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Spawn viability in edible mushrooms after freezing in liquid nitrogen without a cryoprotectant☆

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Abstract

Five strains of edible mushrooms (*Lentinula boryana*, *Lentinula edodes*, *Pleurotus djamor*, *Pleurotus pulmonarius*, and *Volvariella volvacea*) were studied. Spawn were prepared from sorghum seeds and then incubated for 14 days under optimum conditions for each species. Once covered by mycelia, the sorghum seeds were placed in polycarbonate vials for freezing in liquid nitrogen. The effect of adding a cryoprotective solution before freezing (either 10% glycerol v/v or 5% dimethylsulfoxide v/v) was evaluated as a function of mycelial growth and percent viability. Three main treatments were undertaken: (1) freezing with a glycerol or dimethylsulfoxide cryoprotectant, (2) freezing with water and (3) freezing without cryoprotectant or water. Samples were maintained frozen for a week, after which time they were thawed (10 min at 30 °C) and the seeds placed in Petri dishes with a culture medium. A recovery rate of 96.8% was obtained for the total number of samples summed over all strains and treatments. In contrast, 99.2% of the samples frozen without cryoprotectant were recovered. The recovery of frozen mycelia was delayed with respect to a control group, which was not frozen. However, no difference was observed in percent recovery and mycelial diameter when a new series of spawn was prepared from mycelia that had been previously frozen. Results obtained from this experiment demonstrate that an adequate recovery of mycelia can be obtained without using a cryoprotectant. This capacity might enable large quantities of commercial mushroom strains to be handled at reduced production costs. It is suggested that the mycelia survived freezing without cryoprotectants because they were embedded and protected within the sorghum seeds used to elaborate the spawn.

Keywords: Edible mushroom cultivation; *Lentinula*; *Pleurotus*; *Volvariella*; Germplasm conservation; Liquid nitrogen

One of the principal objectives for maintaining strain collections of edible mushroom is the long-term preservation of desirable characteristics. Continuous re-seeding in culture media (the traditional method) provides an effective, short-term option for preservation. Yet, this system can increase the risk of accidental contamination and/or changes in the morphological and physiological characteristics of the organisms [4]. Liquid nitrogen is the preferred method for storing strains that do not sporulate in the mycelial stage

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[1], however, this process must be thoroughly understood if mycelia are to be successfully frozen and adequately recovered. In particular, samples frozen in liquid nitrogen can vary in viability depending on the: (1) mushroom species, (2) age of the mycelium, (3) mycelial growth conditions, (4) type of cryoprotectant being used, (5) rate of cryoprotectant penetration, (6) method and rate of freezing, and (7) thawing time and temperature [1,8].

To avoid ice crystal formation and the cell damage caused by freezing in liquid nitrogen, cryoprotective solutions (cryoprotectants) are used. The cryopreservation of superior mushroom strains is generally undertaken by cutting agar blocks from growing cultures and then immersing them in a cryoprotectant. After immersion in the cryoprotective solution, agar blocks are gradually cooled from ambient temperature to −40 °C at a rate of 1–10 °C/min [12,13] and then placed in liquid nitrogen. Both the gradual freezing of samples and the use of cryoprotective solutions have been considered absolutely necessary for the adequate recovery of mycelia [1,10]. On the other hand, the utilization of water instead of a cryoprotective solution has been found to damage samples and inhibit their recovery [14].

Good recovery of *Agaricus* mycelia has been noted for spawn prepared from gramineous seeds, when a pre-freezing procedure was used [2,3,5, 11,15]. Mycelia have also been recovered for *Volvariella volvacea*, *Pleurotus* spp., and *Lentinula* spp. provided that the spawn were instantly frozen in cryoprotective solutions [6–9]. In the majority of these cited cases, mycelial recovery and new growth were initiated from seed hilae or from fissures on the surface of the seeds. These results suggest that the seeds might have acted as mycelial protectors. In particular, although cellular contents are known to crystallize with rapid freezing (often observed as a darkening or granulation) [14], neither immediate freezing of the spawn nor the absence of cryoprotective substances appears to have been lethal in these studies.

The present study attempted to evaluate mycelial recovery from thawed spawn previously frozen without the use of cryoprotectants, in order to evaluate survival possibilities under these conditions. Such a methodology, if successful, might be cost effective when a large number of specimens are being handled.

Materials and methods

Strains

Five strains of edible mushrooms belonging to the following species were studied: (1) *Lentinula edodes* (Berk.) Pegler, the Japanese Shiitake (IE 40); (2) *Lentinula boryana* (Berk. & Mont.) Pegler, the American Shiitake (IE 154); (3) *Pleurotus djamor* (Fr.) Boedijn, the Pink Oyster Mushroom (IE 111); (4) *Pleurotus pulmonarius* (Fr.) Quélet, the Indian Oyster Mushroom (IE 4); and (5) *V. volvacea* (Bull.: Fr.) Singer, the Paddy Straw Mushroom, (IE 106). All strains were identified and specimens have been deposited at the Instituto de Ecología in the Herbarium XAL. Mycelia are currently being maintained on malt extract agar (MEA) and/or potato-dextrose agar (PDA). Reference numbers for each strain appear above and are preceded by the abbreviation IE.

Spawn preparation and the freezing and thawing of samples

Mushroom strains were pre-cultured for 7 days on MEA (for *Pleurotus* and *Volvariella* species) or PDA (for *Lentinula* species), and spawn were prepared from pre-treated sorghum seeds (i.e., seeds of *Sorghum vulgare* Pers. were hydrated to 65% and then sterilized at 121 °C for 1 h).

Sorghum seeds were placed in Petri dishes; then each dish was inoculated with a pre-cultured mycelium, plus its agar disc (±0.5 cm in diameter). Inoculated dishes were sealed with elastic plastic film and incubated in darkness for 14 days (at 25 °C for *Lentinula* and *Pleurotus* species and at 28 °C for *V. volvacea*), allowing mycelial growth to completely cover the sorghum grains.

Experimental design

''T'' series: freezing and thawing spawn, with or without cryoprotective solutions

In order to determine the effects of cryoprotection during the freezing process, fully-incubated sorghum seeds were placed in sterile, polycarbonate (NALGENE) vials (25 seeds per vial), each vial containing a cryoprotective solution appropriate to the species being tested [6,8,9] (Fig. 1). Sample vials were divided among the following treatments. T1 represented either the cryoprotective solution 10% glycerol v/v (1.5 ml/vial) for *Pleurotus* and *Lentinula*, or 5% dimethylsulfoxide v/v (1.5 ml/vial) for *V. volvacea*. T2 represented a treatment of sterile, distilled water (1.5 ml/vial) that was used for all strains and all species. T3 was a ''no cryoprotection'' treatment because neither cryoprotectant nor water was utilized. Three vials were prepared per treatment per strain. Seeds for T1 and T2 remained in contact with the cryoprotective solutions or water for 1 h, and then all three seed treatments in their polycarbonate containers were directly frozen in liquid nitrogen. One week later, vials were removed from the liquid nitrogen and thawed by submergence in distilled water at 30 °C for 10 min [8]. Once thawed, vials were cleaned for 1 min with an alcohol solution (70% v/v). After cleaning, seeds were removed and placed in Petri dishes with adequate agar media (MEA or PDA) in order to evaluate the effect of freezing with or without cryoprotective solutions, on mycelial recovery and growth.

''C'' series: not freezing spawn, with or without cryoprotective solutions

In order to assess the effects of freezing on mycelial growth, a series of non-frozen, control samples were prepared. The samples ''non-frozen'' were prepared at the same time as treatments 1, 2, and 3. Except for the absence of freezing, these controls were subjected to the same treatment protocol described for the frozen samples. C1 refers to the control for the cryoprotective solutions,

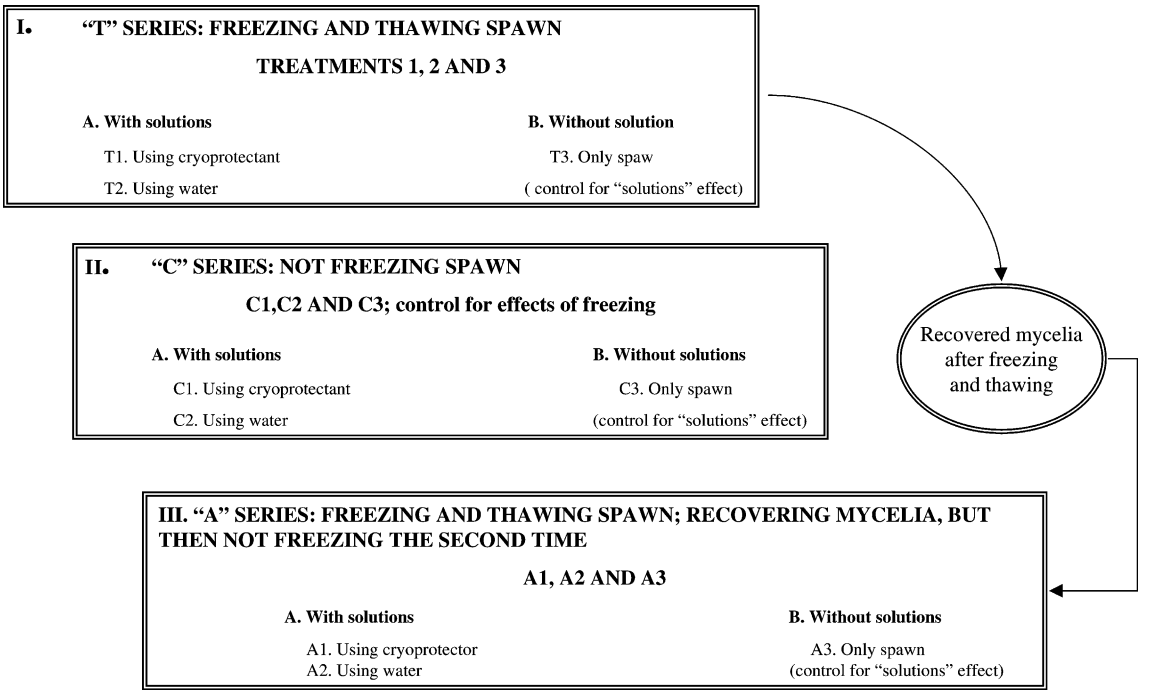

Fig. 1. Experimental design used for testing effects of freezing on mycelial growth and recovery of five strains of edible mushrooms (*L. boryana*, *L. edodes*, *P. djamor*, *P. pulmonarius*, and *V. volvacea*).

C2 indicates the water treatment control, and C3 represents the "neither-cryoprotectant-nor-water" control.

"A" series: freezing and thawing spawn, with or without cryoprotective solutions; followed by not freezing recovered spawn, with or without cryoprotective solutions

Secondly, frozen mycelia were thawed and recovered from T1, T2, and T3, cultured on MEA or PDA for 14 days, and then a new set of spawn was prepared from them. This additional set of spawn was re-submitted to the protocol described above, but this time without freezing. A1 refers to the cryoprotective solution, with no freezing; A2 indicates the water treatment, without freezing; and A3 represents the "neither-cryoprotectant-nor-water" treatment, without freezing. This series was considered important in establishing whether freezing damaged mycelia.

Mycelial growth and viability tests

After thawing, seeds were placed in Petri dishes with appropriated culture media (MEA or PDA). The percent viability of samples for all series and treatments was evaluated by direct, daily observation. Samples were considered to be viable if mycelial growth was detected on the seeds using a stereomicroscope. Mycelial recovery viability was evaluated for 50 seeds per treatment (450 seeds per strain).

Mycelial growth was determined from single seeds incubated in Petri dishes. Appropriate culture medium was used to grow each strain (see above). Ten seeds were prepared per treatment (90 seeds per strain). Seed samples were incubated in darkness at 25 °C for *Pleurotus* and *Lentinula*, and at 28 °C for *V. volvacea*. Mycelial diameters were estimated after four days of incubation for *V. volvacea*, and after seven days of incubation for *Pleurotus* and *Lentinula* species. Mycelial diameters were estimated for each seed sample by projecting and drawing onto each Petri dish cover two perpendicular lines (whose intersection was established at the center of the seed) that represented two different samples of mycelial diameter. These two measurements were then averaged to produce a single estimate of diameter per Petri dish.

Statistical analysis of the data

Tukey's multiple-range tests (95% confidence level) were used to analyze differences among average mycelial diameters for the different treatments.

Results

After thawing, all sorghum grains from T1 (freezing, with cryoprotectant) and T2 (freezing, with water) had lost the external mycelial layers that covered them. In both of these treatments, mycelia from all samples were recovered from seed hilae, or from fissures on the seed surfaces. In T3 (freezing, with neither cryoprotectant nor water), mycelia were never seen to be detached from the seed surface.

Percent recovery in seed samples varied according to treatment and species (Table 1). Strains IE 111 (*P. djamor*) and IE 154 (*L. boryana*) showed 100% recovery for all treatments. Recovery was nearly total for strains IE 106 (*V. volvacea*) and IE 4 (*P. pulmonarius*). Recovery was less for strain IE 40 (*L. edodes*), especially for T2. The freezing of samples delayed recovery times. Samples frozen with glycerol (T1) were recovered between 3 and 10 days after thawing; samples frozen with water (T2) were recovered between 5 and 10 days after thawing; and samples frozen without a cryoprotective solution or water (T3) were recovered between 1 and 10 days after thawing (Table 1). Strain IE 106 (*V. volvacea*) showed the most rapid recovery (5 days), whereas strain IE 40 (*L. edodes*) showed the least (10 days).

With respect to mycelial growth (Table 2), mycelial diameters for T1, T2, and T3 were smaller than mycelial diameters for C1, C2, and C3 (control group; no freezing) and for A1, A2, and A3 (freezing and thawing, followed by no freezing). The difference in growth diameters was significant ($\alpha = 0.05\%$) for each strain. However, for *Pleurotus* (IE 4 and IE 111) and *L. edodes* (IE 40), mycelial diameters associated with C1, C2, and C3

Table 1
Number and total percentage of mycelial samples recovered from five species and strains[a] of edible mushrooms. Mycelia were recovered from samples associated with different treatments[b] that had been applied before or after freezing in liquid nitrogen

Strain and treatments	Time to recovery (days)										% Total
	1	2	3	4	5	6	7	8	9	10	
IE 4											
T1	13	36	50								100
T2	0	1	16	32	42	45	48				96
T3	50										100
IE 111											
T1	30	46	50								100
T2	0	0	24	41	47	48	50				100
T3	50										100
IE 40											
T1	0	0	0	0	15	23	36	45	46	48	96
T2	0	0	0	0	2	5	20	31	35	36	72
T3	0	5	12	29	33	35	40	42	46	48	96
IE 154											
C1	50										100
C2	43	50									100
C3	35	50									100
T1	0	13	31	43	48	49	50				100
T2	0	0	0	13	35	44	50				100
T3	37	43	45	48	49	50					100
IE 106											
T1	6	17	30	39	49						98
T2	2	11	31	42	50						100
T3	34	44	50								100

Note. Treatments A1, A2, and A3 showed 100% recovery of all strains on day 1 (data not shown). With the exception of *L. boryana* (strain IE154), 100% recovery was also obtained on day 1 for all samples submitted to treatments C1, C2, and C3.

[a] IE 4, *P. pulmonarius*; IE 111, *P. djamor*; IE 40, *L. edodes*; IE 154, *L. boryana*; IE 106, *V. volvacea*.

[b] T1, freezing seeds, plus a cryoprotective solution; T2, freezing seeds, plus water; T3, freezing seeds, without cryoprotectant or water; C1, not freezing seeds, plus a cryoprotective solution; C2, not freezing seeds, plus water; C3, not freezing seeds, without cryoprotectant or water; A1, not re-freezing spawn recovered after the initial freezing treatment, plus a cryoprotective solution; 1; A2, not re-freezing spawn recovered after the initial freezing treatment, plus water; A3, not re-freezing spawn recovered after the initial freezing treatment, without cryoprotectant or water.

were not significantly different from diameters associated with the A1, A2, and A3 treatments. On the other hand, only slight differences were found for *L. boryana* (IE 154) and *V. volvacea* (IE 106) between the diameters for the controls (C1, C2, and C3) and the A1, A2, and A3 treatments. The greatest mycelial growth was obtained for *Pleurotus* (IE 4 and IE 111) and the least growth occurred in the *L. boryana* (IE 154). Results suggested that the smaller diameters observed for treatments T1, T2, and T3 were due to delays in the initiation of mycelial growth caused by freezing.

In this study, a grand total of 2250 samples were evaluated. Of these, 2180 (96.8%) specimens were adequately recovered. The total number of samples frozen without a cryoprotectant was 250. Of these samples, 248 (99.2%) specimens were successfully recovered.

Discussion

Results obtained from this experiment demonstrate that an adequate recovery of mycelia (99.2%) can be obtained without using a

Table 2
Average mycelial diameter (mm) for five species and strains[a] of edible mushrooms following different treatments[b] before and after freezing in liquid nitrogen

Strain	IE 4	IE 111	IE 40	IE 154	IE 106
Treatment					
C1	90.0 (0.0) d	88.7 (2,0) d	64.8 (3.6) d	22.2 (4.1) d	55.1 (3.9) e
C2	89.8 (0.6) d	88.3 (1.8) d	63.9 (4.5) d	21.5 (2.7) d	51.9 (8.5) de
C3	89.4 (0.9) d	88.8 (5.4) d	64.5 (2.3) d	23.2 (3.9) d	48.2 (5.4) cd
T1	31.7 (12.6) b	47.3 (10.2) b	57.2 (7.9) bc	16.3 (2.8) c	18.1 (4.2) a
T2	10.6 (4.8) a	21.6 (10.9) a	60.9 (7.8) cd	8.8 (1.7) a	11.4 (2.8) a
T3	44.4 (5.8) c	65.3 (9.0) c	51.2 (6.3) a	13.2 (3.0) b	28.4 (12.1) b
A1	87.0 (4.2) d	88.7 (2.1) d	70.2 (2.9) d	26.4 (3.3) e	42.1 (13.9) c
A2	88.6 (2.2) d	89.1 (3.1) d	69.8 (3.0) d	24.2 (3.0) cde	55.4 (7.5) e
A3	87.2 (3.7) d	84.7 (6.0) d	72.5 (2.2) d	25.3 (3.0) de	54.4 (16.4) e

Note. Values in each cell represent the averages and standard deviations (in parentheses) 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.

[a] IE 4, *P. pulmonarius*; IE 111, *P. djamor*; IE 40, *L. edodes*; IE 154, *L. boryana*; IE 106, *V. volvacea*.

[b] T1, freezing seeds, plus a cryoprotective solution; T2, freezing seeds, plus water; T3, freezing seeds, without cryoprotectant or water; C1, not freezing seeds, plus a cryoprotective solution; C2, not freezing seeds, plus water; C3, not freezing seeds, without cryoprotectant or water; A1, not re-freezing spawn recovered after the initial freezing treatment, plus a cryoprotective solution; 1; A2, not re-freezing spawn recovered after the initial freezing treatment, plus water; A3, not re-freezing spawn recovered after the initial freezing treatment, without cryoprotectant or water.

cryoprotectant. This capacity might enable large quantities of commercial mushroom strains to be handled at reduced production costs.

Factors such as the age and physiological state of the hyphae, as well as its cytoplasmic contents, may have affected the capacity of mushroom cells to resist freezing and thawing [14]. Our results suggest that mycelia may survive freezing without cryoprotectants because of the protection offered by sorghum seeds. Yet, it is unclear if this protection was related to the above-mentioned factors. Instead, this protection might have been associated with a physical attribute of the sorghum seed that allowed the mycelia to survive. Alternatively, the mycelia might have received nutrition from the seeds. Yet another possibility is that a seed compound, such as starch or sugar, may have acted as non-permeating cryoprotectant [4], thereby reducing the injuries associated with freezing and thawing.

Although these preliminary results were gratifying, it is necessary to further ascertain if mycelia frozen without the use of cryoprotectants are really free of damage after thawing and recovery. Nevertheless, previous studies [6,8] show that treating spawn stocks with a cryoprotective solution of glycerol, without pre-freezing, resulted in 100% recovery of *Pleurotus* and *Lentinula* strains. Furthermore, no differences in mushroom production were observed.

In conclusion, we suggest that additional experiments with longer freezing times be conducted to elucidate the mechanisms by which mycelia are protected in the sorghum seed. We also suggest that techniques utilized in this study might be adapted to the freezing and recovery of other microorganisms.

Results obtained from this experiment demonstrate that an adequate recovery of mycelia can be obtained without using a cryoprotectant. This capacity might enable large quantities of strains to be handled at reduced production costs. It is likely that seeds used to elaborate spawn were able to protect the mycelia that were embedded within them.

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