ORIGINAL PAPER

The effect of substrate on cryopreservation of the mycelium of wood decay basidiomycetes

Svitlana V. BONDARUK[1,2] | Galeb A. AL-MAALI[1,2]

Affiliation
M.G. Kholodny Institute of Botany, National Academy of Science of Ukraine, Kyiv, Ukraine
Enamine Ltd., Kyiv, Ukraine

Correspondence:
Svitlana Bondaruk
svitlana.bondaru@gmail.com

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ABSTRACT

Question: How can the pure cultures of basidiomycetes from the IBK Mushroom Culture Collection be preserved for a long time?
Location: IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine
Materials and methods: standard mycological methods of cultivation of the pure culture of fungi
Nomenclature: https://www.mycobank.org/
Results: Culture collections are essential for characterizing and studying fungal species; however, the primary goal of these collections remains the preservation of fungal species diversity and their gene pool. The IBK Mushroom Culture Collection is the largest official specialized culture collection of macromycetes in Ukraine, preserving more than 1,500 strains of edible and medicinal mushrooms. Since there is a need for a reliable method for long-term storage of pure cultures of fungi, the cryopreservation of nine different strains of five wood decay fungi was investigated in this work. Wood-decay fungi have various applications in diverse fields. In particular, studied in this work, *Fomitopsis pinicola* 361, two strains of *Ganoderma tsugae* 1848 and 2566, three strains of *Laricifomes officinalis* 2497, 2498, and 5004, *Pleurotus ostreatus* 297, and two strains of *Schizophyllum commune* 1768 and 1769 are valuable species of wood decay fungi due to their contents of various biologically active compounds. They have diverse applications in pharmacology, biotechnology, and bioremediation due to their enzyme activities. According to this, these species should not change during long-term storage; however, it is known that during long-term storage of cultures on culture medium, certain changes in the activity of enzymes may occur. Storage at low temperatures protects the culture from those changes. Barley, pearl barley, wheat, and four versions of the wooden sticks were studied as substrates for the mycelium storage at a 10% solution of glycerol at low temperatures. Covered by mycelium substrates were stored for one year at – 80°C. After this period, the recovery of mycelium growth was studied on the Petri dishes with glucose-yeast-peptone agar medium at 26 °C. All studied strains revived and grew after storage. Such research was performed for the first time for *F. pinicola* and *G. tsugae*. The obtained results demonstrate a better revival of the mycelium on the grains as a substrate. However, the strains *F. pinicola* 361 and S. commune 1769 are an exception due to their better growth rate after storing on the wooden sticks. The advantages of the described method of storing pure cultures of the fungal species are no need for constant reseeding, which reduces the risk of contamination and excludes the possibility of accumulation of genetic changes.

KEYWORDS
biodiversity, cryoconservation, filamentous fungi, long-term storage, viability, wood decay basidiomycetes

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INTRODUCTION

Basidiomycetes are well-known sources of bioactive metabolites with different properties (Vieira Gomes *et al.* 2019). Therefore, fungi of the Basidiomycota division are actively used in the food and pharmaceutical industries. However, some species with important properties are rare in the environment. This could limit the use of fungi, but pure cultures have no such disadvantages, and their mycelium remains a source of biologically active compounds (Mykchaylova *et al.* 2017, Pasailiuk 2020, Sułkowska-Ziaja *et al.* 2025). Additionally, pure fungal cultures enable the preservation of biodiversity. However, it is necessary to search for a method for the long-term preservation of pure cultures of fungi due to their importance for research. The standard technique is to preserve the fungal culture at 4 °C for 3–4 months; however, it is known that genetic changes occur during long-term cultivation of the culture on the same culture medium (Sakurai *et al.* 2019, Silar 2019).

The primary goal in conservation is to preserve viability without contamination, genetic variation, or deterioration. In general, the technique aims either to minimize the risk of changes and eliminate frequent transfer by extending the periods between subcultures or to bring cellular activity to a halt (Jong & Birmingham 2001). There are several methods for preserving pure cultures of fungi; however, a method that works for all groups of fungi has not been described yet. Among the well-known methods of storing fungi are using wood chips, cereal grains, perlite, agar strips, filter papers, preservation in soil, sterile distilled water, or mineral oil (Singh *et al.* 2018). For basidiomycetes, the preservation on wood chips or cereal grains is the most effective, but it still requires additional research. This work aims to investigate the cryopreservation of mycelium from nine strains of five basidiomycete species on grain and wood sticks.

MATERIALS AND METHODS

The object of the study was nine strains of five species of basidiomycetes: *Fomitopsis pinicola* (Sw.) P. Karst. 361 (it was accepted by NCBI with accession number PQ460591), *Ganoderma tsugae* Murrill 1848, *G. tsugae* Murrill 2566, *Pleurotus ostreatus* (Jacq.) P. Kumm. 297, *Schizophyllum commune* Fr. 1768 (PQ482381), *S. commune* Fr. 1769 (PQ482382), *Laricifomes officinalis* (Vill.) Kotl. and Pouzar) 5004 (MF952886), *L. officinalis* 2498 (PQ363511), and *L. officinalis* 2497 (PQ368547). All studied cultures were received from the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine (Bisko *et al.* 2024). Initially, cultivation of mycelium was performed in Petri dishes using sterile glucose-yeast-peptone agar (GYPA) medium. GYPA medium has the following composition, (g/l): glucose – 25; peptone – 3; yeast extract – 3; KH_2PO_4 – 1; K_2HPO_4 – 1; $MgSO_4 \cdot 7H_2O$ – 0.25; agar – 20; H_2O – for a final volume of 1 L; pH – 6.

The grains of wheat, barley, and pearl barley were pre-boiled for 20 minutes and then autoclaved at 121 °C for 20 minutes. We also used wooden sticks made from deciduous trees as a substrate for cryopreservation, which were pre-treated in 4 different ways:

- wooden sticks were autoclaved at 121 °C for 20 min, after that they were kept in a sterile 2.5% solution of glucose for 24 h (wooden sticks №1);
- wooden sticks were pre-boiled in a 2.5% solution of glucose and autoclaved in this solution at 121 °C for 20 min (wooden sticks №2);
- wooden sticks were autoclaved in a 2.5% solution of glucose at 121 °C for 20 min (wooden sticks №3);
- wooden sticks were autoclaved in a 5% solution of malt at 121 °C for 20 min (wooden sticks №4).

The prepared grains or wooden sticks were put on Petri dishes with fungal mycelium and incubated at 26 °C until completely covered with mycelium. The obtained grains or wooden sticks with mycelium of fungi were transferred to sterile Nalgene® cryogenic vials (Thermo Fisher Scientific Inc.) with a 10% sterile solution of glycerol and soaked for 24 h. The vials were put into Mr. Frosty™ Freezing Container (Thermo Fisher Scientific Inc.). Then they were transferred to the Thermo Scientific ULT1786-6-A43 freezer cryogen for storage at – 80 °C. After 24 h, vials were transferred into cryo boxes.

The growth recovery and growth rate after cryopreservation were examined at 6 and 12 months after freezing. The grains or wooden sticks with mycelium were transferred to Petri dishes with GYPA medium. For comparison, the mycelium, which was stored at 4 °C in the fridge on wort agar medium (which has the following composition, g/l: wort – 20; agar – 20; H_2O – for a final volume of 1 L; pH – 6), was transferred to Petri dishes with GYPA medium. The cultures were observed to fully overgrow the Petri dishes in triplicate. The mycelial growth diameters were measured for the investigation of viability. The obtained results were analyzed with Excel statistical functions using the Microsoft Office XP software. Data were presented as means ± SD (standard deviation).

RESULTS AND DISCUSSION

The influence of wooden sticks and grains on the cryopreservation technique. Four ways of preparing wooden sticks and three different sorts of grain were used in the investigation to compare the mycelial growth of nine strains of five species of basidiomycetes on various substrates. The choice of wooden sticks as a substrate is because the studied strains belong to wood decay fungi. At first, wooden stick №1 was used for all strains, but the absence of mycelium growth of the two strains of *S. commune* and one strain of *F. pinicola* was observed during 6 days. Therefore, additional types of preparation of wooden sticks were used for these three strains. As a result, a better rate of mycelium growth was observed for the wooden sticks №2, but the process of covering the substrate by mycelium proceeded for at least 6 days. The better results of these species with other types of sticks, in our opinion, may be related to the influence of temperature on the wood structure, which led to a better absorption of glucose and wort in wooden sticks. However, this requires additional, more detailed studies for a better understanding of this process. On the other hand, grain is more actively used as a substrate for cryopreservation, even for wood decay fungi, because they have strong enzyme systems.

The faster result of mycelium overgrowth was observed on both wooden sticks №1 and grain substrate within two days for *P. ostreatus* 297. The mycelium of *G. tsugae* 2566 covered the wooden sticks №1 and wheat within two days, but barley and pearl barley were covered within four days. The other strain, *G. tsugae* 1848, covered barley the best for 2 days, compared to other substrates that yielded results similar to those of the previous strain. The duration of substrate covering by mycelium for *F. pinicola* 361 ranged from 5 (pearl barley) to 8 (wooden sticks №3 and №4) days. A difference of two times was also found between the duration of overgrowth of wooden sticks and grains for both strains of *S. commune* 1768 and 1769. A certain strain specificity was also noted for this species, as *S. commune* 1769 covered all kinds of grains at the same rate, while the other strain more quickly covered the barley with mycelium. The strain specificity of this process was also observed during the overgrowth of substrates by the mycelium of *L. officinalis*, also known as *Fomitopsis officinalis* (Vill.) Bondartsev and Singer. *L. officinalis* is retained as its current name according to Mycobank (www.mycobank.org). The overgrowth of wooden sticks №1 by the mycelium of three strains of *L. officinalis* lasted 12 days, while all three types of grains were covered by the mycelium of two strains of *L. officinalis* 2497 and 2498 during 10 days and by the mycelium of *L. officinalis* 5004 during three days.

According to the obtained results, the use of grain for cryopreservation is more effective than the use of wooden sticks. The grain is an excellent substrate for mycelium growth and is actively used for cryopreservation of basidiomycetes (Zaghi Júnior *et al.* 2018, Bertéli *et al.* 2022). Also, using grain provides additional physical protection during cryopreservation due to the mycelium growth within the capillaries of the grain (Mantovani *et al.* 2012). The duration of overgrowth of the substrate by fungal mycelium for long-term storage depends on the growth rate of the strain (Zalesky *et al.* 2024). It is necessary to study various substrates to select those that are characterized by a higher rate of mycelial growth, especially when studying species such as *L. officinalis*, which is extremely valuable and belongs to slow-growing fungi (Flores *et al.* 2023). As a result, the growth rate and characteristics identified for wheat, barley, and pearl barley indicate that these grains are better substrates for cryopreservation than wooden sticks for the studied strains.

The estimation of mycelium growth after cryopreservation of basidiomycetes. The measurement of mycelium growth was carried out after both 6 months and 1 year of storage at – 80 °C. The obtained results were similar, so the results of longer storage are demonstrated in TABLE 1. For all the studied strains, prosperous cryopreservation was observed over one year, with high mycelial viability for all basidiomycetes. In this work, cryopreservation of both fast-growing (*P. ostreatus*) and slow-growing (*L. officinalis*) species was investigated. The cultures that were stored at 4°C by the standard technique for the IBK Mushroom Culture Collection were used as a control. *F. pinicola* 361, *G. tsugae* 2566 and 1848, *S. commune* 1768 and 1769 began to grow after 3 days of incubation, *P. ostreatus* after 2 days, and three strains of *L. officinalis* after 6 days. It means that the growth rate of some strains is slower compared to the samples, that were stored at + 4 °C. After cryopreservation, these strains need some time for acclimatization to the new temperature. For most strains, the mycelium preserved on the grains showed better mycelial growth recovery than the same mycelium preserved on the wooden sticks. However, there were exceptions for the strains *F. pinicola* 361 and *S. commune* 1769 (TABLE 1).

TABLE 1. The investigation of fungal mycelial growth diameter after one year of storage at – 80°C on different substrates, mm

№	Strain	Control	Wheat	Barley	Pearl barley	Wooden sticks №1	Wooden sticks №2	Wooden sticks №3	Wooden sticks №4
on the 7th day									
1	*Fomitopsis pinicola* 361	5.8±0.4	3.5±0.1	3.8±0.2	4.5±0.2	x	5.2±0.2	4.3±0.3	4.6±0.3
2	*Ganoderma tsugae* 1848	6.0±0.3	8.4±0.5	7.5±0.3	6.7±0.4	5.7±0.5	–	–	–
3	*Ganoderma tsugae* 2566	5.4±0.3	7.2±0.2	5.0±0.2	5.0±0.3	6.0±0.3	–	–	–
4	*Pleurotus ostreatus* 297	8.8±0.3	8.4±0.5	7.5±0.5	9.0±0.0	9.0±0.0	–	–	–
5	*Schizophyllum commune* 1768	8.3±0.2	9.0±0.0	9.0±0.0	9.0±0.0	x	8.0±0.3	7.3±0.2	9.0±0.0
6	*Schizophyllum commune* 1769	8.0±0.5	7.5±0.2	7.3±0.3	7.5±0.5	x	8.7±0.2	8.5±0.1	7.8±0.3
on the 28th day*									
7	*Laricifomes officinalis* 5004	9.0±0.0	9.0±0.0	7.9±0.3	8.1±0.4	7.0±0.5	–	–	–
8	*Laricifomes officinalis* 2497	5.3±0.2	–	6.7±0.1	6.0±0.4	5.2±0.4	–	–	–
9	*Laricifomes officinalis* 2498	5.8±.2	6.5±0.3	6.6±0.3	5.7±0.2	5.1±0.3	–	–	–

x – The mycelium of these mushroom species did not grow on this kind of substrate for six days.
"–" – The research on this sort of substrate for cryopreservation and this basidiomycetes strain was not carried out.
* – The growth estimation of *L. officinalis* was carried out on the 28th day due to the species' characteristics and slow growth.

According to TABLE 1, the growth rate of most strains after cryopreservation matched the growth rate of the control. The wheat was established as the best substrate for both strains of *G. tsugae* and *L. officinalis* 5004 (FIGURE 1). The barley was established as the best substrate for two strains of *L. officinalis* 2497 and 2498. The mycelium of strain *S. commune* 1768 grew on wheat, barley, pearl barley, and wooden sticks №4 at the same fast rate; on the other hand, strain *S. commune* 1769 grew on wooden sticks better than grains, and the highest result was obtained for the mycelium which was stored on wooden sticks №2. This type of pre-treatment of wooden sticks was the best for the mycelium of *F. pinicola* 361. The mycelium of *P. ostreatus* 297 had the best growth recovery after storage on pearl barley and wooden sticks №1. According to the obtained results, individual selection of substrate is recommended for long-term storage, especially for rare and valuable species.

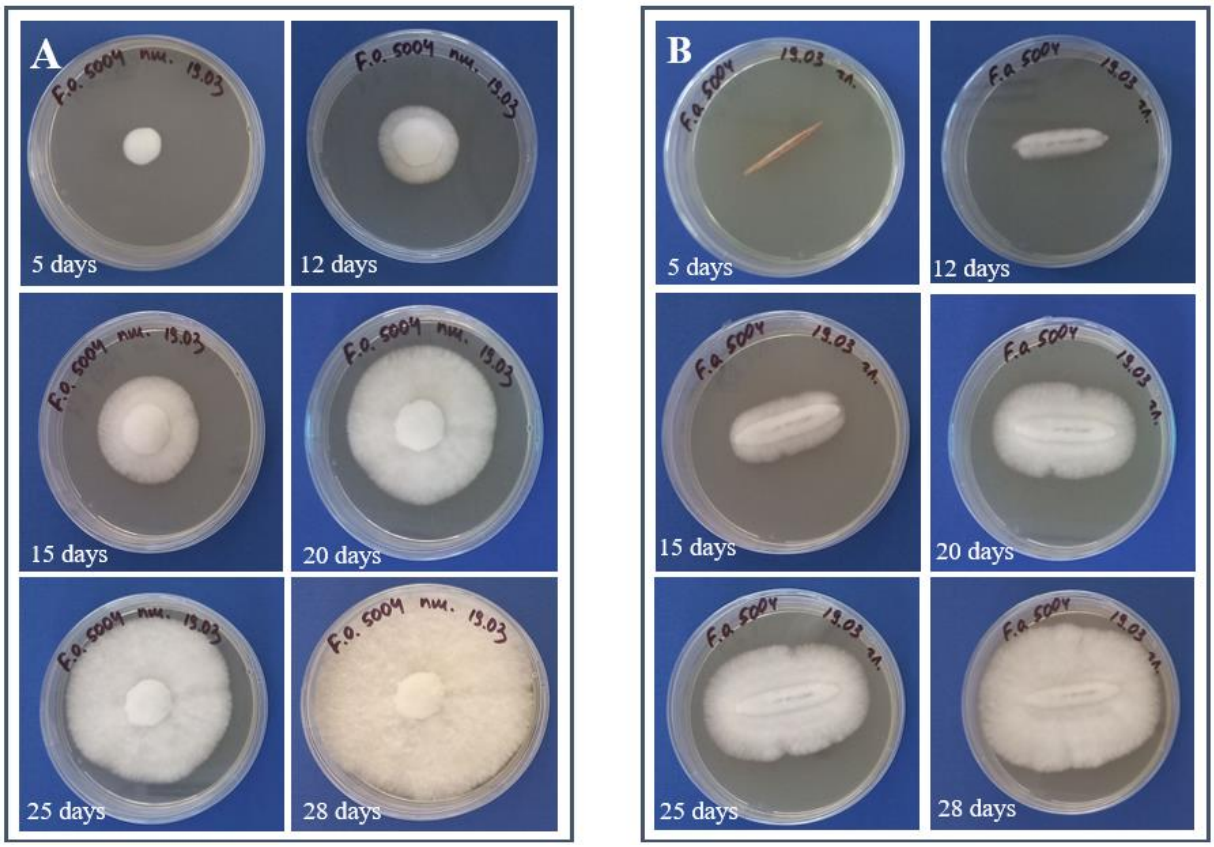

FIGURE 1. Growth result of the mycelium of the strain *L. officinalis* 5004, which was preserved on the wheat grain (A) and wooden sticks №1 (B) during incubation in GYPA medium at 26˚C for 5 to 28 days of incubation.

An investigation was conducted into the storage of *P. ostreatus* mycelium on plastic straws at – 70 °C (Eichlerová *et al.* 2015). The recovery of mycelium growth was detected in 2 days, which was also observed in our work. For this species, wheat has already been investigated as a substrate before cryopreservation (Mantovani *et al.* 2012), but barley and pearl barley have never been used before our work. The cryopreservation of mycelium on the wheat was also described for *S. commune* (Zaghi Junior *et al.* 2020), but 4% glucose was used as a cryoprotectant agent compared to 10% glycerol in our work. Both cryoprotectant agents are effective. For *L. officinalis*, the storage of mycelium as a suspension with a 15% solution of glycerol was conducted (Cartabia *et al.* 2022). Although this method is effective, however it is also more traumatic compared to the storage of mycelium on the grain (Mantovani *et al.* 2012). The fungal growth of the mycelium preserved on the wheat is shown in FIGURE 1. At the same time, the investigation of the long-term storage of the mycelium of *F. pinicola* and *G. tsugae* was carried out for the first time.

It is well known that storage at – 80 °C offers some benefits compared to storage at – 20 °C, because it is the most viable technique for preserving basidiomycetes (Linde *et al.* 2018). Basidiomycetes can be stored at such temperatures as an agar disk with fungal mycelium in a cryotube containing 10% (v/v) glycerol and 5% (v/v) trehalose (Ilyas & Soeka 2019). However, this method can have a problem related to cells being injured during storage. On the other hand, storing the mycelium on grains or wooden sticks has no such disadvantage. Storing the mycelium on grains even has an advantage. The positive effect may be related to the grain's carbohydrates and proteins that are capable of binding water to the grain. It reduces free water and the formation of intracellular ice crystals (Linde *et al.* 2018). According to the above information and the results of this research, it can be concluded that grain is the most optimal substrate for storing the mycelium of basidiomycetes at – 80 °C. All three types of grain studied in this work can be used for such a goal.

CONCLUSIONS

This work demonstrates the effectiveness of using both various grains and wooden sticks for long-term preservation of fungal mycelium. No difference in mycelial growth was observed when cultures were stored for six months and twelve months. After storing for a year at a temperature of – 80 °C, all studied strains retained their viability. Only the mycelium of strain *F. pinicola* 361 showed slower growth after cryopreservation compared to the control, while the mycelium of other strains did not differ from the corresponding controls. The obtained results extended the data on methods of cryopreservation of such valuable species as *F. pinicola*, *P. ostreatus*, *G. tsugae*, *S. commune*, and *L. officinalis*. The paper reports for the first time the use of barley and pearl barley as a substrate for cryopreservation of mushroom mycelium. The received results also confirm a certain species specificity in the selection of substrate for storing the fungal mycelium. Thus, the species *F. pinicola* and strain *S. commune* 1769 were faster at restoring the growth of mycelium, which was preserved on the wood substrate, than on the grain. At the same time, the opposite results were established for other studied strains. This suggests the need to investigate the impact of the substrate on mycelial storage for various fungal species and strains.

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РЕЗЮМЕ

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Колекції культур є важливими для характеристики та вивчення видів грибів, проте основною метою даних колекцій залишається збереження видового різноманіття та генофонду грибів. Колекція культур шапинкових грибів ІВК є найбільшою офіційною спеціалізованою колекцією культур макроміцетів в Україні, яка зберігає понад 1500 штамів їстівних та лікарських грибів. Оскільки існує потреба в надійному методі тривалого зберігання чистих культур грибів, у цій роботі було досліджено кріоконсервацію дев'яти штамів п'яти видів дереворуйнівних грибів. Такі гриби мають різне застосування у різноманітних галузях, зокрема, досліджені в даній роботі *Fomitopsis pinicola* 361, два штами *Ganoderma tsugae* 1848 та 2566, три штами *Laricifomes officinalis* 2497, 2498 та 5004, *Pleurotus ostreatus* 297 та два штами *Schizophyllum commune* 1768 та 1769 є цінними видами дереворуйнівних грибів завдяки наявності різних біологічно активних речовин. Вони мають різноманітне застосування у фармакології, біотехнології та біоремедіації завдяки своїй ферментативній активності. Тому ці види не мають зазнавати змін при тривалому зберіганні. Однак відомо, що під час тривалого зберігання культур методом субкультивування можуть відбуватись певні зміни активності ферментів. Зберігання за низьких температур захищає культуру від таких змін. Ячмінь, перлова крупа, пшениця та чотири варіанти підготовлених за різними технологіями дерев'яних паличок досліджували як субстрат для зберігання грибного міцелію в 10% розчині гліцерину за низької температури. Вкриті міцелієм субстрати зберігались протягом одного року за температури – 80 ℃. Після цього періоду відновлення росту грибного міцелію вивчали на чашках Петрі з глюкозо-пептонно-дріжджовим агаризованим середовищем за температури 26 ℃. Міцелій усіх досліджуваних штамів відновив ріст після зберігання. Таке дослідження було проведено вперше для видів *F. pinicola* та *G. tsugae*. Отримані результати демонструють краще відновлення росту міцелію після зберігання на зерні у якості субстрату. Однак штами *F. pinicola* 361 та *S. commune* 1769 є винятками, оскільки характеризуються кращим ростом міцелію після зберігання на дерев'яних паличках. Перевагами описаного методу зберігання чистих культур грибів є відсутність необхідності постійного пересіву, що знижує ризик контамінації та виключає можливість накопичення генетичних змін.

Ключові слова: біорізноманіття, тривале зберігання, кріоконсервація, життєздатність, дереворуйнівні гриби.

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