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Research article

Low-cost preservation protocol for *Lentinus* mushrooms

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Abstract
Preservation maintains the viability of an organism within a certain period. Mycelia are preserved through sub-culturing; however, it is laborious and maintains cultures for a short period. Hence, this study was conducted to establish an alternative low-cost preservation technique. The efficiency of wood sticks for the preservation of *Lentinus tigrinus*, *Lentinus sajor-caju*, and *Lentinus swartzii* was determined. The viability and the mycelial growth of the preserved cultures were assessed for 18 months. The efficiency of the method was verified by evaluating the fruiting body production. The incubation period, size of the fruits, yield, and biological efficiency were evaluated. Wood sticks were suitable for the three mushrooms which demonstrated 100% viability. The mycelial growth of the preserved cultures of *L. swartzii* and *L. tigrinus* are comparable to the control while a significantly longer mycelial diameter was recorded in the preserved culture of *L. sajor caju*. The control group of *L. tigrinus* and *L. sajor-caju* showed faster mycelial growth in fruiting bags while preserved cultures of *L. swartzii* showed a shorter incubation period. Fruit size, yield, and biological efficiency were all comparable. Therefore, this low-cost preservation method can be utilized to preserve *L. tigrinus*, *L. sajor-caju*, and *L. swartzii* for extended periods.

Keywords
Lentinus sajor caju, *Lentinus swartzii*, *Lentinus tigrinus*, mycelial growth, fruiting body production

Introduction

Mushrooms are rich sources of compounds with nutritional, medicinal, industrial, and environmental significance (Wani et al., 2010; Adenipekun and Lawal, 2012). In the wild, their survival depends on their mycelia which is greatly affected by climatic changes, global warming, and some human activities. For instance, *Collybia reineckeana* Henn., which was first documented in Puncan, Carranglan Nueva Ecija, after an Earthquake in 1990 was no longer observed in the area (Reyes et al., 1997). This phenomenon led to the Center for Tropical Mushroom Research and Development's

(CTMRD) continuous efforts in collecting and preserving wild strains of mushrooms all over the country. Currently, these mushrooms are being preserved in potato dextrose agar (PDA) bottles at room temperature (28–30 °C) and are maintained every three months through sub-culturing. However, this technique is time-consuming, costly, and maintains fungal cultures for a short period which is not recommended for large collections (Maia et al., 2012). Aside from this, continuous sub-culturing can be of high risk of contamination and possible deterioration of the genetic characteristics of the cultures (Nakasone et al., 2009; Homolka, 2014). The biological efficiency of mushrooms also decreases and they become less aggressive as they are continuously sub-cultured, resulting in the reduction of yield or inability to produce fruiting bodies at all (Chen et al., 2019). Thus, there is a need to undertake other preservation techniques to maintain the quality and purity of these resources for future generations.

Preservation is the process used to maintain the viability of culture organisms for a long period by inactivating their metabolism, thus permitting their survival and maintaining their genetic, morphological, and physiological stability (Hubalek, 2003; Chang and Miles, 2004). Mycelia preservation began in the 1960s when various preservation strategies were utilized such as the use of different substrates like distilled water, mineral oil, paraffin oil, grains, paper, sawdust, and soil (Maia et al., 2012; Homolka, 2014; Bermeo-Escobar et al., 2020; Castro-Rios and Bermeo-Escobar, 2021). Recently, mushroom cultures have been successfully preserved in low temperatures through cryopreservation, lyophilization, and the use of a sophisticated freezer (Singh et al., 2004; Piattoni et al., 2017; Leonardi et al., 2018). Among these techniques, the most reliable one is cryopreservation which involves the immersion of cultures into liquid nitrogen, previous incorporation of cryoprotectants such as glycerol, glucose, sucrose, sorbitol, and dimethyl sulfoxide (DMSO) to protect them from potential damage (Piattoni et al., 2017; Singh, 2017). Studies have shown that this technique can support the viability of mushrooms for 3 to 8 years (Mata et al., 2004; Mantovani et al., 2012). However, it is a delicate method based on the constant addition of liquid nitrogen, which is expensive, dangerous to handle and requires safety transport procedures, proper storage room, staff training (Liquid Nitrogen Safety Guidelines), and continuous monitoring (Kitamoto et al., 2002; Nakasone et al., 2004). Hence, the development of a low-cost and practical protocol for the preservation of mushrooms has to be established.

Low-cost preservation refers to the techniques of preserving mycelia using materials and methods that are cost-effective and friendly. Similar to advanced techniques, these methods of preservation aim to minimize the practice of periodic sub-culturing while maintaining the qualities of the organism. They are usually simple methods that can be easily implemented and have wide adaptability since they can be carried out without the need for specialized procedures. For example, Maia et al. (2012) successfully preserved *Agaricus brasiliensis* Fr. for 12 months using a low-cost technique with paddy rice as the substrate material, and distilled water as preservation solution stored at room temperature.

Still today, however, most mushrooms that have undergone preservation methods are usually cryopreserved. *Pleurotus* and *Agaricus*, among others, are the most studied mushrooms and have been successfully cryopreserved for an extended period (Linde et al., 2018), as well as the delicate hypogenous fungus *Tuber borchii* Vittad. (Piattoni et al., 2017). However, other mushrooms with exceptional medicinal properties such as those from the genus *Lentinus* have never been studied except for the report in 1996 on *Lentinus lepidu*s Fr. which survived after 15 years of storage in the

cryobank (Ito and Nakagiri, 1996) Therefore, it is of utmost importance to subject these mushrooms to preservation studies to ensure their long-term viability.

In this study, the effectiveness of a low-cost and expensive equipment-free method for the preservation of *Lentinus* mushrooms was determined. The efficiency of the preservation method was determined in terms of mycelial growth performance, fruiting body quality, and mushroom biological efficiency.

Materials and Methods

Source of mushroom cultures

Three *Lentinus* species were considered in this study: *Lentinus tigrinus* (Bull.) Fr. (BP32), *Lentinus sajor-caju* (Fr.) Fr. (C005), and *Lentinus swartzii* Berk. (BIL4618). The mushrooms were previously identified morphologically and confirmed molecularly using rDNA-ITS sequence analysis by Kalaw et al. (2021). The pure culture of the mushrooms was obtained from the Center for Tropical Mushroom Research and Development, Department of Biological Sciences, College of Science, Central Luzon State University, Science City of Muñoz, Nueva Ecija (Philippines). This study was carried out from October 2021 to July 2023.

Preparation of the substrates

The method for the preparation of the substrate was adapted from Nakasone et al. (2004) and Singh et al. (2018) with modifications. Wood sticks measuring 115 mm by 10 mm were washed and soaked in potato dextrose broth (pH 5.2) for 24 h. Three wooden sticks were placed in 300 mL clear bottles with caps. The bottles were subjected to sterilization using an autoclave (Vertical Pressure Steam Sterilizer, LS-B75L-I) at 121 °C, 15 psi for 1 hour. The bottles were then cooled, aseptically inoculated with one 10-mm mushroom mycelial disc, tightly closed, and incubated at 28-30 °C.

Evaluation of mycelial viability

The viability of the preserved mycelia was monitored every 6 months for 18 months. The method used for the evaluation of viability was adapted from the study of Camelini et al. (2012). One wood stick ramified with mycelia was aseptically transferred into sterile PDA (pH 5.6) bottles. The bottles were stored closed at 28-30 °C to allow mycelial ramification. Mycelial growth on PDA was monitored and recorded. When ramification reached 80% of the media, it was considered successful.

Evaluation of mycelial growth performance of the preserved mycelia

Following the method of Kalaw et al. (2016) the mycelial growth performance of the preserved cultures (PS) was determined in PDA and compared with their respective periodically transferred (PT) cultures (sub-cultured every three months). PDA was prepared and sterilized in an autoclave for 30 minutes then poured on a sterile 90-mm petri dish and solidified. One 10-mm mycelial disc from the 7-day-old PS and 7-day-old PT cultures was placed in the middle of each plate separately. The daily mycelial growth was measured using a Vernier caliper. The density of the mycelia was also evaluated and rated as either very thick, thick, thin, or very thin.

Evaluation of fruiting body performance

Grain spawn preparation
Rice (*Oryza sativa* L.) grains were used as spawning material. The spawn was prepared based on the method described in Dulay et al. (2017). Rice seeds were washed, soaked in water for 12 h, and boiled for 30-40 min. The moisture of the grains was maintained at 65% then 40 g of the grains were placed in 6 cm by 4 cm polypropylene (PP) bags. The grains were sterilized in an autoclave at 121 °C, 15 psi for 1 h. The grains were allowed to cool, then aseptically inoculated with one 10-mm 7-day-old mycelial disc of PS and PT cultures and incubated at room temperature (28-30 °C) until full mycelial ramification.

Fruiting substrate preparation
Fruiting bags were prepared following the method of Kalaw et al. (2001). Rice straw was soaked in tap water for 3 days, washed, and then transferred to a screen to drain the excess water and covered for 5 days to prevent drying and allow the decomposition process. The rice straw was then manually cut into 30–50 mm pieces and mixed with coconut (*Cocos nucifera* L.) sawdust with a ratio of 7 RS:3 SD. Five hundred grams of the substrate were placed in a 6 cm by 12 cm PP bag. 25-mm by 10 mm polyvinyl chloride (PVC) pipe was used as the neck of the bag tied with a rubber band and then closed with cotton waste. The bags were pasteurized in a box-type pasteurizer for 8–12 h. Cooled bags were inoculated with 40 g grain spawn from each mushroom. This experiment was replicated 10 times. The number of days of full ramification of the bags, cap and stipe size, and yield per bag (total fresh weight of fruiting bodies obtained throughout the fruiting period) were recorded. The biological efficiency of the mushrooms was computed by dividing the total fresh yield by the original weight of the substrate (De Leon et al., 2017).

Statistical analysis
A T-test was used to compare the difference in response between the 2 groups (PS and PT) at a significance level of 5%.

Results

Mycelial viability of preserved Lentinus *mushrooms*
Wood sticks were found to be a suitable substrate for *L. tigrinus*, *L. sajor-caju*, and *L. swartzii*. The substrate maintained the viability of the mushrooms for 18 months as indicated by the mycelial growth on PDA bottles (Fig. 1). All mushrooms showed 100% viability since the substrates were fully colonized by mycelia.

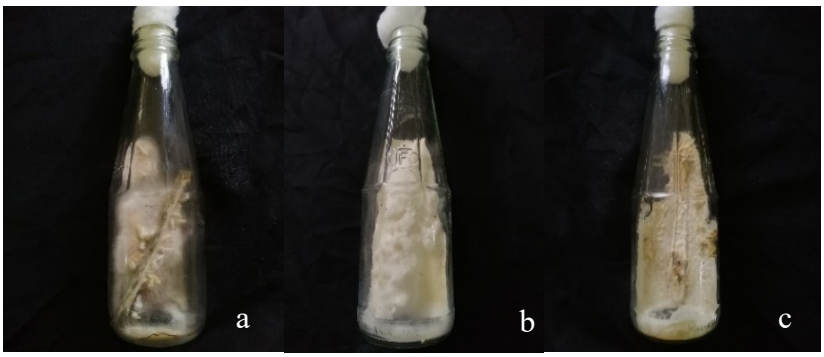

Fig. 1 − Mycelia of the three *Lentinus* mushrooms after 18 months in wood sticks (a) *L. tigrinus*, (b) *L. sajor-caju*, and (c) *L. swartzii.*

Mycelial growth performance of the preserved mycelia

The mycelial growth performance of the three mushrooms was evaluated on PDA plates after 6, 12, and 18 months of preservation to wood sticks. The mean mycelial diameter of the three mushrooms after 5 days in three observation periods is presented in Table 1. In all observation periods, the mycelial growth of the PS cultures of *L. swartzii* and *L. tigrinus* is comparable to the control (PT). However, a higher mean mycelial diameter was observed in the PS culture of *L. sajor-caju* compared to its PT culture. In terms of mycelial diameter, thick mycelia were observed in all cultures (Fig. 2).

Table 1 − Mean mycelial diameter of the three mushrooms after 5 days of incubation to PDA in three observation periods.

Maintenance period (months)	Mushroom	Mycelial diameter (mm)	
		PS	PT
6	*L. tigrinus*	58.58±0.28[a]	65.46±6.76[a]
	L. sajor-caju	74.05±3.96[a]	59.10±2.79[b]
	L. swartzii	70.03±3.84[a]	66.35±0.39[a]
12	*L. tigrinus*	58.83±0.39[a]	65.63±6.60[a]
	L. sajor-caju	74.42±4.52[a]	60.50±2.27[b]
	L. swartzii	71.20±5.27[a]	69.01±0.82[a]
18	*L. tigrinus*	57.29±1.96[a]	62.79±3.93[a]
	L. sajor-caju	69.86±1.98[a]	58.54±2.05[b]
	L. swartzii	71.19±2.31[a]	68.01±2.69[a]

[a, b] = Means of PT and PS for the same mushroom for each maintenance period bearing different letters are significantly different from each other ($P<0.05$).

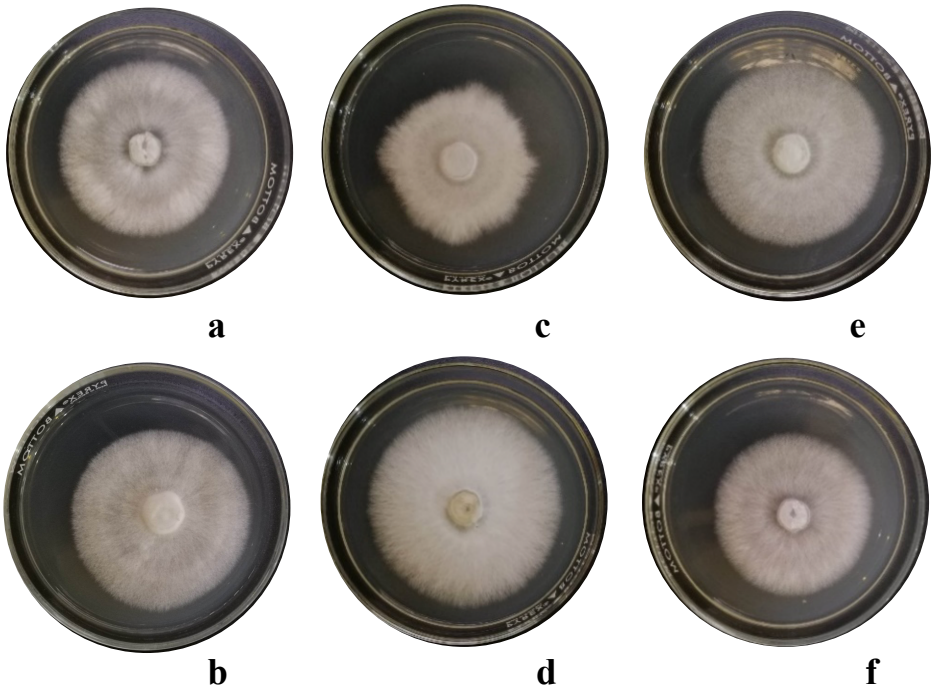

Fig. 2 - Mycelial growth of 18-month-old preserved cultures of *L. tigrinus*, *L. sajor-caju* and *L. swartzii* in PDA after 5 days: (a) *L. tigrinus* PT, (b) *L. tigrinus* PS, (c) *L. sajor-caju* PT, (d) *L. sajor-caju* PS, (e) *L. swartzii* PT, and (f) *L. swartzii* PS.

Fruiting body performance of the preserved Lentinus *mushrooms*

The fruiting body production of the PS cultures of *L. tigrinus, L. sajor-caju,* and *L. swartzii* on rice straw and sawdust substrate was investigated after 18 months. The different parameters gathered for the fruiting body production are shown in Tables 2 and 3. In the fruiting bags, the PT cultures of *L. tigrinus* and *L. sajorcaju* exhibited a significantly faster mycelial growth, showing a fully colonized substrate within 12 and 14.25 days, respectively (Table 2). Contrastingly, preserved cultures of *L. swartzii* showed a significantly shorter incubation period (12.50 days) compared to its PT culture which took 17.25 days to fully ramify the substrate. The results for the size of the stipe and pileus of the fruiting bodies indicate no significant differences in cap sizes and stipe lengths between the PS and PT cultures of the three mushrooms. The mean yield per bag and the mean percentage of biological efficiency throughout the fruiting period are shown in Table 3. The preserved cultures showed similar productivity since comparable yields and biological efficiency were recorded.

Table 2 - Mean incubation period, cap size, and stipe length of 18-month-old preserved culture *L. tigrinus*, *L. sajor-caju*, and *L. swartzii.*

Mushroom	Incubation period (days)		Cap size (mm)		Stipe length (mm)	
	PS	PT	PS	PT	PS	PT
L. tigrinus	13.00±0.82[b]	12.00±0.00[a]	67.70±21.80[a]	64.65±18.50[a]	29.60±4.90[a]	25.93±5.60[a]
L. sajorcaju	17.25±0.50[b]	14.25±0.50[a]	122.13±11.80[a]	128.63±9.80[a]	35.50±5.20[a]	35.75±3.10[a]
L. swartzii	12.50±1.00[b]	17.25±0.50[a]	36.80±17.40[a]	44.50±11.20[a]	25.20±10.50[a]	21.60±5.30[a]

[a, b] = Means of PT and PS for the same mushroom bearing different letters are significantly different from each other ($P < 0.05$).

Table 3 - Mean yield and biological efficiency of 18-month-old preserved cultures of *L. tigrinus*, *L. sajor-caju*, and *L. swartzii.*

Mushroom	Yield/bag (g)		Biological efficiency (%)	
	PS	PT	PS	PT
L. tigrinus	25.81±0.58[a]	23.33±3.78[a]	5.12±0.08[a]	4.70±0.87[a]
L. swartzii	35.64±6.31[a]	36.68±8.81[a]	7.12±1.26[a]	7.72±1.76[a]
L. sajorcaju	28.90±2.33[a]	30.07±2.40[a]	5.87±0.46[a]	6.02±0.42[a]

Discussion

Preservation of mycelia is important as it maintains the vital characteristics of mushrooms for future generations, thus ensuring biodiversity, which is a matter of public concern, nowadays. The present results show that wood sticks can ensure the viability of the three *Lentinus* species considered. In the wild, these mushrooms are typically growing on twigs and branches of any dead hardwood tree. Wood is a lignocellulosic material; it is considered a suitable substrate for growing mushrooms. Different types of wood contain 27.55–34.08% lignin, 44.79–46.76% cellulose, 15.32–16.29% hemicellulose, and 1.30–3.44 % ash (Badu et al., 2011; Boadu et al., 2023). The presence of these components supports the growth and development of mushroom mycelia, allowing their viability for a long period. Also, sawdust is one of the suitable substrates that can preserve mycelial viability for more than two years under ultralow temperatures (Linde et al., 2018). In our case, it is possible that the standardized environmental conditions of incubation (28-30 °C) and the sealed ones might have favoured *Lentinus* development, as found also by Dulay et al. (2021, 2021b, 2012). These favorable

conditions possibly prevented mycelia from drying, thus permitting their survival for extended periods.

To date, there are no published reports regarding preservation methods suitable for *L. tigrinus*, *L. sajor-caju*, and *L. swartzii* to compare with our results. Concerning the setting of the ideal incubation temperature, similar conditions (30 °C) were found favourable for maintaining the viability of other mushrooms like *Ganoderma lucidum* (Curtis) P. Karst., *Pleurotus sajor-caju* Fr., *Pleurotus florida* Singer, *Agaricus brasiliensis* Fr., and *Calocybe indica* Purkay. & A. Chandra within 12 months (Veena and Pandey, 2010; Maia et al., 2012). In some cases, the proper interval of monitoring for the optimum maintenance period is also an important factor that needs to be further investigated. As it was for *Agaricus blazei* which period of cryopreservation resulted to be cryoprotectant-dependent (Colauto et al., 2012). Other mushrooms like *Auricularia* polytricha (Mont.) Sacc.and *Volvariella volvacea* (Bull.) Singer also showed a decline in viability after being cryopreserved for an extended period (Linde et al., 2018). The result obtained in this study suggests that wood sticks can be used as an alternative substrate for preserving species of mushrooms specifically *L. tigrinus*, *L. sajor-caju,* and *L. swartzii* for 18 months highlighting the importance of substrate selection, storage temperature, and incubation duration in achieving successful preservation.

The mycelial growth performance of mushrooms can be used to determine their aggressiveness. *Lentinus* mushrooms can grow luxuriously in different substrates as well as in a wide range of pH concentrations; and can adapt to extreme environmental conditions (Dulay et al., 2021). In our study, the observed mycelial characteristics of the preserved cultures demonstrate preservation in wood sticks and the growing conditions (pH 6.0) did not affect the qualities of the mushrooms. This finding allows to get around the risks due to re-culturing for long periods which can change the morphology and the genotypic attributes of the culture (Mata and Perez-Merlo, 2003) as well as is liable to affect mycelia the mycelial growth rate and mycelial biomass production of *V. volvacea* (Zhao et al., 2022). On the other hand, culture of *A. brasiliensis* which consistently undergoes sub-culturing did not show any changes in mycelial growth (Maia et al., 2012). Concluding that the viability depends on the genus involved, our results suggest that the preservation of the *Lentinus* mycelia to wood sticks for 18 months has no negative effects on the mushrooms since the mycelia developed and grew normally. Thus, this result suggests that wood stick is an effective substrate that can maintain the viability of these mushrooms within 18 months.

The quality and productivity of the preserved cultures are important parameters to verify the efficiency of the preservation method. The quality of a fruiting body is assessed based on morphological characteristics such as the size of the pileus or cap and the length of the stipe. It is good to note that there are no undesirable changes observed in the quality of the fruits like anomalous form of growth or deformations, which is an indication of a successful and efficient preservation method. The normal development of fruiting bodies observed in this study indicates that the cultures remain in good condition despite prolonged preservation. Our results align with Bermeo-Escobar et al. (2020) with *Pleurotus ostreatus* (Jacq.) P. Kumm. preserved in mineral oil, distilled water, and saline solution, where no changes in terms of biological efficiency were observed after 4 months; with Singpoonga et al. (2019) with *Cordyceps militaris* (L.) Fr. where no morphological changes on the fruit and similar yields were observed after 4 months of preservation in rice grains at 35 °C or higher temperatures of incubation and with Kitamoto et al. (2002) with *Flammulina velutipes* (Curtis)

P. Karst. there were no variations occurred in the quantity and weight of fruits produced after preservation in sawdust for three years.

On the other hand, also with cryopreservation, no morphological differences were observed by Singh et al. (2004) on 30-month-old cultures of *Agaricus bisporus* (J.E. Lange) Imbach, *Agaricus bitorquis* (Quél.) Sacc., *Pleurotus flabellatus* Sacc., and *P. ostreatus*, where genetic stability of the mushrooms was even confirmed by no DNA fragment variation in banding patterns. Similarly, these findings were further supported by Homolka et al. (2010), who verified that no physiological changes were verified in cryopreserved mycelial cultures of 30 basidiomycetous fungi. Thus, given that it is advisable to avoid continuous sub-culturing for the risk of the late formation of primordia as well as the inability to produce fruiting bodies, as it occurred with *V. volvacea* (Zhao et al., 2022) and considering that both cryopreservation and low-cost and friendly preservation protocols may provide similar goals, our results confirm that our cost-effective method is reliable in preserving the studied mushrooms.

The study demonstrates that wood sticks serve as a suitable substrate for preserving *L. tigrinus*, *L. sajor-caju*, and *L. Swartzii* and that room temperature and sealed conditions are ideal for their preservation. Altogether, these factors contribute to the effective preservation of these mushrooms which produce consistent mycelial growth, fruit quality, and yield. It is recommended to further investigate alternative substrates based on their environmental preferences and to test longer periods of preservation.

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