Viability of fungal cultures from the GUFCC and induction of sporulation in the viable fungal cultures

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DECLARATION BY STUDENT

I hereby declare that the data presented in this dissertation entitled, **"Viability of fungal cultures from the GUFCC and induction of sporulation in the viable fungal cultures"** is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Prof. Bernard F. Rodrigues and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation **"Viability of fungal cultures from the GUFCC and induction of sporulation in the viable fungal cultures"** is a bonafide work carried out by **Ms. Sukhada Uttam Salkar** under my guidance in partial fulfilment of the requirements for the award of the degree of **M. Sc.** in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University.

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INTRODUCTION

Conservation of biological diversity is one of the significant challenges of the present day. The Convention on Biological Diversity addresses biodiversity at multiple levels, from species to ecosystems to genetic resources. At all of these levels, fungi are known to be an essential component of biodiversity. Yet, until recently, as compared to plants or animals, fungi were rarely considered in conservation issues, and they were frequently neglected in international biodiversity agreements. However, over the last two decades, there has been a noticeable increase in awareness of fungal conservation (Bisko *et al*., 2018).

Fungal culture collections are essential to biologists, mycologists, microbiologists, epidemiologists, and other health and natural science professionals. Because fungi and other microorganisms are important in medicine, the techniques and their isolation and preservation methods have improved. The continuous isolation of new strains and the need to keep such strains for pathological, taxonomic, and industrial studies have motivated various scientific and industrial research centres to maintain extensive collections of living microorganisms (Roseli *et al*., 2011).

Isolating pure cultures from specimens and their maintenance for future research is challenging. There are several methods for their isolation, some of which are complicated. Excellent procedures should be able to separate the most strains possible from a specimen and obtain the desired taxon while excluding all others. Sporulating cultures are essential to describe the morphological characters of relevant fungi, but these characteristics are often missing or hard to detect on natural substrates. It is also hard to connect the same fungi based on their sexual and asexual forms. Moreover, cultures are employed for DNA extraction, mating or cultivation research, sporulation, and metabolite extraction. Hence, preserving cultures and specimens is crucial (Senanayake *et al*., 2020).

Continuous transfer on agar slants and storage in sterile water are two of the simplest procedures for maintaining fungal cultures for research (Burdsall and Dorworth, 1994). The advantages and disadvantages of the different methods of maintaining fungal cultures over long periods have been thoroughly discussed by Smith and Onions (1983) and Dana *et al* (2010). The method of preserving fungus on an agar slant is a classical method. Repeated subculturing, commonly used for preservation, involves transferring the cultures regularly to a suitable solid substrate based on the microorganisms and the room conditions (de Moraes Borba and Rodrigues, 2000). It is, however, time-consuming, prone to contamination, and does not prevent genetic and physiological changes during long-term maintenance. To overcome these drawbacks, various storage methods have been developed. Besides lyophilization (which is ineffective for most basidiomycetes), cryopreservation at low temperatures is a very efficient way to achieve this goal. These techniques, however, have their own set of drawbacks (Homolka, 2014).

Many reviews have been on preserving microorganisms, including basidiomycetes (Onions, 1971; Heckly, 1978; Smith and Onions, 1994; Ryan *et al*., 2000; Smith, 2001), but an update is required due to recent advancements in the field. Stored strains should be viable for long periods but dormant to prevent the accumulation of mutations causing morphological and biochemical changes (Borman *et al*., 2006). So far, no ideal method has been developed. As a result, it is critical to create new or improve existing preservation methods that combine the advantages and eliminate the disadvantages of individual techniques (Homolka, 2014).

Various fungi are commercially used in industries to produce products such as acetic acid, antibiotics, food processing, bio-pest control, *etc*. So, preserving them for long-term use is crucial for the industrial economy. Long-term fungal culture preservation is extremely time-consuming. This is usually accomplished through the continuous subculture method and storage at 4°C (Kitamoto *et al*., 2002). Lowering metabolism through cold storage is one

simple way to reduce the frequency of subculturing. The cultures are stored in a standard refrigerator at 4-8°C. For the preservation of fungi, repeated subculturing is a common practice. Though not practical for storing large numbers of cultures, this technique is inexpensive, simple, and universally applicable and is currently used in the majority of culture collections with limited funds, as well as in many collections as a secondary method of cryopreservation or lyophilization. (Homolka, 2014).

Mineral or paraffin oil preservation (Perrin, 1979; Johnson and Martin, 1992) is inexpensive, simple, and recommended for laboratories with limited resources. Cultures can be kept at room temperature or 15-20°C for several years. This method suits mycelial or non-sporulating cultures that cannot be frozen or freeze-dried. Furthermore, oil reduces mite infestations. Nonetheless, the procedure is unsuitable for maintaining basidiomycetes cultures, as tested earlier by Homolka and Lisá (2008). The main disadvantage of mineral oil maintenance is that the fungi continue to grow, which may result in a selection of mutants that can grow under adverse conditions.

Storage in sterile water (Richter and Bruhn, 1989) is another low-cost method for preserving fungal cultures that have recently gained popularity. This method has successfully preserved many basidiomycetes (Croan *et al.*, 1999).

Drying suspends fungal metabolism to a point where it is either reduced or, preferably, stopped, but the culture can be revived. This preservation method is helpful for cultures that produce spores or other resting structures and doesn't require expensive equipment. The disadvantages include the possibility of culture degeneration or strain drift (Nakasone *et al.*, 2004). By removing the water and preventing rehydration, the cultures can be dried using a variety of carriers, including soil, silica gel, sand, glass beads, paper strips, grains, and other materials. These methods, along with storage in mineral or paraffin oil, sterile water, or cold

storage in a standard refrigerator at 5-8°C, may be appropriate. Fungi have been successfully stored on silica gel for up to 11 years (Smith and Onions 1983). Many practical methods have been developed for preserving fungi on various organic substrates, such as wood chips, cereal grains, straw, filter paper, and insect or plant tissues (Nakasone *et al.*, 2004). Some wood-inhabiting basidiomycetes and ascomycetes can be stored on wood chips for up to ten years (Delatour 1991). Nuzum (1989) described a method for drying fungal cultures on agar strips. With this method, some basidiomycetes species survived for 18 months, whereas ascomycetes and their mitosporic forms survived for 3-5 years.

Freezing methods are widely used nowadays. Most fungi can be preserved in standard freezers or liquid nitrogen (LN), with or without cryoprotectants. One sophisticated and efficient technique is freeze-drying (lyophilization), in which fungal cultures are frozen and dried under a vacuum. The method is highly effective with spore-producing cultures. Freeze-drying and freezing below -139°C are excellent methods for long-term preservation. Storage at low and ultra-low temperatures (in freezers and LN), i.e., cryopreservation, is currently the most important technique to preserve fungi, including basidiomycetes. Cryopreservation is versatile and useful for many fungi, including those that cannot be lyophilized. However, both methods require the use of specialized and costly equipment. The disadvantages of cryopreservation include cell and tissue damage caused by ultra-freezing shock, and the cultures cannot be revived (Corbery and Le Tacon, 1997). To fix this problem, cryoprotectants such as glycerol, DMSO, ethylene glycol, and others are used (Nagpal *et al.*, 2012). However, this does not completely protect the culture. Also, the risk of injury to the user when handling LN is a major issue. The availability, facility, and cost of LN for small laboratories located in remote areas pose a significant challenge (Ryan *et al.*, 2000).

Fungal sporulation is one of the key criteria for correctly identifying and confirming periodically maintained fungal cultures by different preservation techniques. The formation

of spores or conidia from vegetative cells is referred to as fungal sporulation or conidiation (Adams *et al.*, 1998). It is influenced by environmental and endogenous biological processes (Su *et al.*, 2012). Fungal sporulation is most common in unfavourable growth conditions (Dahlberg and Etten, 1982).

Asexual sporulation may be an adaptive response that allows the organism to survive in harsh environments such as radiation, extreme heat or cold, and a lack of nutrition. Spores are thick-walled, dormant structures that preserve the genetic content of an organism. Many experiments have been carried out to understand the molecular mechanisms of conidial sporulation (Roncal and Ugalde, 2003; Sun *et al.*, 2012). Fungal spore and conidial morphology are important in fungal taxonomy (Kirk *et al.*, 2008). However, many isolates are unable to sporulate on standard artificial media (Su *et al.*, 2012).

Major environmental factors influencing sporulation include nutrient levels, host substrate, and light (Su *et al.*, 2012). Various conditions and techniques have been used in commercial and scientific research to facilitate fungal sporulation (Xu *et al.*, 2012). These methods include pre-incubation in the dark, low-temperature treatment, brushing mycelia, and exposing mycelia to UV light (Guo *et al.*, 2003; Wang *et al.*, 2005; Xu *et al.*, 2012). Other factors that influence fungal sporulation include mycelium destruction (Su *et al.*, 2012), temperature (Prasad *et al.*, 1973), pH (Yazdany and Lashkari, 1975), and humidity adjustments (Paul and Munkvold, 2005). According to Kashket and Cao (1995), some fungi lose their sporulation ability *in vitro* due to repeated transfers.

Mycelia exposed to ultraviolet (UV) radiations for a short period can successfully induce conidial sporulation (Su *et al.*, 2012).

Many types of fungi need light for sporulation. For instance, blue-ultraviolet light stimulates *Trichoderma viride* sporulation, which is sensitive to low light intensities (Gressel and

Hartmann, 1968). To develop conidia, *Aspergillus ornatus* must be exposed to light for at least three hours (Hill, 1976). Several species of ascomycetes have successfully triggered sporulation after exposure to bright fluorescent or blue light for 18 to 24 hours (Crous *et al.*, 2006). Light radiation, however, may prevent nocturnal sporulators such *Alternaria, Choanephora, Helminthosporium, Peronospora, and Stemphylium* from sporulating (Dhingra and Sinclair, 1985).

In neutral to alkaline media, the fungal vegetative phase develops well, and an increase in acidity may promote sporulation (Tatiana *et al.*, 2010). While many experts agree that most fungi can sporulate at room temperature, others contend that low temperatures are as effective at causing sporulation (Shahin and Shepard, 1979; Tatiana *et al.*, 2010).

Various culture media are used in fungal isolation and culturing. The main nutrients for the successful development of fungi are carbon (C), nitrogen (N), and other microelements (Su *et al.*, 2012). Most regularly used media offer suitable fungal growth conditions (Su *et al.*, 2012), although they typically struggle to induce sporulation (Li *et al.*, 2007). Hence, artificial media with low nutrient content, such as water agar, half- or 1/4-strength PDA, and synthetic nutrient-poor agar, induce sporulation. Starvation or nutritional depletion frequently increases sporulation (Wulandari *et al.*, 2009; Braun *et al.*, 2011).

The transmission of false signals to the cells on the status of the environmental nutrients is one potential route for sporulation stimulation (Adams *et al.*, 1998). Records of the sporulation of some *Mucor* species have been found in synthetic mucor agar, whereas Kickxellomycetes members sporulate on cornmeal agar or half-strength malt extract-yeast extract agar (Swathi-Sri and Subrahmanyam, 2017). Hence, the induction of spores and maintenance of sporulating fungi through various appropriate fungal preservation techniques is challenging yet crucial for fungal identification and conservation.

The present study aimed to revive the fungal cultures currently available in the Goa University Fungus Culture Collection (GUFCC) and to induce sporulation in non-sporulating viable fungal cultures.

The present study aimed to study the following objectives:

1. To assess the viability of the fungal cultures presently available in the Goa University Fungus Culture Collection (GUFCC).
2. To sub-culture the viable cultures on suitable culture medium.
3. To induce sporulation in non-sporulating viable fungal cultures by exposing them to different treatments.
4. To study the macro- and micro-morphological characteristics of viable cultures.

Borba and Rodrigues (2000) evaluated the viability and sporulating capability of 45 Coelomycetes. They maintained the subcultures under mineral oil, in soil, and on agar slant for varying periods *viz.*, 50 years, 39 years, and 2 years, respectively. Out of the 34 strains preserved under mineral oil, 20 strains were viable, and one strain of *Pestalotiopsis guepinii* sporulated after 3 years covered by 0.3 cm mineral oil. From the 16 strains in the soil, only the *Septoria lycopersici* strain showed viability but failed to sporulate. Also, 11 endophytic strains preserved on corn meal agar slants were viable and showed sporulation. One endophytic strain of *Phomopsis* sp. recorded viability after 8 years of storage under mineral oil but failed to sporulate after several attempts.

Kaur *et al.*, (2011) analysed the physiological (linear growth and biomass production), biochemical (β-1,4 endoglucanase production), and fruiting behaviour of two temperate mushrooms *Agaricus bisporus* and *Pleurotus florida*. They preserved these cultures in 10% (v/v) glycerol and aimed to establish the recovery/changes in these fungi when stored at different temperatures *viz.*, room temperature (25-35°C), −20°C and −196°C for 6 months. Studies concluded that both species showed good viability and recovery rate when stored in liquid nitrogen for longer periods. However, he observed that the conventional sub-culturing method is more appropriate for regular use.

Su *et al.*, (2012) investigated the effects of nutrition, host tissue, and light on fungal sporulation in artificial media, employing five strategies (1/10-strength PDA, $CaCO_3$ water agar, pine needle medium, mulberry agar, and near-ultraviolet light irradiation) to induce these strains to sporulate, with a 62% success rate. The most suitable technique was pine needle medium, which induced sporulation in 40% of the recalcitrant strains.

Karaduman *et al.*, (2012) compared storage methods for a newly isolated basidiomycetes culture of *Schizophyllum commune*. The origin of the culture media (mycelium-colonized wheat seeds, mycelium-colonized agar plugs), the protectant type [sterile distilled water (SDW), glycerol (15%), trehalose], and the preserving temperature (4°C, 20°C, and -20°C) were all different among the 12 methods used. The viability of the fungal cultures and their biological activity (mycelial recovery ratio, mycelial growth rate, mycelial biomass weight, and enzyme activity) were monitored for one year. The strain's vitality was successfully preserved, and mycelial growth rates and biomass weight were comparable across storage methods. However, enzymatic activity varied across the storage methods. The study revealed that agar plugs stored in sterile water at 4°C and 15% (v/v) glycerol at 20°C are suitable methods for preserving *S. commune*. Other suitable options include mycelium-colonized wheat seeds in sterile water at 4 °C and agar plugs in sterile water at 20°C.

Bisko *et al.*, (2018) described some methods and listed various nutrient agar media to preserve the viable vegetative mycelium of macromycetes pure cultures from the IBK Culture Collection in Ukraine. Morphological features of the fungal strains on agar nutrient media and microstructures of vegetative mycelium by optical and scanning electron microscope were characterized. Many macromycetes were investigated for the production of biologically active compounds, including melanin, polysaccharides, lipids, cytokinins, antibacterial activity, and heavy metal sorption capacity. The main parameters of nutrient media, plant bio stimulants, and cultivation conditions for certain types of macromycetes are characterized. The fungal strains having the potential production of biologically active substances were selected and their cultivation conditions, the composition of nutrient media, and methods for stimulating the synthesis of biologically active compounds have been established. High-yielding edible and medicinal mushrooms strains for fruit bodies' production on the selective plant substrates were demonstrated.

Al-Bedak *et al.*, (2019) introduced a novel method for long preservation and fast revival of filamentous fungi using sterile cotton balls (CB). A total of 135 strains representing 16 fungal genera and 47 species were preserved on cotton balls moistened with Potato Dextrose Agar (PDA) and incubated at 25 ± 2°C until sufficient growth was obtained, then stored at 17-20°C for 36 months. Every 6 months, the fungal growth on PDA was monitored. After 2 years of long-term preservation, the CB method successfully revived 135 fungal strains, with a revival rate of 100%. While 76 strains were revived after 3 years of preservation and the revival rate was 59%. Even after 3 years of preservation, the new method did not affect viability or mycelial morphology making it a simple, cost-effective, and convenient method for preserving filamentous fungi for long periods without risk of contamination.

Susanna *et al.*, (2019) studied the growth and micromorphological characteristics of bracket fungus *Ganoderma adsperum* on Malt-Extract Agar (MEA) and Potato-Dextrose Agar (PDA) media for 6 days and submerged culture for 14 days at varied temperatures (25, 30, 35, 38 °C). The optimum temperature for growth of *G. adspersum* was found to be 25- 30°C. Temperature above 30°C suppressed mycelial growth of *G. adspersum*.

Surja *et al.*, (2020) assessed and compared the viability, morphology, contamination, and antifungal susceptibility profile of fungi *Candida* sp., *Trichophyton mentagrophytes*, and *Aspergillus* sp. preserved in sterile water or paraffin oil versus periodic subculture for 6 months. Both these methods retained the vitality of most of the fungal species except *T. mentagrophytes* did not survive in sterile water during preservation. In all methods, the morphology was preserved without contamination. Sterile water and paraffin oil methods showed a decrease in the inhibition zone against the antifungal drug. The study revealed that for the preservation of fungal viability, the paraffin oil method was preferred over the sterile water method making it more convenient to use in a simple laboratory.

Senanayake *et al.*, (2020) discussed on culture and specimen preservation techniques of different microfungal groups. Also described the standardized procedures in the taxonomic study and general practices of phenotypic approaches in fungi. Various staining, mounting, and slide preparation techniques were covered that are necessary for fungal identification. Detailed methods for isolating various groups of micro fungi, as well as techniques for inducing sporulation in various fungal groups, were also analysed.

Mahalakshmi *et al.*, (2021) investigated the effect of different media, temperature, and pH on the growth, cultural, and morphological characteristics of the pathogen *Alternaria solani* using the agar plate method for successful crop disease management. The study revealed that among the media, PDA and PD broth were best in supporting the mycelial growth (88.67mm) and mycelial dry weight (289.51mg) of the pathogen. Furthermore, the temperature of 30°C was suitable for *A. solani*, recording a maximum mycelial growth (69.52mm). The optimum pH for pathogen growth was 6.5, with a maximum mycelial growth of 71.52mm.

MATERIALS AND METHODS

In the present study, fungal cultures from GUFCC were sub-cultured separately on Malt Extract Agar (MEA) medium and Potato Dextrose Agar (PDA) medium to check their viability.

Preparation of Potato Dextrose Agar (PDA) medium:

39.0g of Potato dextrose agar powder and 5.0g of Agar powder were dissolved in 1 litre of distilled water (de Farias *et al.*, 2010). The pH was adjusted to 5.6. The media was boiled to dissolve the constituents completely. Then it was autoclaved at 121°C for 15 minutes. Streptomycin Sulphate (0.4 mg/ml) and Ampicillin (0.4 mg/ml) (Bills, 1996) were added to the autoclaved media aseptically.

Preparation of Malt Extract Agar (MEA) medium:

Malt Extract (30g), Peptone (5g), and Agar powder (18g) were weighed and suspended in 1 litre of distilled water. The pH was adjusted to 5.4. The media was boiled to dissolve the constituents completely. Then it was autoclaved at 121°C for 15 minutes.

The media and the glass vials were autoclaved at 15psi for 15 minutes, and the slants were prepared under sterile conditions.

The fungal cultures from GUFCC were first cultured on MEA and PDA plates and incubated at 25°C for 15 days to 3 months (depending on the growth rate of different fungi). The cultural characters of the studied cultures were compared with those of the original cultures (on the same medium and grown under the same conditions) to confirm their viability further.

The viable fungal cultures were successfully sub-cultured in triplicate on MEA slants, incubated at room temperature for five days, and then stored in a BOD incubator. The

cultures were then checked periodically for fungal growth. Both macroscopic and microscopic characters of viable cultures were studied.

Taxonomic identification:

Once mycelial growth and/or sporulation appeared, the cultures were confirmed viable and then identified using a light microscope. Slides were prepared using lactophenol cotton blue stain (Leck, 1999) and identified at the genus level based on spore shape, size, texture, and shape of fungal hyphae using the identification key (Seifert *et al.*, 2011) (The genera of Hyphomycetes, Netherlands).

Induction of sporulation in the non-sporulating viable fungal cultures:

The fungal cultures that sporulated were identified by preparing slides. The non-sporulating fungal cultures were exposed to the following treatments:

UV exposure

The non-sporulating fungal cultures were cultured on MEA plates and incubated at room temperature for five days. The plates were exposed to UV light (250-260 nm) for 4 hours and then kept in the dark for 8 hours.

Low temperature

The non-sporulating fungal cultures were grown on MEA plates, and after the appearance of fresh mycelial growth, the fungal isolates were incubated in a cold room at 4°C.

pH

MEA media was prepared, and pH was adjusted to 4, 6.5, and 9. The non-sporulating fungal cultures were cultured aseptically on the medium and incubated at room temperature. After 15 to 30 days of the treatments, the cultures were observed under a microscope for the appearance of sporulation using lactophenol cotton blue stain.

RESULTS AND DISCUSSION

In all, 51 fungal isolates were assessed for viability. After many observations, over a few days to months, it was observed that most of the cultures took a long time to revive, whereas few cultures showed mycelial growth after a few days of sub-culture. It is presumed that this may be due to the culture's age and various other factors. The non-viable cultures (8 to 10 years old) were assumed to have lost their reviving capacity. Therefore, developing better, more affordable techniques for maintaining fungal cultures over a long period is important.

Of the 51 cultures undertaken for the study, 24 fungal isolates showed viability, and 27 were non-viable. Of the 24 viable isolates, nine fungal isolates have a unique GUFCC number and are listed in **Table 1**. Four isolates were not identified but had GUFCC numbers and are listed in **Table 2**., while eleven isolates without GUFCC numbers were identified and are listed in **Table 3**.

The inventory of all 27 non-viable isolates is given in **Table 4**. The viability percentage was calculated to be 47.05%, while the non-viability percentage was calculated to be 52.94%.

In the second objective of the study, 12 fungal cultures **(Table 6)** out of a total of 24 viable cultures that failed to sporulate on MEA and PDA media after three months were selected for the induced sporulation study. All the 12 fungal cultures were cultured on MEA plates and exposed to exogenous spore-inducing factors, *viz.*, UV light exposure (250-300nm) for 4 hours, growth on MEA medium with varied pH *viz.*, 4, 6.5, and 9, and treatment at 4°C. The study revealed that sporulation was recorded only in four (33.33%) fungal cultures. Nine (66.67%) of the fungal cultures failed to sporulate even after exposure to exogenous spore-inducing factors.

Table 1: Viable cultures identified and with GUFCC number.

Sr. No.	GUFCC No.	Name of the fungi	Original date
1	15024	*Coprinus plicatilis*	-
2	6960	*Ganoderma lucidum*	07-11-2012
3	9045	*Stereum* sp.	23-05-2013
4	6331	*Boletus* sp.	28-12-2018
5	6935	*Omphalotus* sp.	28-12-2018
6	15022	*Ganoderma lucidum*	10-01-2019
7	6950	*Daldinia* sp.	01-02-2019
8	6942	*Omphalotus* sp.	30-05-2019
9	6109	*Pleurotus cystidiosus*	30-09-2019

Table 2: Viable cultures unidentified and with GUFCC number.

Sr. No	GUFCC No.	Original date
1	6965	07-11-2012
2	9037	07-11-2012
3	1989 VIC- 113	11-01-2019
4	6326	29-05-2019

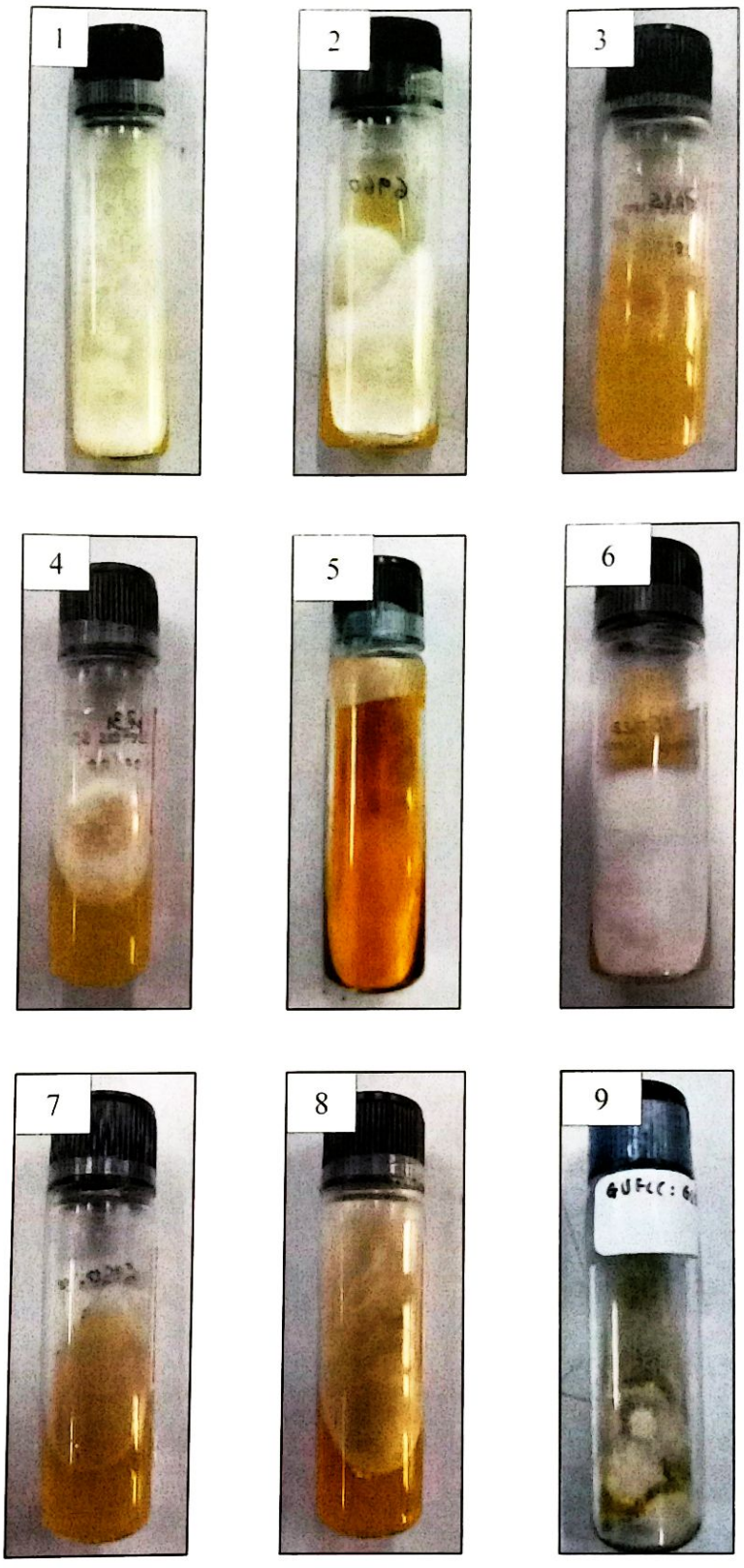

PLATE 1: VIABLE CULTURES (Identified and with GUFCC number)

1-15024, **2**-6960, **3**-9045, **4**-6331, **5**-6935, **6**-15022, **7**-6950, **8**-6942, **9**-6109.

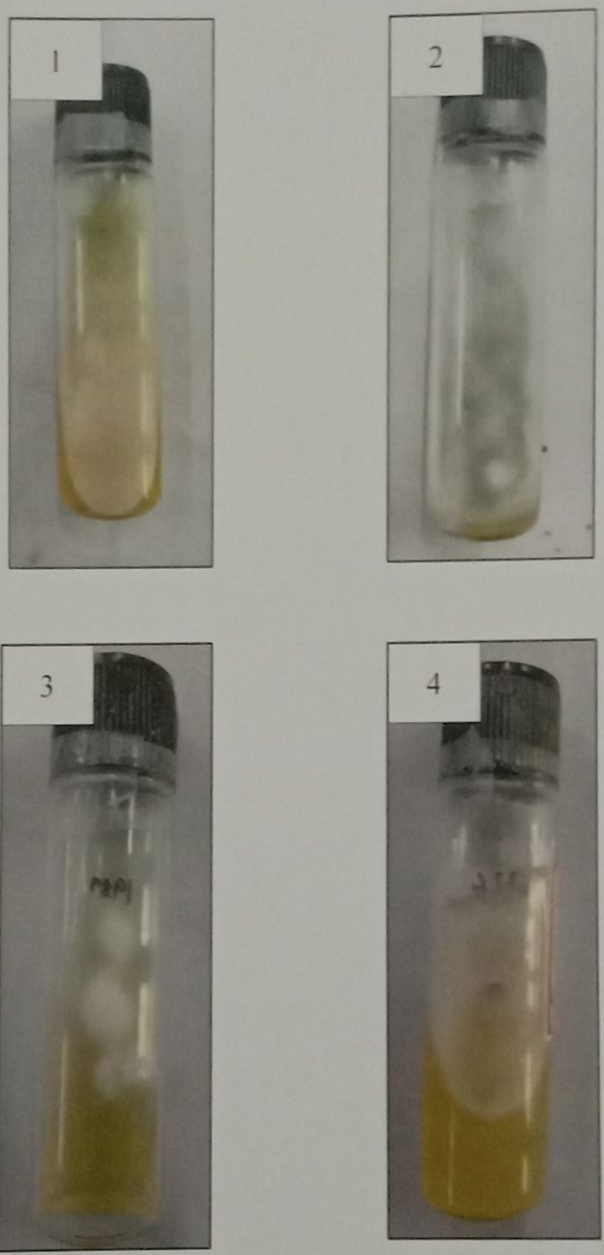

PLATE 2: VIABLE CULTURES (Unidentified and with GUFCC number)

1-6965, **2**-9037, **3**-1989 VIC- 113, **4**-6326

Table 3: Viable cultures identified and without GUFCC number.

Sr. No.	Name of the fungi	Original date
1	*Fusarium* sp.1	07-11-2012
2	*Pleurotus cystidiosus*	28-12-2018
3	*Penicillium* sp.1	28-12-2018
4	*Ganoderma lucidum*	10-01-2019
5	*Aspergillus* sp.1	18-01-2019
6	*Fusarium* sp.2	01-02-2019
7	*Paecilomyces* sp.	01-02-2019
8	*Penicillium* sp.2	28-02-2019
9	*Aspergillus* sp.2	20-05-2019
10	*Aspergillus* sp.3	29-05-2019
11	*Fusarium* sp.3	30-05-2019

PLATE 3: VIABLE CULTURES (Identified and without GUFCC number)

1- *Fusarium* sp.1, **2**- *Pleurotus cystidiosus*, **3**- *Penicillium* sp.1,

4- *Ganoderma lucidum*, **5**- *Aspergillus* sp.1, **6**- *Fusarium* sp.2,

7- *Paecilomyces* sp., **8**- *Penicillium* sp.2, **9**- *Aspergillus* sp.2,

10- *Aspergillus* sp.3, **11**- *Fusarium* sp.3

Table 4: Non-viable cultures.

Sr. No.	GUFCC No.	Name of the fungi	Original date
1	-	*Omphalina* sp.	-
2	NC- 184	*Craterellus* sp.	-
3	NC- 173	*Craterellus* sp.	-
4	NC-165	*A. polytricha*	-
5	NC- 39	*A. polytricha*	-
6	6307 NC-16	*G. lucidum*	-
7	6962	*Boletus* sp.	-
8	6968	-	-
9	15015 NC-210	*Polyporus arcularius*	-
10	15023	*Agaricus campestris*	-
11	6338	*A. campestris*	07-11-2012
12	6967	*Boletus* sp.	07-11-2012
13	15021	*M. rhacodes*	07-11-2012
14	13010	*Pleurotus sajor-caju*	30-05-2013
15	6947	*Stereum* sp.	10-06-2013
16	6894	*S. ruber*	28-12-2018
17	9017	*Ganoderma boninense*	28-12-2018
18	6907	*Lentinus lepideus*	10-01-2019
19	9050	*Ganoderma lucidum*	11-01-2019
20	-	*Chlorophyllum molybdites*	01-02-2019
21	NC- 177	*Volvariella bombycina*	01-02-2019
22	6946	*Boletus* sp.	28-02-2019
23	9042	*Panellus* sp.	20-05-2019
24	6905	*Lentinus lepideus*	29-05-2019
25	-	*Ganoderma lucidum*	30-05-2019
26	9001	*Auricularia polytricha*	30-05-2019
27	9013	*Polyporus* sp.	07-06-2019

Table 5: Sporulating viable cultures.

Sr. No.	GUFCC No.	Sporulating fungi
1	-	*Penicillum* sp.1
2	-	*Penicillum* sp.2
3	-	*Aspergillus* sp.1
4	-	*Aspergillus* sp.2
5	-	*Aspergillus* sp.3
6	-	*Fusarium* sp.1
7	-	*Fusarium* sp.2
8	-	*Fusarium* sp.3
9	-	*Paecilomyces* sp.
10	6326	-
11	6965	-
12	9037	-

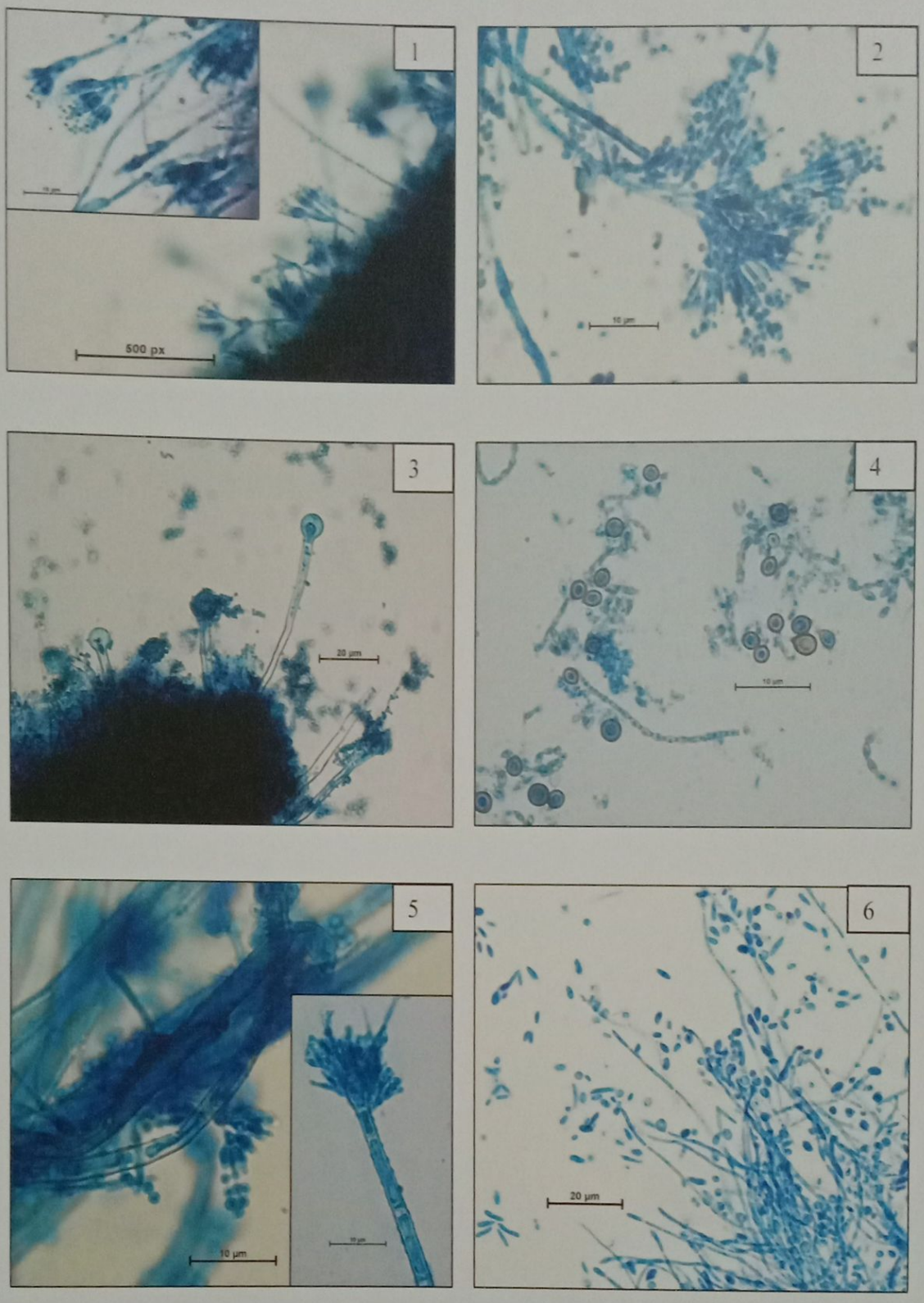

PLATE 4: SPORULATING VIABLE CULTURES (with and without GUFCC number)

1- *Penicillum* sp.1, **2**-*Penicillum* sp.2 **3**- *Aspergillus* sp.1, **4**- *Aspergillus* sp.2, **5**- *Aspergillus* sp.3, **6**- *Fusarium* sp.1

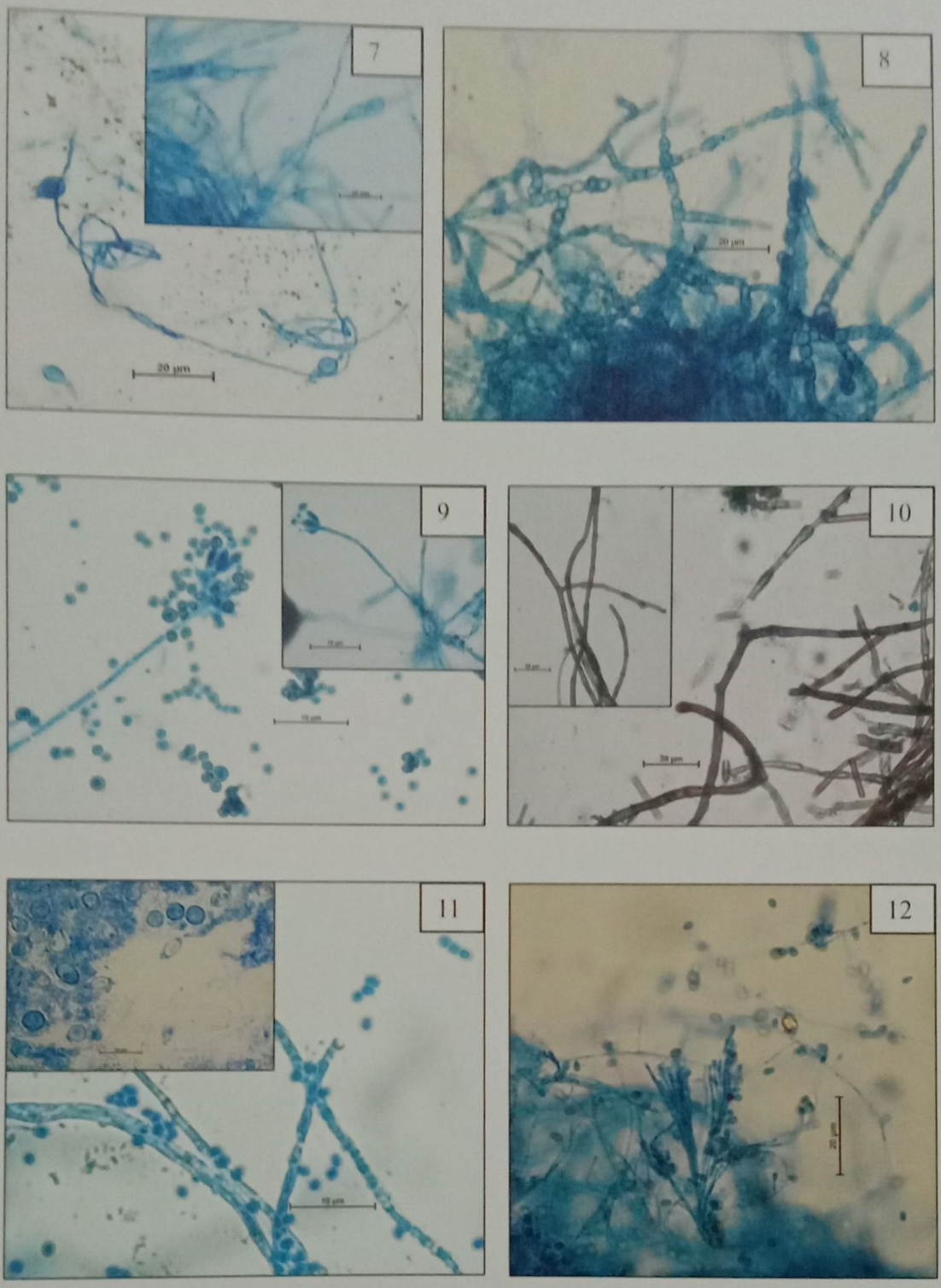

PLATE 5: SPORULATING VIABLE CULTURES (with and without GUFCC number)

7- *Fusarium* sp.2, **8**- *Fusarium* sp.3, **9**- *Paecilomyces* sp., **10**-6326, **11**-6965, **12**-9037

Table 6: Non sporulating viable cultures.

Sr. No.	GUFCC No.	Non- sporulating fungi
1	1989 VIC- 113	-
2	-	*Ganoderma lucidum*
3	-	*Pleurotus cystidosus*
4	6109	*Pleurotus cystidosus*
5	6935	*Omphalotus* sp.
6	6942	*Omphalotus* sp.
7	6950	*Daldinia* sp.
8	6960	*Ganoderma lucidum*
9	9045	*Stereum* sp.
10	15022	*Ganoderma lucidum*
11	15024	*Coprinus plicatilis*
12	6331	*Boletus* sp.

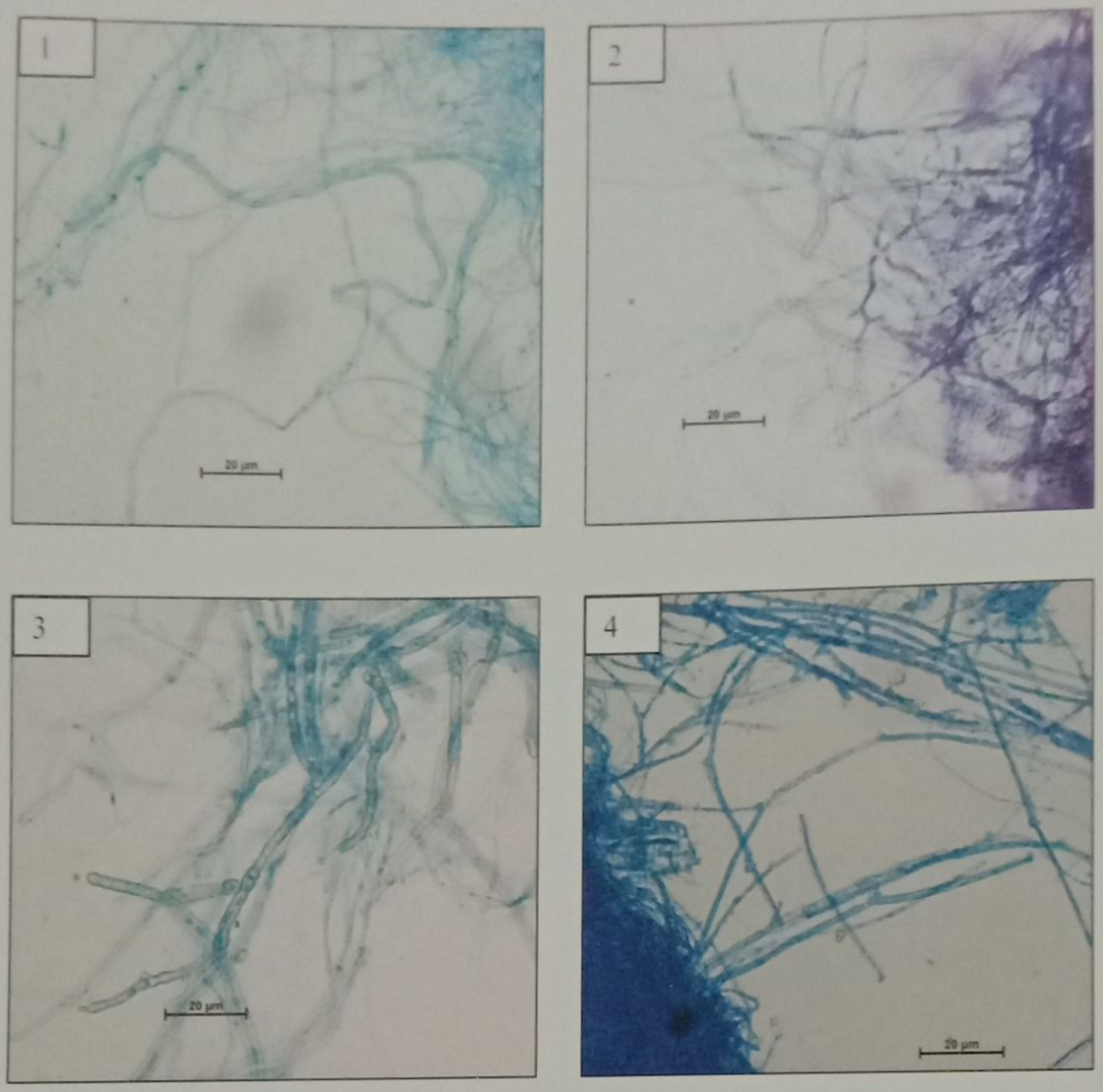

PLATE 6: NON-SPORULATING VIABLE CULTURES (with and without GUFCC number) showing hyphae:

1-1989 VIC- 113, **2**- *Ganoderma lucidum*, **3**- *Pleurotus cystidosus*,

4-6109.

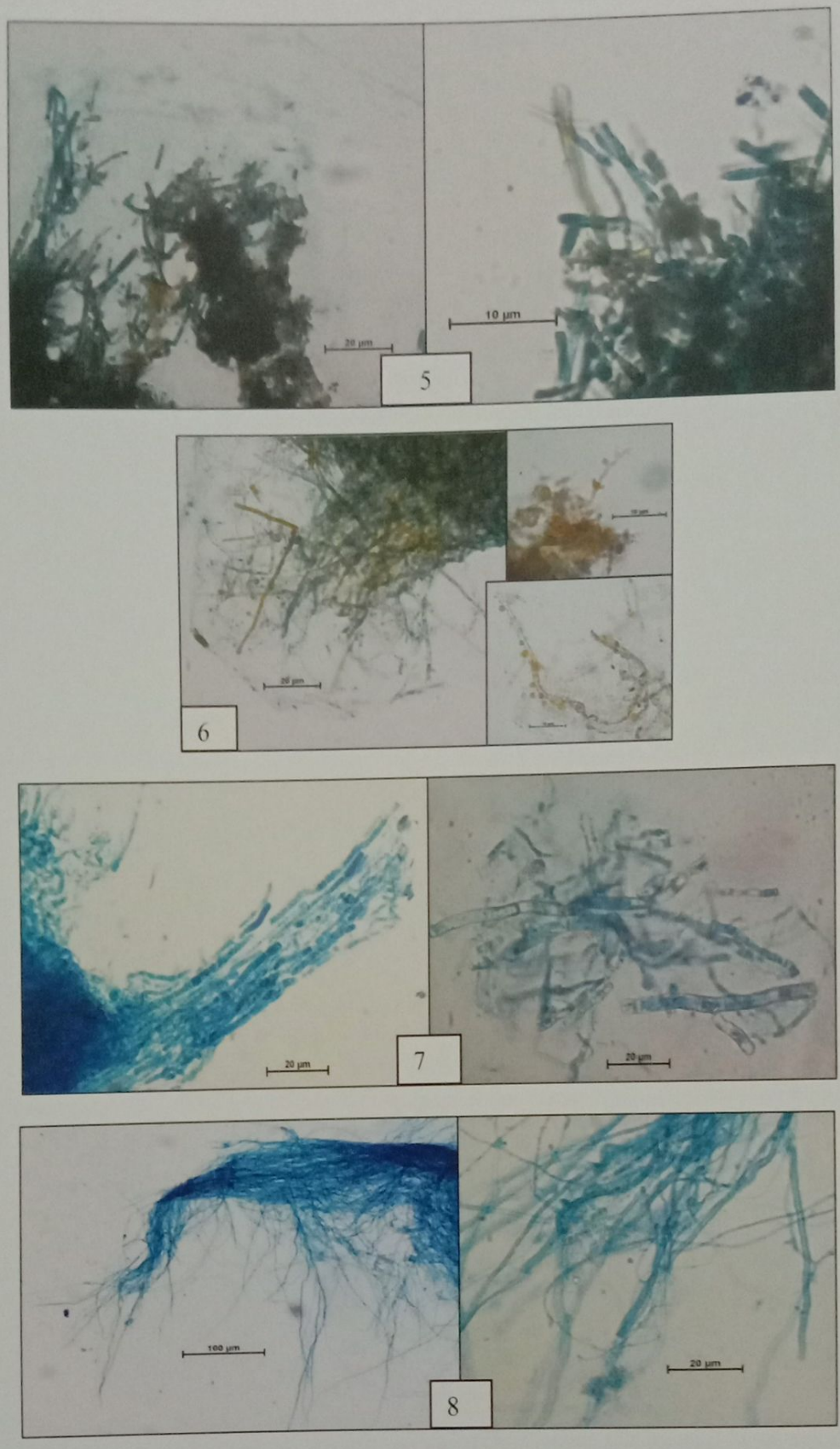

PLATE 7: NON-SPORULATING VIABLE CULTURES (with and without GUFCC number) showing hyphae

5-6935, **6**-6942, **7**-6950, **8**-6960.

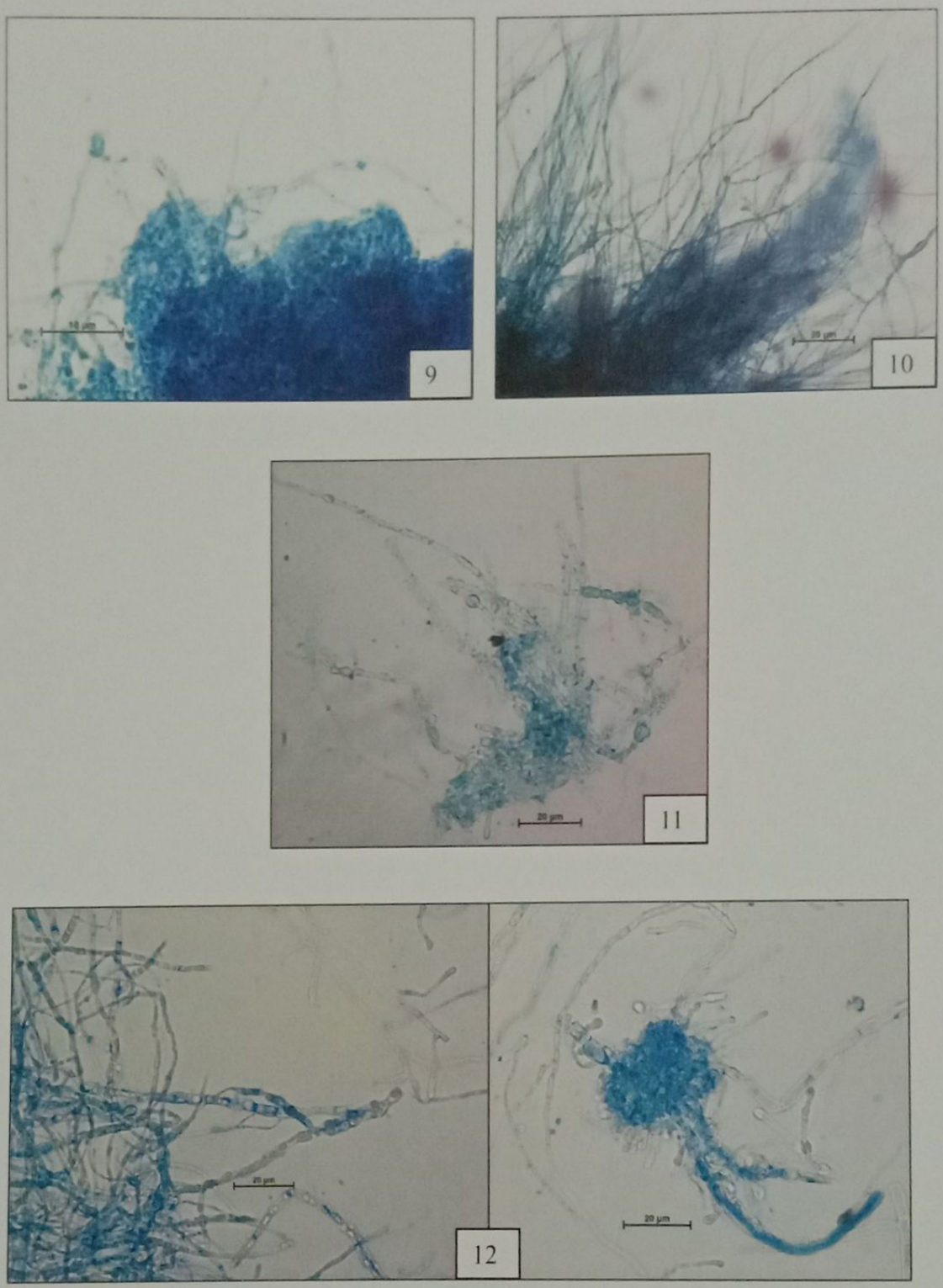

PLATE 8: NON-SPORULATING VIABLE CULTURES (with and without GUFCC number) showing hyphae

9-9045, **10**-15022, **11**-15024, **12**-6331.

Growth was slow in all the cultures at pH 4, while it was normal at pH 9 and 6.5. Similarly, slow growth was noticed in all cultures exposed to low temperatures, whereas it was normal in UV-treated cultures. Low temperature at 4°C in this study was not effective in spore induction compared to UV exposure.

In the UV-exposed and alkaline pH-treated cultures, two *Pleurotus cystidiosus* species showed the formation of sclerotium-like structures, which are resting bodies of a compact mass of fungal mycelium containing food reserves grown to survive in the environmental stresses. While the other two cultures, *Stereum* sp. and *Coprinus plicatilis* showed the formation of chlamydospores, thick-walled asexual resting spores formed within hyphae or at hyphal tips. This indicates that exposing the fungal cultures to these unfavourable conditions produced asexual chlamydospores and resting bodies in the medium.

It was observed that most of the macro-fungi did not sporulate, indicating that the exogenous spore-inducing treatments were not suitable or that a combination of treatments is required to induce sporulation. Hence, more research is needed in this area.

The macrofungal cultures failed to sporulate even after different treatments. Their macro-morphological characters were compared with the cultural characters of the original cultures (grown on the same medium and under the same conditions) to confirm the cultures' viability further.

Fungal sporulation is a highly complex process that is regulated by both endogenous and external stimuli. Endogenous factors may include competence, where a fungus grows and becomes capable of sporulation. A fungus cannot sporulate until this phase is complete, despite having all the necessary environmental requirements. Exogenous factors such as nutrition, light, pH, temperature, humidity, and host tissue all impact fungal sporulation. However, the impact of a given component or factors on the sporulation of a certain fungus

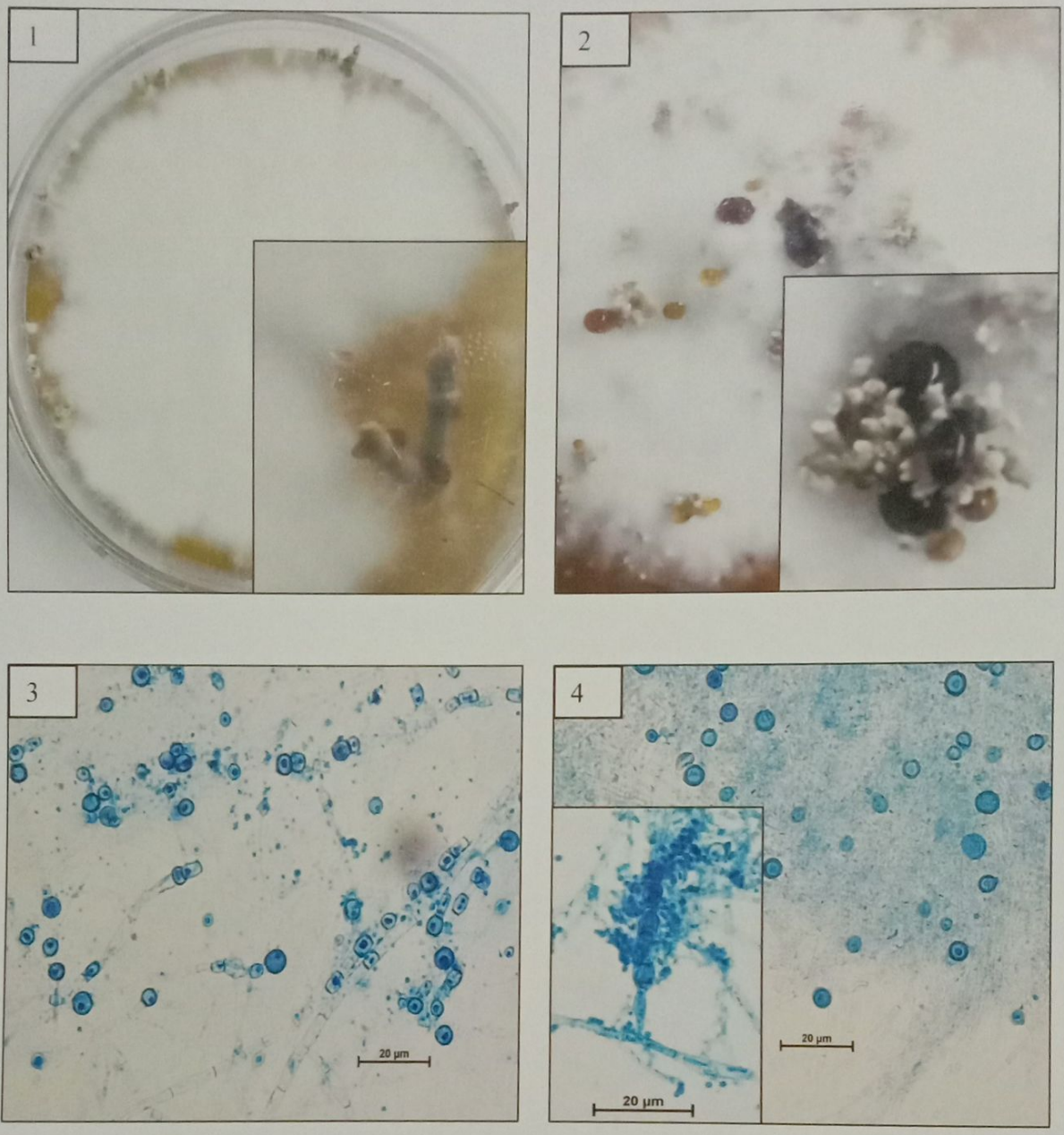

PLATE 9: FUNGAL CULTURES SPORULATED AFTER TREATMENTS

1-*Pleurotus cystidosus* showing sclerotium, **2**-6109 showing sclerotium,

3-9045 showing chlamydospores, **4**-15024 showing chlamydospores.

differs due to different requirements and complex sporulation behaviour (Mattoo and Nonzom, 2022).

The macro-morphological characters of the viable genera were recorded:

1. *Aspergillus*

Colonies restricted to fast-growing, white blue, blue-green, green, yellowish green, brown, fawn, ochraceous to black, zonate or azonate, velvety, floccose, lanose or funiculose; mycelium mostly submerged.

2. *Boletus*

A dense network of branched hyphae, colourless or white; mostly submerged into the substratum.

3. *Coprinus*

Hyphae grow as mashy, sticky, butyrous mass, white when young becoming yellow or brown in later stages.

4. *Daldinia*

White felt colonies, blackish grey colouration on the reverse side; blackish or coloured stromata 3-5cm in diameter and varying shapes.

5. *Fusarium*

Colonies are usually fast growing, pale to bright coloured; aerial mycelium, felty or diffuse fruit body when present a sporodochium; sporogenous cells arise directly from vegetative hyphae to form conidiophores.

6. *Ganoderma*

Mycelia forms as a white cottony mass, developing into stable, spherical aggregates consisting of dense, branched and partially intertwined networks of hyphae.

7. ***Omphalotus***

Fast-growing fungi, hyphae yellowish to red-brown but becoming dark brown to blackish towards the base; surface turns in butyrous slimy, sticky hyphal mass: reverse brown to black.

8. ***Paecilomyces***

Colonies grow fast; aerial mycelium is never typically green, funiculus, conidiophore well developed, erect; mostly sporogenous cells are borne more or less directly on the vegetative hyphae.

9. ***Penicilium***

Grows as compact, dense colonies, colonies variously coloured, usually blue-green, texture variable, zonate or azonate; vegetative hyphae creeping; reverse uncoloured or variously coloured; conidiophores usually conspicuous, more or less erect, hyaline.

10. ***Pleurotus***

Fast-growing mycelial growth, hyphae hyaline, submerged into the substratum; sporogenous cells are sometimes borne more or less directly on the vegetative hyphae.

This assessment gives the data for the easy finding and long-term maintenance and storage of the cultures for further use in various studies.

CONCLUSION

The present study was initiated to check the viability of various fungal cultures available in the Goa University Fungal Culture Collection (GUFCC) and to induce sporulation in the non-sporulating fungal isolates.

To assess their viability, the previously isolated and maintained micro- and macro-fungal cultures from the GUFCC were sub-cultured. It was concluded that out of the selected 51 fungal isolates, 24 were viable, and 27 were non-viable.

Of the 24 viable fungal isolates, only 12 cultures showed sporulation, making identifying and matching their characters with the original cultures easy. But rest 12 isolates failed to sporulate on the media and could not be identified. These cultures were subjected to different spore-inducing treatments, *viz.*, UV exposure, pH, and low temperature.

In response to the UV exposure and slightly acidic and alkaline pH treatments, two out of four cultures, i.e., *Stereum* sp. and *Coprinus plicatilis* showed the production of asexual chlamydospores. Whereas sclerotium-like resting bodies were observed in two *Pleurotus cystidiosus* species. The low-temperature treatment was not effective in inducing sporulation. The remaining eight macro-fungi did not respond to the treatments which were later identified based on the similarities in their macro-morphological characteristics to the original cultures. Hence, different fungal cultures sporulate only in response to specific individual or combination treatments of spore-inducing factors. As a result, more knowledge and study are necessary for this field.

The induction of sporulation allows for easier identification, which leads to a better understanding of natural fungal communities. Several external parameters, such as low-nutrition media, host tissue, light irradiation, temperature, and pH, should be considered to induce sporulation in artificial media. These factors vary depending on the fungal cultures.

In addition, the isolation and preservation of fungal cultures provide a foundation for taxonomic, medical, and pathological research. Besides, they have industrial applications that include solvents, antibiotics, enzymes, vitamins, amino acids, polymers, and many other valuable compounds through fermentation.

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