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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*.

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

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*.

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Agaricus subrufescens Peck, also named *Agaricus blazei* Murrill *sensu* Heinemann, *Agaricus rufotegulis* Nauta or *Agaricus brasiliensis* Wasser, M. Didukh, Amazonas & Stamets¹¹ 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 Ludwig²¹ and Cappelli⁵ and the fact that the homonym *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 Hwang³¹ 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 Pérez Merlo²³ 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

Table 1
Studied strains with their original registration number and their geographical origin.

Strain number	Inecol reference	CGAB reference	Species	Origin	Original number	Collector or [collection]	Date
1	IE 623	Bs 687	<i>A. bisporus</i>	Mexico, wild	IE 623	G. Mata, P. Callac	2001
2	IE 754		<i>A. bisporus</i>	Spain, cultivar	Mis Paj 365	G. Mata	2007
3	IE 789	CA 603	<i>A. subrufescens</i>	Mexico, wild	IE 789	G Mata, C Billette	2008
4	IE 829	CA 454	<i>A. subrufescens</i>	Brazil, wild	ATCC76739	Wei Jen Chen	
5	IE 830	CA 487	<i>A. subrufescens</i>	France, wild	CA 487	JC Blanchard	2006
6	IE 831	Bs 527	<i>A. bisporus</i>	France, cultivar	30A	[Euromycel]	1997
7	IE 832	CA 454-3 × CA 487-100	<i>A. subrufescens</i>	Hybrid produced at INRA, France	CA 454-3 × CA 487-100	[CGAB]	2009
8	IE 872	CA 276	<i>A. subrufescens</i>	Cultivar		Wei Jen Chen	
9	IE 873	CA 438 A	<i>A. subrufescens</i>	Spain, wild	ARAN559	P. Arrillaga et al.	2005
10	IE 875	CA 561	<i>A. subrufescens</i>	Brazil, cultivar	ABL-99/30	DC Zied	1999
11	IE 876	CA 562	<i>A. subrufescens</i>	Brazil, cultivar	ABL-03/44	DC Zied	2003
12	IE 877	CA 563	<i>A. subrufescens</i>	Brazil, cultivar	ABL-04/49	DC Zied	2004
13	IE 878	CA 564	<i>A. subrufescens</i>	Brazil, cultivar	ABL-05/51	DC Zied	2005
14	IE 879	CA 565	<i>A. subrufescens</i>	Brazil, cultivar	ABL-03/48	DC Zied	2003
15	IE 880	CA 570	<i>A. subrufescens</i>	Brazil, cultivar	ABL-01/29	DC Zied	2001
16	IE 881	CA 571	<i>A. subrufescens</i>	Brazil, cultivar	ABL-98/11	DC Zied	1998
17	IE 882	CA 572	<i>A. subrufescens</i>	Brazil, cultivar	ABL-07/58	DC Zied	2007
18	IE 883	CA 574	<i>A. subrufescens</i>	Brazil, cultivar	ABL-07/60	DC Zied	2007
19	IE 884	CA 643	<i>A. subrufescens</i>	France, wild	CA 643	J Guinberteau	2008
20	IE 885	CA 646	<i>A. subrufescens</i>	Belgium, cultivar	7700	[Mycelia]	
21	IE 886	CA 647	<i>A. subrufescens</i>	Belgium, cultivar	7703	[Mycelia]	

were incubated in darkness at 25 °C for 14 days and the mycelial diameters were measured on two perpendicular axes.

Statistical analysis of data

Data recorded for mycelial diameter were analysed 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

Recovery rates of the samples varied between the treatments and between strains in some treatments. They were 100% in controls (C) and treatment at 4 °C (R). No sectoring was observed during the fungal colony extension. Most samples were recovered in one day after being placed in the culture medium. However, in some sampling times the delay for total recovery of the samples was between 2 and 3 days. In controls only two strains required two days to reach full recovery, they both were *A. bisporus*. In the treatment R, one *A. bisporus* strain out of three and five *A. subrufescens* strains out of 18 had recovery delays higher than one day, and one of them was recovered completely only on the third day (Table 2). Samples stored in liquid nitrogen showed recovery rates between 84 and 100% depending on the strain and the sampling date. It is noteworthy that the lower rate was for the Mexican strain of *A. bisporus* after four weeks of storage and required between 8 and 11 days for total or partial recovery, while some other strains required up to 13 days (Table 2). In all strains and conditions tested, the recovered mycelia showed normal appearance in color and texture, with no apparent alteration.

The average of mycelial diameter for all the *A. subrufescens* strains after incubation for 14 days was statistically different between the 3 treatments when the recovering times were grouped. Mean values for LN treatment reached 80% of the control value, and 88% of that of R treatment (Table 3). However, for half of the strains the differences between controls and refrigerated mycelia were not significant (Table 4). For the other half, higher diameters were recorded in the control treatment than in R. The

two Brazilian cultivars 14 and 16 were the only *A. subrufescens* strains with a significant lower mycelial diameter in LN than in R. There was no significant correlation between the recovery rates and the mycelial growth ability which appeared to be a characteristic of strains. The strain with the slowest growth was strain 3 (wild Mexican isolate) with a mean diameter of 16.4 mm, and the fastest growing strain was strain 17 (one of the more recent Brazilian cultivar) with 68.2 mm (Table 3). It is noteworthy that the wild strains of *A. subrufescens* had the significantly lowest mycelial diameters after 14 days of incubation in each treatment.

For *A. bisporus*, the three strains had different reactions to the treatments (Tables 3 and 4). Whereas the Mexican wild strain was significantly gradually affected by the low temperatures, the observed differences in the cultivars were not or slightly significant. The mycelial diameters after 14 days of incubation were in the same range than that of the wild *A. subrufescens* strains.

The highest growth ability was observed in the first's weeks of storage in treatments C and R, thereafter with the passage of time, mycelial diameters decreased gradually reaching significant lower values in both treatments. *A. bisporus* strains showed no significant difference in mycelial growth between weeks 4 and 8 in C treatment, while in R treatment it did not differ between weeks 4 and 6. The growth inhibition at week 10 in treatment C was 34.3%, while in treatment R was 35.5% at week 8 (Table 5). Moreover strains of *A. subrufescens* showed their greatest mycelial growth at week 4 in both C and R treatments. The observed growth from week 6 was significantly different and growth inhibitions were obtained up to 38.7% at week 10 of treatment C and 42.5% at week 24 of treatment R (Table 5).

Discussion

For the preservation of genetic resources and breeding programs, the longevity of the strains is an important question. Whereas the mycelial cultures of *A. subrufescens* in liquid or agar media are known to suffer damage when exposed for prolonged periods at temperatures of 4 °C or lower,¹¹ storage of mycelium on sorghum grain at 4 °C did not affect the resilience of mycelia. The final recovery observed during 24 weeks of storage was always

Table 2
Number of samples recovered in treatments C, R and LN and percentage of recover.

Species	Strain number	Control						Refrigeration						Liquid nitrogen			% Recover
		Week						Week						Week			
		4	6	8	10	12	24	4	6	8	10	12	24	4	12	24	
<i>Agaricus bisporus</i>	1	*	*	*(2)	*	*	*	*	*	*	*	*	*	27 (11)	49 (10)	*(8)	84
	2	*	*	*(2)	*	*	*	*	*	*	*(2)	*	*	*(6)	39 (13)	48 (9)	91
	6	*	*	*	*	*	*	*	*	*	*	*	*	*(8)	47 (12)	48 (7)	97
<i>Agaricus subrufescens</i>	3	*	*	*	*	*	*	*	*	*	*(2)	*	*	*(9)	*(5)	*(2)	100
	4	*	*	*	*	*	*	*	*	*	*	*	*	*(3)	*(13)	*(7)	100
	5	*	*	*	*	*	*	*	*	*	*	*	*	*(6)	46 (13)	43 (7)	95
	7	*	*	*	*	*	*	*	*	*	*	*	*	40 (13)	*(13)	*(7)	95
	8	*	*	*	*	*	*	*	*	*	*	*	*	*(2)	*(13)	49 (9)	99
	9	*	*	*	*	*	*	*	*	*	*	*	*	45 (13)	*(13)	*(6)	97
	10	*	*	*	*	*	*	*	*	*	*	*	*	*(12)	*(12)	49 (8)	99
	11	*	*	*	*	*	*	*	*	*	*	*	*	*(13)	*(10)	41 (7)	94
	12	*	*	*	*	*	*	*	*	*	*	*	*	49 (13)	49 (12)	*(8)	99
	13	*	*	*	*	*	*	*(3)	*	*(2)	*	*	*	*(11)	46 (8)	48 (9)	96
	14	*	*	*	*	*	*	*	*	*	*(2)	*	*	*(12)	49 (9)	46 (9)	97
	15	*	*	*	*	*	*	*	*	*	*	*	*	*(13)	48 (8)	45 (7)	95
	16	*	*	*	*	*	*	*	*	*	*(2)	*	*	*(3)	46 (12)	40 (8)	91
	17	*	*	*	*	*	*	*	*	*	*	*	*	*(11)	*(10)	44 (7)	96
	18	*	*	*	*	*	*	*	*	*	*	*	*	*(3)	*(10)	48 (8)	99
19	*	*	*	*	*	*	*	*	*	*	*	*	*(12)	39 (13)	43 (7)	88	
20	*	*	*	*	*	*	*	*	*	*	*	*	*(11)	*(10)	39 (7)	93	
21	*	*	*	*	*	*	*	*	*	*(2)	*	*	47 (11)	*(12)	*(8)	98	

Numbers in parentheses are days for recovery.

Without number in parentheses means that all samples were recovered in 1 day.

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

Table 3
Average of mycelial diameter in mm and standard deviations in treatments combining all the recovery times.

Species	Treatment							
	C		R		LN		C + R + LN	
	Strain	Mean (mm) ± SD	Strain	Mean (mm) ± SD	Strain	Mean (mm) ± SD	Strain	Mean
<i>A. subrufescens</i>	8	72.9 ± 14.4 a	11	67.5 ± 15.5 a	17	72.1 ± 22.9 a	3	16.4
	14	69.0 ± 14.8 ab	17	66.0 ± 21.7 ab	18	62.6 ± 27.8 ab	4	58.8
	17	68.4 ± 18.9 ab	15	66.0 ± 15.9 ab	15	61.7 ± 24.1 ab	5	48.3
	20	68.0 ± 20.8 ab	16	61.0 ± 15.1 ab	20	60.0 ± 29.0 ab	7	52.6
	13	67.9 ± 16.4 ab	4	60.6 ± 17.4 ab	21	59.2 ± 20.2 ab	8	61.6
	18	66.1 ± 17.9 ab	18	59.6 ± 17.2 ab	8	54.8 ± 32.4 ab	9	48.0
	10	65.4 ± 14.4 ab	14	57.8 ± 15.9 ab	12	50.2 ± 26.5 bc	10	58.3
	15	64.8 ± 13.9 ab	20	57.4 ± 13.7 ab	16	49.4 ± 23.6 bc	11	60.7
	11	64.1 ± 19.0 ab	21	56.7 ± 12.7 ab	13	48.8 ± 23.0 bc	12	56.0
	21	63.7 ± 16.3 ab	10	56.6 ± 21.4 ab	10	47.4 ± 17.9 bc	13	59.0
	4	63.6 ± 16.8 ab	8	55.1 ± 23.0 bc	4	46.6 ± 21.2 bc	14	58.6
	12	61.7 ± 13.6 ab	13	54.6 ± 20.1 bc	5	46.4 ± 20.4 bc	15	64.7
	9	58.5 ± 17.9 bc	7	53.4 ± 19.5 cd	7	46.1 ± 19.7 bc	16	56.8
	16	55.5 ± 18.8 cd	12	52.9 ± 15.2 cd	11	40.5 ± 22.4 cd	17	68.2
	7	55.0 ± 20.6 cd	5	47.9 ± 18.3 de	14	40.3 ± 21.9 de	18	62.8
	5	49.5 ± 19.3 fg	19	44.4 ± 21.2 ef	9	34.7 ± 10.5 fg	19	40.4
	19	42.1 ± 19.2 gh	9	43.0 ± 22.6 fg	19	26.1 ± 10.5 gh	20	62.2
	3	16.6 ± 8.2 i	3	18.7 ± 3.6 i	3	11.1 ± 1.9 j	21	60.0
	Mean	59.6 ± 13.1 A		54.3 ± 11.2 B		47.6 ± 14.2 C		
	<i>A. bisporus</i>	1	54.0 ± 22.2 de	1	43.3 ± 21.3 fg	6	37.3 ± 5.8 ef	1
6		51.4 ± 17.3 ef	2	41.6 ± 20.4 gh	1	27.6 ± 6.5 hi	2	35.0
2		36.3 ± 20.7 h	6	41.0 ± 18.3 h	2	20.3 ± 4.3 ij	6	43.9
Mean		47.2 ± 7.8 A		42.0 ± 0.9 A		28.4 ± 7.0 B		

Different letters in the same column indicate significant differences between strains in mycelial diameters using Tukey's multiple range test ($p = 0.05\%$). Different capital letters on a line indicate significant differences in mycelial diameters between the treatments for each species.

100% and mostly in a single day, even if some strains showed a slightly greater recovery time. These results suggest that mycelia can survive at sub-lethal temperature, provided that they are embedded within and protected by the sorghum seeds in the spawn. Using paddy rice grains and 5 strains, Maia et al.²² reported no mycelial recovery after storage for 3 months at 4 °C, whereas at room temperature good mycelial recovery was obtained until 6 months but the ability decreased after. The higher longevity obtained here with sorghum grain might be due to differences in physical attributes of the seeds that allow the mycelia to survive. The round shape and the low size of sorghum might contribute to a higher physical protection of the mycelium. With *A. bisporus* on wheat straw, Mata and Rodríguez-Estrada²⁴ observed that mycelia only penetrated the external layer of the seed. After cryopreservation without cryoprotectant, mycelia were always recovered from seed hila, or from fissures on the seed surface, supporting the idea that seeds offer protection to mycelia. Sorghum seed hilum is an easy gate for mycelial penetration and the protective effect of the hull of the individual rice kernels might be less efficient than the sorghum pericarp. Sorghum seeds had previously been used for cryopreservation of other mushrooms.²³

For improving the efficiency of damage protection during the cryoconservation in liquid nitrogen, spawn grains were immersed in 10% glycerol. As above such a treatment was reported to be inefficient with paddy rice by Maia et al.,²² whereas recovery percentages ranged from 88 to 100% for the 18 *A. subrufescens* strains studied. Fast freezing from ambient temperature to -196 °C was used. Such a freezing process was inefficient using rice whereas a slow freezing from 8 to -80 °C allowed to efficiently cryopreserving 5 *A. subrufescens* strains at -80 °C for one year with 100% recovery.⁷ One can expect to improve the recovery efficiency after storage in liquid nitrogen by using a slow freezing process with the protecting sorghum grains. However, consistently with the present observations, the mycelial growth of cryopreserved cultivar strains of *A. subrufescens* (-80 °C) was recovered after nine days of incubation in Colauto et al.⁷ The delayed recovery observed in liquid nitrogen treatment could be linked to the damage suffered by the

mycelium during the thawing process of the spawn and the difficulty to clean the samples from the remains of glycerol. This delay at the beginning of mycelial growth was observed when spawn was frozen in liquid nitrogen using cryoprotectant solutions.^{23,24} Therefore, the differences between growth in LN treatment and treatments C and R could be linked directly to the delayed onset of mycelial growth rather than negative effects caused by the treatment itself.

The behavior of the 18 strains of *A. subrufescens* was similar to that of three *A. bisporus* strains that are less susceptible to low temperatures, either at 4 °C or at -196 °C. The recovery percentages and the delays observed after storage in liquid nitrogen with *A. bisporus* were in the same range than for *A. subrufescens*, whereas germplasm of this species is preserved efficiently in liquid nitrogen (on spawn or agar media) for many years in different laboratories, since cryogenic preservation for the maintenance of *A. bisporus* was proposed by San Antonio and Hwang.³⁰ Despite their initial susceptibility difference to freezing, both species can be cryopreserved efficiently as spawn on sorghum grain, showing the interest of this preservation method.

Our chief interest is the longevity of the strains, but effects imposed by cold stress cannot be ignored. Despite the good results on the recovery of strains, the mycelial growth measured after incubation for 14 days showed a significant decrease with increasing storage time. This was observed for all the strains both with storage at 4 °C and at 25 °C. This phenomenon could be linked to an aging process of the mycelium independent of the temperature in a tested range. Changes over time are frequently observed in *A. bisporus* and are expressed as sectors of different growth rates and aerial mycelium density in cultures on agar media.¹⁸ The observed changes in mycelial growth could be linked to the instability of the strains and frequently are considered as irreversible degeneration of the strains. Due to the large standard deviation observed, it is expected that during the recovering process, one can select intentionally or not, the variant keeping their original vitality and productivity.

Table 4
Significant differences by each strain in the tested treatments.

Species	Strain	Treatment		
		C	R	LN
<i>A. bisporus</i>	1	a	b	c
	2	a	a	a
	6	a	b	b
<i>A. subrufescens</i>	3	a	a	b
	4	a	a	b
	5	a	a	a
	7	a	a	a
	8	a	b	b
	9	a	b	b
	10	a	b	b
	11	a	a	b
	12	a	b	b
	13	a	b	b
	14	a	b	c
	15	a	a	a
	16	ab	a	b
	17	a	a	a
	18	a	a	a
19	a	a	b	
20	a	b	ab	
21	a	a	a	

Different letters in the rows indicate significant differences in mycelial diameters using Tukey's multiple range test ($p = 0.05\%$).

Table 5
Mycelial diameter average and standard deviation in all samples of treatments C and R in different weeks.

Species	Controls at 25 °C			Refrigerated at 4 °C		
	Week of storage	Mean diameter (mm) ± SD	% growth inhibition	Week of storage	Mean diameter (mm) ± SD	% growth inhibition
<i>A. bisporus</i>	8	63.2 ± 14.7 a		4	61.9 ± 17.5 a	
	6	61.1 ± 20.2 a	3.3	6	51.8 ± 10.0 ab	16.3
	4	55.0 ± 10.0 a	13.0	10	48.7 ± 20.3 bc	21.3
	10	41.5 ± 26.3 b	34.3	8	39.9 ± 13.7 c	35.5
	24	30.8 ± 8.2 bc	51.3	12	25.1 ± 9.5 d	59.5
	12	21.0 ± 5.2 c	66.7	24	20.0 ± 6.2 e	67.7
<i>A. subrufescens</i>	4	71.0 ± 13.2 a		4	73.2 ± 14.3 a	
	8	65.1 ± 20.8 b	8.3	6	59.8 ± 17.9 b	18.3
	24	62.7 ± 25.2 b	11.7	12	56.9 ± 19.3 b	22.3
	6	62.1 ± 19.6 b	12.5	8	48.5 ± 22.7 c	33.7
	12	51.8 ± 17.6 c	27.0	10	42.6 ± 15.0 d	41.8
	10	43.5 ± 16.4 d	38.7	24	42.1 ± 15.9 d	42.5

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

However, even with the delayed recovery in the liquid nitrogen treatment, high recoveries of all the strains were obtained and that is encouraging to protect the genetic material of *A. subrufescens* through freezing spawn on sorghum grain. Several studies^{10,14,31} have shown an efficient recovery of *A. bisporus* spawn without affecting their productivity. Similarly spawn has been used successfully for the conservation of *Pleurotus* strains,²⁷ *Lentinula edodes* and *Lentinula boryana*,²⁶ and *Volvariella volvacea*.²⁹ These results are encouraging and suggest that conservation of *A. subrufescens* genetic resources on sorghum grain for long periods of time could be a viable option even if experiments will be necessary for testing longer freezing times in order to evaluate the possible effects of freezing on the productivity of the strains.

The results obtained in this work suggest that spawn of *A. subrufescens* remains viable even under refrigeration, however, diminishes their ability to grow in relatively short periods of time. This has important implications for the management of spawn on commercial terms as the storage periods cannot be too long. The spawn should be used in an appropriate physiological time, this is particularly important when using cultivation substrates without a good selectivity because rapid growth of the mycelium to avoid competition with antagonistic organisms is required.

Conflict of interest

The authors declare that there is no conflict of interest.

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