquid nitrogen without a cryoprotectant

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Three strains of the White Button Mushroom (*Agaricus bisporus*) were studied. First, spawn was prepared from wheat seeds covered with mycelia, and then placed within polycarbonate vials for freezing in liquid nitrogen. The effect of adding a cryoprotective solution before freezing was evaluated as a function of mycelial growth and percent viability. Two treatments were undertaken: 1) freezing with a glycerol-based cryoprotectant and 2) freezing without a cryoprotectant. Samples were maintained frozen for two weeks, after which time they were thawed and the seeds placed in Petri dishes containing a culture medium. A recovery rate of 96% was obtained for all combined samples in the experiment, whereas 95.8% of the samples frozen without a cryoprotectant were recovered. These results suggest that mycelia can survive frozen storage without cryoprotectants, provided that they are embedded within and protected by the wheat seeds in the spawn. In addition, no *significant* differences were observed in recovery rates and mycelial diameters between the cryoprotection and non-cryoprotection treatments when a new series of spawn was prepared from the originally-frozen mycelia.

Key words: *Agaricus bisporus*, edible mushroom cultivation, germplasm conservation, liquid nitrogen, white button mushroom

Introduction

Agaricus bisporus (Lange) Imbach, commonly known as the “white button mushroom”, is the most commonly cultivated edible mushroom in the world. Estimated production in 2001 was approximately 2.4 million tons (Van Griensven, 2003). The maintenance of genetic diversity in *A. bisporus* species, as well as advances in culture preservation, allow scientists to enhance productivity and thereby meet world market demands. Laboratory germplasm should be periodically replenished, as continued subculturing (the common

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method for preserving strains) also increases mutation probabilities (Mata *et al*., 2000). Cryogenic storage is the most stable, long-term means of preserving germplasm currently available. However, this process still needs to be thoroughly studied in order to ensure the freezing and recovery of viable mycelia.

The viability of samples frozen in liquid nitrogen can vary depending on: 1) the species of mushroom being used, 2) the age of the mycelium, 3) growth conditions, 4) the type of cryoprotectant used, 5) the rate of cryoprotectant penetration, 6) the method and rate of freezing, and 7) thawing time and temperature (Chvostová *et al*., 1995; Mata *et al*., 2000).

To avoid cell damage due to freezing in liquid nitrogen, cryoprotective solutions (cryoprotectants) are commonly used. The cryopreservation of mushroom is generally undertaken by cutting agar blocks from growing cultures, and then immersing them in a cryoprotectant. Following immersion, the agar blocks are cooled from ambient temperature to -40°C at a rate of 1 to 10°C/min (Smith, 1998), and then placed in liquid nitrogen for storage.

Both the gradual freezing of samples and the use of cryoprotective solutions have been considered indispensable for the adequate recovery of mycelia (Roquebert and Bury 1993; Chvostová *et al*., 1995). *Agaricus* mycelium has been recovered from spawn prepared with gramineous seeds, when a pre-freezing procedure was used (Hwang and San Antonio, 1972; Kneebone *et al*., 1974; Jodon *et al*., 1982; San Antonio and Hwang, 1982). Nevertheless, satisfactory recovery rates have also been observed in *Volvariella volvacea*, *Pleurotus* spp. and *Lentinula* spp. when spawn were frozen, without pre-freezing procedure, in cryoprotective solutions (Pérez and Salmones 1997; Mata *et al*., 2000, 2004). In recent experiments, mycelia were also successfully recovered from spawn that had been frozen without the use of any cryoprotective treatment (Mata and Pérez-Merlo, 2003). In the majority of these cases, the recovery and growth of mycelia were initiated either from seed hila or else from fissures on the seed surface. These results suggest that the seeds might have protected the mycelia from damage. In particular, although the cellular contents of mycelia are known to crystallize with rapid freezing (a condition often observed as a darkening or granulation of the mycelia) (Smith and Thomas, 1998), neither the immediate freezing of spawn nor the absence of cryoprotective substances appears to have been lethal.

The present study evaluates mycelial recovery and survival rates in thawed *A. bisporus* spawn, following their preparation, freezing, and cold-storage without the use of cryoprotectants. Three *A. bisporus* strains were studies: two commercially available lines and one wild strain obtained in Mexico.

Materials and methods

Strains

Three strains of *Agaricus bisporus* were studied. Two of them, IE 272 and IE, 273 are commercial strains ("white" varieties) that were provided by a Mexican mushroom farm. The third strain, IE 623 (a wild, "brown" type collected by G. Mata and P. Callac) was found in Tlaxcala, Mexico where it was growing on the litter of *Cupressus benthamii* (Mata *et al*., 2002). Mycelia of all three strains are currently being maintained in the Strain Collection of the Institute of Ecology (IE) at Xalapa, Mexico.

Spawn preparation and the freezing and thawing of samples

Mushroom strains were pre-cultured for 7 days on malt extract agar medium (MEA), after which time spawn were prepared from pre-treated wheat seeds. Wheat grains were washed with water, cooked for 15 minutes, left standing in hot water for an additional 10 minutes, and then drained. The grains were added with a 0.5 % (dry weight) of a 1:1 proportion of calcium carbonate and calcium sulfate. These were autoclaved at 121°C for 90 minutes in polyethylene bags. After that, the sterilized wheat seeds were placed in Petri dishes and then each dish was inoculated with a pre-cultured mycelium (MEA disc ± 0.5 cm in diameter). Inoculated dishes were sealed with parafilm and incubated in darkness for 14 days at 24°C, a sufficient amount of time to allow mycelial growth to completely cover the wheat grains.

Treatment series

"T" Series: Freezing and subsequently thawing and recovering spawn, with or without a cryoprotective solution. In order to determine the effects of cryoprotection during the freezing process, fully-incubated wheat seeds were placed in sterile, polycarbonate (NALGENE) vials (20 seeds per vial), each vial containing a cryoprotective solution (10% glycerol v/v. 1.5 ml/vial). Sample vials were divided between two treatments: T1 represented a cryoprotection treatment and T2 was a "no cryoprotection" treatment. Three vials were prepared per treatment per strain. Seeds belonging to T1 remained in contact with the cryoprotective solution for 1 hour. Seeds in both treatments were directly frozen in liquid nitrogen within their polycarbonate containers. Two weeks later, vials were removed from the liquid nitrogen and thawed by immersion in distilled water at 30°C for 10 minutes (Mata *et al*., 2000). Once

thawed, vials were cleaned for 1 minute with an ethanol solution (70% v/v). After cleaning, seeds were removed and placed in Petri dishes with MEA in order to evaluate the effect of freezing, with or without cryoprotective solutions, on the recovery and growth of mycelia.

"C" Series: Non-frozen spawn, with or without a cryoprotective solution. In order to assess the effects of freezing on mycelial growth, a series of non-frozen, control samples was prepared. These "non-frozen" samples were prepared at the same time as the T1 and T2 treatments. Except for the absence of freezing, these controls were prepared according to the same protocol as described above for the frozen samples. Specifically, the C1 treatment refers to the control group for the cryoprotective solutions and C2 represents the "no cryoprotectant" control group.

"A" Series: Freezing and subsequently thawing and recovering spawn, with or without a cryoprotective solution; followed by a second round with recovered spawn, but this time without freezing. Mycelia recovered from T1 and T2 were cultured on MEA for 14 days, and then a new set of spawn was prepared from them. This additional spawn was treated according to the same protocol described for the "C" Series, but this time without freezing. A1 refers to a cryoprotective treatment and A2 indicates no cryoprotection. This series was important in establishing whether or not freezing damaged the mycelia.

Viability tests and mycelial growth

After thawing, seeds were placed in Petri dishes containing MEA. The percentage of viable samples for all series and treatments was evaluated by direct, daily observation. Samples were considered to be viable if mycelial growth was detected on seeds using a stereomicroscope. Fifty seeds per treatment (300 seeds per strain) were used in the evaluation of mycelial recovery and viability. Additional thawed specimens from each series and treatment group were examined with a scanning electron microscope to detect changes in seed structure and/or other factors related to mycelial survival.

Mycelial growth was determined from single seeds incubated in Petri dishes with MEA. Ten seeds were prepared per treatment (60 seeds per strain). Seed samples were incubated in darkness at 24°C. After fourteen days of incubation, the mycelial diameter of each seed sample was estimated from two perpendicular lines that were projected and drawn onto each Petri dish cover, whose intersection in addition was arbitrarily constrained to be the projected

center of the seed. In this way, each line represented two different samples of mycelial diameter. These two measurements were then averaged to produce a single estimate of mycelial diameter per Petri dish. Tukey's multiple-range test (95% confidence level) was used to analyze differences among average mycelial diameters for the different treatments.

Results

After thawing, all wheat grains from T1 (freezing, with a cryoprotectant) had lost their external mycelial layers. In this treatment, mycelia from all samples were recovered from seed hila and fissures on the seed surface (Figure 1). On the other hand, in T2 (freezing without a cryoprotectant) mycelia were never observed to be detached from the seed surface. However, as with T1, the mycelia from T2 were also recovered from seed hila and fissures.

Percent recovery in seed samples varied according to treatment and strain (Table 1). Strain IE 623 showed the highest percent recovery followed by strains IE 272 and IE 273. Freezing delayed recovery times. Samples frozen with a glycerol-based cryoprotectant (T1) were completely recovered between 9 and 16 days after thawing, whereas samples frozen without a cryoprotective solution (T2) were completely recovered between 3 and 14 days after thawing (Table 1). All samples belonging to T2 were recovered from the IE 623 and IE 272 strains. Strain IE 623 (the wild isolate) showed the most rapid recovery rates for all strains, being 9 days for T1 and 3 days for T2.

With respect to mycelial growth (Table 2), the diameters for mycelia in T1 and T2 were smaller than those encountered in C1 and C2 (control groups; no freezing) and in A1 and A2 (freezing and thawing, followed by no freezing). The difference in diameters was significant ($\alpha = 0.05\%$) for each strain. However in strains IE 623 and IE 272, the mycelial diameters for C1 were not significantly different from those associated with the A1 and A2 treatments, and only slightly different from the C2 treatment. The greatest mycelial growth was obtained in the IE 623 control samples, whereas the least growth was observed for the IE 272 strain. Results suggested that the smaller diameters observed for treatments T1 and T2 were due to delays in the initiation of mycelial growth that were caused by freezing.

In this study, mycelia recovery was tested for a grand total of 900 specimens (including the three tested strains and two treatments with series T, C and A), from which 864 (96.0%) were adequately recovered. In addition, of these 900 total specimens, 450 were frozen without a cryoprotectant and 431 (95.8 %) successfully recovered.

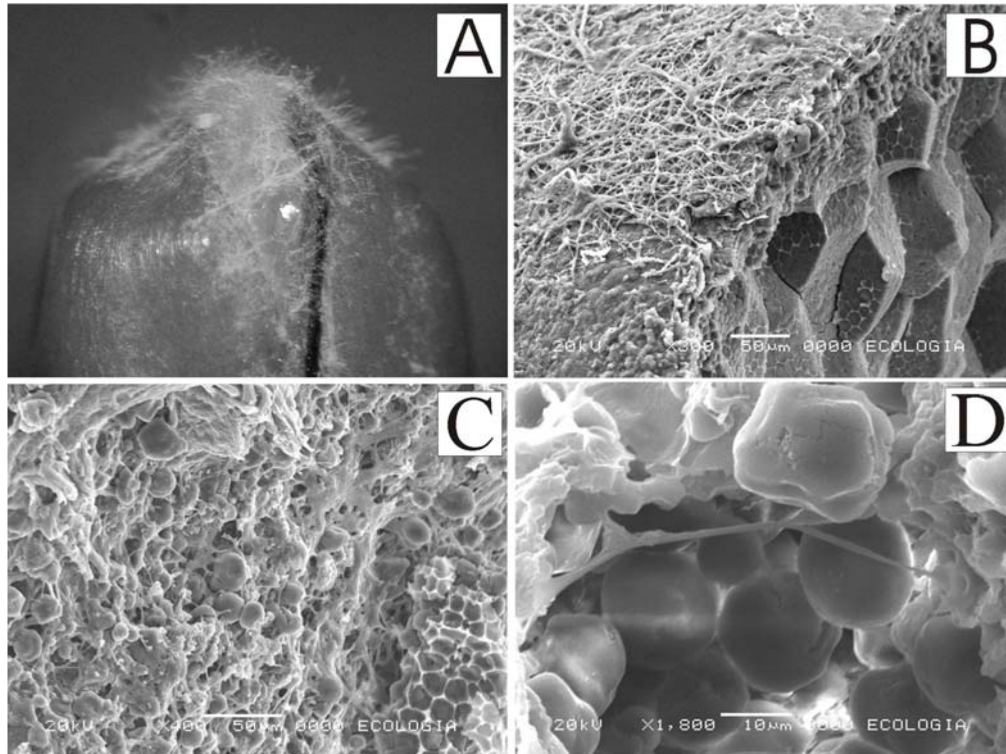

Fig. 1. Recovered spawn samples of *Agaricus bisporus* after freezing in liquid nitrogen without a cryoprotectant. **A.** A recovered mycelium growing in a seed fissure along a hilium. (Note that the seed surface has no living mycelium attached to it.) **B.** A treated wheat seed showing an internal structure and a mycelium emerging to the seed surface from the most external layer of the seed (× 300). **C.** The external layer of the seed showing structural modifications due to the presence of the mycelium (× 400). **D.** A mycelium living on the modified external layer of the seed (× 1800).

Discussion

The ability of mushroom cells to resist freezing and thawing may be affected by factors such as the age and physiological state of the hyphae, as well as by the nature of cytoplasmic contents (Suman and Jandaik, 1991). Results obtained from this experiment suggest that mycelia may survive freezing, without the use of cryoprotectants, because of the protection offered by wheat seeds. However, it is unclear if this protection might be related to the above-mentioned factors. Instead, this protection may be associated with a physical attribute of the wheat seed that allows the mycelia to survive. For

Table 1. Number and total percentage of mycelial samples recovered from three strains of *Agaricus bisporus*. Mycelia were recovered from samples associated with different treatments* that had been applied before or after freezing in liquid nitrogen.

Strain	Treatments	Time to recovery (days)																% Total
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	
IE 623	**T1**	0	0	2	12	18	6	4	3	1								92
	T2	19	26	5														100
IE 272	**T1**	0	0	1	2	4	4	5	2	3	6	2	5	6	0	1		82
	T2	7	18	14	9	1	0	1										100
IE 273	**T1**	0	0	0	0	2	0	3	3	3	3	3	2	2	7	2	1	62
	T2	1	1	5	11	6	3	6	5	3	2	1	0	0	2			92

*T1 = seeds frozen with a cryoprotective solution; T2 = seeds frozen without a cryoprotectant.

Note: The following treatments showed 100% recovery for all strains on day 1 (data not shown): C1 = non-frozen seeds, with a cryoprotective solution; C2 = non-frozen seeds, without a cryoprotectant; A1 = spawn recovered from initial freezing with a cryoprotective solution, but not subsequently re-frozen in the second round of tests; and A2 = spawn recovered from initial freezing without a cryoprotective solution, but not subsequently re-frozen in the second round of tests.

example, this protection might involve some type of structure within the seed that allows for mycelial growth. Alternatively, the mycelium might receive nutrition from the seed. Yet another possibility is that a specific compound in the seed, such as starch or sugar, might act as a non-permeating cryoprotectant (Jong and Davis, 1986), thereby reducing the injuries associated with freezing and thawing.

The fact that mycelia in this study were always recovered from seed hila, or from fissures on the seed surface, supports the idea that seeds offer protection to mycelia. Mycelia only penetrated the external layer of the seed and modified the structure in order to get nutrients. After freezing and thawing in liquid nitrogen, mycelium starts to grow from inside the seeds (Figure 1). In this experiment, the recovery rate for *A. bisporus* mycelia frozen without a cryoprotectant (95.8%) is very near the recovery rates obtained for other edible mushroom species (*Lentinula* spp., *Pleurotus* spp. and *Volvariella volvacea*

Table 2. Average mycelial diameter (mm) for three strains of *Agaricus bisporus* following different treatments* before and after freezing in liquid nitrogen.

Treatments	Strains					
	IE 623		IE 272		IE 273	
T1	16.6	a	14.9	a	12.8	a
T2	36.8	b	29.2	b	28.5	b
C1	59.7	d	50.8	d	46.9	c
C2	49.7	c	39.2	c	52.1	cd
A1	51.9	cd	55.5	d	53.9	de
A2	53.4	cd	50.8	d	58.1	e

Values in each cell represent the averages of mycelial diameters based on 10 seed samples per treatment. Different letters in each column indicate significant differences in mycelial diameters using Tukey´s test.
*T1 = seeds frozen with a cryoprotective solution; T2 = seeds frozen without a cryoprotectant; C1 = non-frozen seeds, with a cryoprotective solution; C2 = non-frozen seeds, without a cryoprotectant; A1 = spawn recovered from initial freezing with a cryoprotective solution, but not subsequently re-frozen in the second round of tests; and A2 = spawn recovered from initial freezing without a cryoprotective solution, but not subsequently re-frozen in the second round of tests.

that were frozen and thawed under similar conditions (Mata and Pérez-Merlo, 2003), however, spawn were prepared from sorghum seeds.

In spite of these preliminary results, it is necessary to further ascertain if mycelia frozen without the use of cryoprotectants are really free of damage following thawing and recovery. Even so, previous studies with *Pleurotus* and *Lentinula* strains (Mata *et al*., 2000, 2004) have shown that treating spawn stocks with a glycerol-based cryoprotective solution, without any pre-freezing, can result in 100% recovery of mycelia and without affecting subsequent mushroom production. We suggest that additional experiments, using longer freezing times, should be conducted in order to elucidate the mechanisms by which mycelia are protected within the wheat seed. Testing different kind of seeds in order to relate grain structure and composition to mycelial recovery rates would also be of interest.

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