Cultural conditions for basidiospore germination of *Lentinus swartzii* and *Lentinus strigosus* and their morphogenesis

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**Abstract**

The effects of liquid culture media, pH, temperature, and illumination on the basidiospore germination of *Lentinus swartzii* and *Lentinus strigosus* as well as their morphogenesis were studied and documented. Coconut water and potato broth with pH of 5.0 – 7.0, incubated at 30°C and total dark, were the optimum nutritional and physical factors in liquid culture condition as activators for successful basidiospore germination of the two *Lentinus* species. A peculiar germination process was observed in which the basidiospores germinated through swelling and elongated to become hyphae. The vegetative phases of both mushrooms occurred in four significant stages: swelling and clumping (a mechanism for plasmogamy), elongation, septation, and branching. On the other hand, their reproductive phases had six distinct stages: mycelial coat formation, browning and hardening stage (only in *L. swartzii*), popcorn-like formation (only in *L. strigosus*), primordia initiation, pinhead stage, pileus expansion stage, and maturation stage. This study established that the two *Lentinus* spp. have the same vegetative phase (i.e., basidiospore germination and development of hypha and mycelia), but have unique cultivation characteristics in their reproductive phase (i.e., formation of basidiocarp). The obtained information on the developmental biology of the two *Lentinus* spp. is very useful not only in the generation of effective mushroom biomass production technologies but also in many fungal biotechnological applications.

**Keywords:** *Lentinus swartzii*, *Lentinus strigosus*, Germinating basidiospores, Morphogenesis, Vegetative and reproductive phases

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Introduction

The Philippines is rich in natural resources, which serve as haven for naturally occurring mycoflora. Many kinds of macrofungi have been listed in our ethno-mycological expeditions in different areas of Luzon Island. These wild macrofungi include the genera *Ganoderma*, *Pleurotus*, * Auricularia, Volvariella, Termitomyces, Coprinopsis, Schizophyllum, Polyporus, Oudemansiella, Trametes, Xylaria, Collybia*, and *Lentinus*. *Lentinus*, which belongs to Family Polyporaceae, is a group of saprophytic, white-rot basidiomycetous mushrooms that naturally grow on lignin- and cellulosic-rich substrate. This genus has significant position in food industry, enzyme production, effluent treatment and medical application (Manjunathan and Kaviyarasan, 2010). As edible and medicinal macrofungal resource, *Lentinus* species contain proteins, carbohydrates, sugars, fiber, lipids, and minerals, and exhibit antioxidant, antibacterial, and anti-hyperglycemic activities (Dulay et al., 2014; Dulay and Pamiloza, 2017). Recently, another species, *L. strigosus* (Bangyeekhun, 2019). Aside from mycelia and fruiting body, extracts of basidiospores and germinating basidiospores of macrofungi have also been reported to demonstrate biological activities (Zhu et al., 2000; Pei-Yu et al., 2012).

With a variety of wild *Lentinus* species in the Philippines, only four (*L. tigrinus, L. sajor-caju, L. squarrosulus, L. strigosus*) have been isolated and maintained in the laboratory using the optimal culture conditions for mycelial growth, and successfully cultivated in the fruiting bags consisting of rice straw and sawdust formulated substrate in growing house conditions (Cuevas et al., 2009; De Leon et al., 2013; Dulay et al., 2017). Recently, another species, *Lentinus swartzii*, was isolated and the mycelial nutritional requirements were already established (Dulay et al., 2020). Apart from the optimal cultural conditions for mycelia growth and fruiting body production, the germination of basidiospore and morphogenesis must also be investigated in order to have a better understanding of the fundamental aspects of growth and development of mushroom, which is very essential in a successful production technology. In our previous work, we have demonstrated the optimal conditions for basidiospore germination and morphogenesis of wild strain of *L. tigrinus*, and found out a peculiar germination process and significant developmental stages (Dulay et al., 2012b). Activation and stimulation of basidiospore germination have been reported in various interesting works. For instance, germination of spores of mycorrhizal fungi such as *Paxillus involutus, Laccaria lacca, Hebeloma crustuliniforme, Lactarius turpis*, and *Amanita fulva* were stimulated by the plant roots and their exudates (Ali and Jackson, 1988). In their earlier study, the fungus *Tritirachium roseum* and bacteria *Micrococcus roseus, Pseudomonas stutzeri*, and *Corynebacterium* spp. isolated from the sporophores of mycorrhiza, soil, and agar plates as natural contaminants were shown to induce the spore germination of certain mycorrhizal fungi (Ali and Jackson, 1989). Moreover, the application of heat and light wavelength to activate germination of basidiospores of higher basidiomycetes has also been described (Vidyapin et al., 2007; Poyedinok et al., 2015). In addition, treatment of mutagen (methanesulfonate methylester) to basidiospores of *Hypsizygus marmoreus* generated new mushroom strains (Lee et al., 2011). Thus, investigation of the morphology, germination, morphogenesis, growth and development, variations, and chemical compositions of basidiospores is very vital in the selection of strains for spawn production and in fungal biotechnology research.

In our objective to extend the optimization studies and documentation of the basidiospore germination and basidiocarp formation to other *Lentinus* species, we investigated herein the *L. swartzii* and *L. strigosus* to understand fully their requirements for existence and their developmental biology in our aim to contribute to the efficient cultural management for biomass production.

Material and Methods

Mushroom strains and basidiospores

Pure cultures were obtained from our culture collections. Fruiting bodies were produced following the established production technology for the two mushrooms (Dulay et al., 2012a; Dulay et al., 2017). Basidiospores were collected from mature healthy fruiting bodies by laying down on sterile white paper
in Petri plate and incubated for 12 hours at ambient room temperature to allow the detachment of the basidiospores from the basidium. A portion of paper with basidiospores was aseptically cut using scissors and pre-soaked in test tube containing 9 mL distilled water. The basidiospore concentration was determined using a haemocytometer and adjusted to $7.5 \times 10^{3}$/mL. Spore suspensions were incubated in a water bath at 40-50°C for 1 h, and 0.1 mg/ml of streptomycin sulphate was added prior to inoculation.

**Evaluation of liquid culture media and pH**

The favorable liquid culture medium for the germination of basidiospores of the two *Lentinus* was determined. Four indigenous liquid media, namely: coconut water from mature coconut (*Cocos nucifera*), rice bran D1 (class A) broth (50g of *Oryza sativa* bran/L of water), yellow corn grit broth (50g of *Zea mays* grit/L of water) and potato broth (250g of *Solanum tuberosum* L of water) were tested in this study. The liquid media were adjusted to pH 6, and 1 mL of medium was dispensed into each tube. Three replicates of each medium were prepared. These were sterilized in an autoclave at 121°C, 15 psi for 30 min. After cooling, the liquid media were inoculated with 0.1 mL of spore suspension. The inoculated liquid media were incubated at 30°C under alternating light and dark conditions. Germination (elongation of the basidiospores) of 100 spores from randomly captured images under a compound microscope was recorded after 7 and 12 h of incubation. Sampling was done three times from each tube. To determine the optimum pH, the most favorable liquid medium was adjusted to varying pH levels (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) using 1 M NaOH and 1 M HCl. Liquid media were dispensed into tubes, sterilized, inoculated with 0.1 mL from the spore suspension, and incubated at 30°C. Percentage germination was recorded after 7 and 12 h of incubation.

**Evaluation of temperature and illumination**

The most suitable liquid medium and optimum pH were used to evaluate the influence of temperature and illumination on basidiospore germination. To determine the optimum temperature, inoculated liquid media were incubated in three temperature conditions: 10°C, 20°C and 30°C. On the other hand, for illumination, inoculated liquid media were exposed to artificial white light (322.92 lumens/m²), while the others were maintained in total darkness by covering with black paper, and both were incubated at optimum temperature. Evaluation was done in triplicate. Percentage germination was recorded after 7 h and 12 h of incubation. Data were analyzed using Analysis of Variance, and treatment means were compared using Tukey’s HSD at 5% level of significance in the SAS System Version 9.0 (SAS Institute Inc. Cary, NC, USA) program. T-test was used to compare the two illumination conditions.

**Documentation of morphogenesis**

Using the established nutritional and physical requirements for basidiospore germination in the optimization study, the germination process and morphogenesis was documented by periodically observing every 2 h from the time that swelling appeared as observed under a compound microscope. The undergoing swelling, clumping, hyphal fusion, elongation, septation, and branching were observed and photo-documented using a camera. For the developmental stages of basidiocarp formation, fruiting bags were prepared, sterilized, inoculated, and incubated (Dulay et al., 2012a; Dulay et al., 2017). After the full mycelial ramification, fruiting bags were opened and watered to allow the emergence of the basidiocarp. The significant developmental stages from mycelial ramification up to basidiocarp maturation were monitored and photo documented.

**Results**

**Influence of nutritional and physical factors**

The percentage germination of basidiospores of *L. swartzi* and *L. strigosus* as affected by nutritional and physical factors is presented in Table 1. Interestingly, all liquid culture media evaluated showed stimulatory effect on the germination of basidiospores of the two mushrooms. However, coconut water and potato broth recorded the highest percentage basidiospore germination of *L. swartzi* and *L. strigosus* in both observation periods. Tukey’s HSD revealed no significant difference between the two liquid media in both mushrooms. The optimum pH of the medium was also determined. Remarkably, basidiospores of both mushrooms germinated at pH 4 to 9, however, the optimum pH range was found at pH 5 to 7. Temperature and illumination significantly influenced the basidiospore germination of the two mushrooms. Noticeably, 30°C and total dark significantly recorded the highest percentage basidiospore germination in both mushrooms.
Table 1. Germination of basidiospores of *L. swartzii* and *L. strigosus* as affected by the nutritional and physical factors after 7 and 12 hours of incubation.

| Nutritional and Physical Factors | *L. swartzii* Basidiospore Germination (%) | *L. strigosus* Basidiospore Germination (%) |
|---------------------------------|-------------------------------------------|-------------------------------------------|
|                                 | 7th Hour | 12th Hour | 7th Hour | 12th Hour |
| Media                           |          |           |          |           |
| Coconut water                   | 82.00 ± 3.00^a | 89.33 ± 2.08^a | 77.00 ± 3.61^a | 84.00 ± 2.65^ab |
| Rice bran broth                 | 68.33 ± 4.04^b | 80.00 ± 3.61^b | 77.67 ± 2.52^a | 82.33 ± 2.08^b |
| Corn grit broth                 | 71.00 ± 2.65^b | 77.00 ± 3.61^c | 68.67 ± 4.04^b | 76.00 ± 1.73^c |
| Potato broth                    | 81.00 ± 3.00^a | 85.00 ± 2.00^ab | 79.33 ± 3.51^a | 86.67 ± 1.53^a |
| pH                              |          |           |          |           |
| 4.0                             | 74.67 ± 1.53^c | 81.33 ± 2.52^c | 67.67 ± 6.03^bc | 71.67 ± 4.73^c |
| 5.0                             | 79.67 ± 2.31^b | 86.33 ± 1.53^ab | 80.67 ± 2.52^a | 85.00 ± 2.00^ab |
| 6.0                             | 83.00 ± 1.00^a | 90.33 ± 2.08^a | 80.33 ± 1.53^a | 85.33 ± 1.53^ab |
| 7.0                             | 81.67 ± 2.31^b | 87.00 ± 2.00^ab | 81.67 ± 2.08^a | 88.33 ± 1.53^a |
| 8.0                             | 73.00 ± 3.00^c | 77.67 ± 5.69^c | 70.67 ± 4.04^bc | 76.00 ± 3.61^c |
| 9.0                             | 48.67 ± 5.13^d | 51.00 ± 6.00^d | 58.33 ± 5.13^c | 63.00 ± 6.24^d |
| Temperature                     |          |           |          |           |
| 30°C                            | 82.67 ± 1.53^a | 90.00 ± 2.65^a | 81.33 ± 2.08^a | 89.67 ± 1.53^a |
| 20°C                            | 66.33 ± 8.62^b | 75.33 ± 5.51^b | 78.67 ± 2.52^a | 86.33 ± 1.15^b |
| 10°C                            | 11.33 ± 2.31^c | 12.33 ± 1.53^c | 26.33 ± 9.02^b | 29.67 ± 7.77^c |
| Illumination                    |          |           |          |           |
| Lighted                         | 76.67 ± 3.51^b | 79.67 ± 3.06^b | 79.00 ± 1.00^b | 86.00 ± 1.00^b |
| Dark                            | 83.67 ± 1.53^a | 91.67 ± 1.53^a | 83.33 ± 1.15^a | 90.33 ± 1.53^a |

Means with the same letters of superscript in each column and factor are not significantly different according to Tukey’s HSD (p < 0.05). Values are the mean ± SD of three replicates.

Morphogenesis from basidiospore to basidiocarp

The morphogenesis of the two *Lentinus* species is shown in Figures 1 and 2. The life cycle is divided into two phases: vegetative phase and reproductive phase. In vegetative phase (basidiospore germination and formation of hypha and mycelia), four significant stages including swelling and clumping, elongation, septation, and branching were observed in both mushrooms. However, these stages appeared differently in the two mushrooms. For instance, elongation appeared after 12-16 hours in *L. swartzii* and after 11-14 hours in *L. strigosus*. Branching of mycelia succeeded after 22-36 hours in *L. swartzii* and after 18-30 hours in *L. strigosus*. The reproductive phase (development of basidiocarp) had six distinct stages including mycelial coat formation, browning and hardening stage (only in *L. swartzii*), popcorn-like formation (only in *L. strigosus*), primordia initiation, pinhead stage, pileus expansion stage, and maturation stage.

![Figure 1. Morphogenesis of Lentinus swartzii.](image-url)
Discussion

Basidiospore germination is the beginning of life of any basidiomycetous fungi. Inactive basidiospores turn into active forms by the formation of germ tubes, and differentiate into mycelia. This process could be stimulated by special environmental factors in their natural habitat. Hence, this study investigated the influence of liquid culture media, pH, temperature and illumination on basidiospore germination of the two Lentinus in liquid culture conditions. The present study found out that the liquid culture media used, especially coconut water and potato broth could induce basidiospore germination of L. swartzii and L. strigosus. Previously, potato broth was identified as the most suitable medium for the germination of basidiospores of L. tigrinus (Dulay et al., 2012b), whereas coconut water was utilized as medium for successful basidiospore germination of Ganoderma lucidum (Magday et al., 2014). The stimulatory effect of the liquid media could be attributed to their nutrient compositions. Borlingame et al. (2009) reported that potato has fructose, sucrose, amylase protein, vitamins such as thiamine, niacin, folic acid and minerals. Coconut water, on the other hand, contains sugars, sugar alcohols, lipids, amino acids, nitrogenous compounds, organic acids, enzymes, vitamins, and minerals (Santoso et al., 1996; Yong et al., 2009). The above-mentioned nutrients were included in the list of nutritional requirements that stimulate basidiospore germination of Schizophyllum commune enumerated by Niederpruem and Dennen (1966).

Apart from the nutrient compositions of the medium or substrate, there are works that demonstrated the use of compound activators. Kikuchi et al. (2007) reported that seven flavonoids including hesperidin, morin, rutin, quercetin, naringenin, genistein, and chrysin have greater stimulating effects compared to the control on basidiospore germination of Suillus bovinus using diffusion gradient assay on water agar plates. Karadeniz et al. (2013) demonstrated that the treatment of basidiospores of G. lucidum with hydrogen peroxide for antiseptic purposes also stimulated germination. On the other hand, germination of basidiospores of four Suillus species was induced after exposure to the extracts/exudates from Scotch pine (Pinus sylvestris L) roots. The effect was due to the isolated and identified diterpene resin acid, abietic acid (Fries et al., 1987). The present study successfully demonstrated the utilization of liquid culture media consisting of complex nutrients as one of the effective techniques to induce basidiospore germination of the two Lentinus species. It is necessary, however, to evaluate different nutritional sources (e. g. carbon, nitrogen, minerals, vitamins, etc.) for basidiospore germination in our intention to design a chemically-defined medium for consistent germination rate and process, which is very important in several biotechnological applications and production of mushroom biomass and metabolites.

To assess the effect of pH on basidiospore germination, coconut water (for L. swartzii) and potato broth (for L. strigosus) were adjusted to six pH levels and inoculated with basidiospores. The optimum pH range obtained in the present work is slightly lower when compared to the pH requirements for basidiospore germination of L. tigrinus, S. commune, and Hebeloma vinosophyllum (Dulay et al., 2012b; Bulsco et al., 2005; Deng and Suzuki, 2008). The low percentage germination at pH 9 indicates that the presence of essential nutrients in coconut water and potato broth is not an indicator to obtain the maximum basidiospore germination, it is also dependent on the favourable pH of the medium.
interesting to note that the optimum pH for mycelial growth of *L. strigosus* that we previously reported (Dulay and Garcia, 2017) is within the optimum pH range for the germination of its basidiospores obtained in this work. The results of the present study strongly suggest that pH is an important factor in the medium that significantly influenced the germination of basidiospores.

After establishing the most favorable liquid culture media and optimum pH, it is also necessary to assess the effects of environmental physical factors such as temperature and illumination. Incubation at 30°C and total dark were found to be the optimum physical conditions for basidiospore germination of both *Lentinus*. The results suggest that temperature and illumination are also significant, thus, incubation at 30°C and total dark could be used as technique to stimulate germination of basidiospores of the two *Lentinus* species. Similarly, basidiospores of most crosses of *Cryptococcus gattii* showed significantly higher germination rates at 30°C than at 23°C (You and Xu, 2018). However, the present result does not agree with the reported optimal temperature of *L. tigrinus, S commune, and P. ostreatus*, which is at 23°C to 28°C (Dulay et al., 2012b; Bulseco et al., 2005; Lin, 2004). In addition, *Coprinus radatus* had significantly greater proportion of germinated basidiospores at higher temperature of 45°C than at 30-35°C (Mills and Eilers, 1973). Based on these findings and our results, it is safe to say that higher basidiomycetes have diverse germination rates as they respond to certain temperature. You and Xu (2018) explained that this diversity might be related to mitochondrial inheritance patterns that are affected by environmental factors such as temperature, which may influence spore viability and energy generation. Moreover, this is also due to variation and uniqueness of individual fungal species and strain.

Although light is an important morphogenetic factor in most macrofungi, the present study proved the inhibitory effect of light on basidiospore germination of the basidiomycetes. The same response was also observed on the vegetative mycelial growth of *L. strigosus* (Dulay and Garcia, 2017). However, present results contradict our previous observations in *L. tigrinus* (Dulay et al., 2012b). Moreover, Poyedinok et al. (2015) reported the stimulating effects of light wavelengths and coherence on basidiospore germination. The most suitable conditions were red coherent and incoherent light of 632.8 nm and 660.0 nm for *Agaricus bisporus, Ganoderma applanatum, P. ostreatus*, red and blue coherent light of 632.8 nm and 488.0 nm for *Flammulina velutipes, G. lucidum* and *Hericium erinaceus*, and both red and blue laser and light-emitting diode (LED) light for *Lentinula edodes*. In addition, spore germination of *Pleurotus sapidus* was strongly inhibited by white and blue lights, but not by red and yellow lights (McCracken, 1982). These findings clearly indicate that the response of basidiospores of several species and strains to light in terms of germination may be either stimulatory or inhibitory. Therefore, germination of basidiospores is not only necessarily dependent on the nutritional attributes and pH of the culture media but also on the combined influence of physical environmental factors such as temperature and illumination.

Since the present work demonstrated the successful germination of basidiospores of *L. swartzii* and *L. strigosus* in their most suitable liquid culture medium and optimum physical conditions, it was therefore necessary to document the germination process and morphogenesis to fully understand their developmental biology, which is very vital in the generation of efficient biomass production technologies. The vegetative and reproductive phases of both mushrooms were studied. The vegetative phases of both mushrooms occurred in four significant stages: swelling and clumping, elongation, septation, and branching. It is interesting to note that the two *Lentinus* had different period of appearance of the above-mentioned stages. The germination process was found faster in *L. strigosus* than in *L. swartzii*. Fries (1984) reported that the period and rate of germination vary depending on species and strain type, among different basidiocarps, and even among different parts of the hymenium. Basidiospore is said to be germinating when a germ tube emerges from the hilum, which is common in *Volvariella volvacea* (Reyes, 1999). However, in this study, a peculiar type of germination process was observed. Liberated basidiospores of the two *Lentinus* germinated through swelling and elongation, where the basidiospore coat became part of the hypha. Clumping or grouping of germinating basidiospores was also evidently observed, which is probably a mechanism for plasmogamy or hyphal fusion. The basidiospores continuously elongated and formed septa to become hyphae, then into primary mycelia. After the course of plasmogamy (dikaryotization), primary mycelia developed into secondary mycelia. In prolonged incubation in liquid culture, massive branching of
mycelia continuously occurred until the formation of thick mycelial mat on the surface of the medium, which indicates a very promising alternative of mycelial biomass production from the basidiospores origin. To document the reproductive phase (basidiocarp formation) of life cycle, substrates in fruiting bags were inoculated and incubated to allow mycelial ramification. After 18-25 days (for L. swartzii) and 20-24 days (for L. strigosus) from the day of inoculation, the entire substrate was fully colonized by mycelia and thick mycelial coats were formed. In L. strigosus, thick mycelial coats developed into popcorn-like swellings after 2-4 days, then into primordia (fruiting initials) after the fruiting bags were opened and watered. However, in L. swartzii, popcorn-like swellings were not observed. Instead, browning and hardening appeared after 3-5 days from the mycelial coat formation and after exposure to water. Then, from brown and hardened mycelia, the primordia initiated after 5 days. In contrast, browning and hardening stage was not observed in L. stigosus. However, both popcorn stage and browning stage were documented in L. tigrinus (Dulay et al., 2012b). The primordia continuously elongated to become the stipe and formed a broad button-like tip, the young pileus. The in-rolled downward margin of young pileus expanded into mature pileus. The dark brown young pileus of L. swartzii turned into light brown, scaly, hairy mature pileus, while the purple young pileus of L. strigosus became pale, hairy, mature pileus. The lamellae or gills underneath the mature pileus produced the numerous basidiospores to be liberated and dispersed for the perpetuation of the species. The present work has shown the successful germination of basidiospores of L. swartzii and L. strigosus in liquid culture using nutrient-rich indigenous media, varying pH levels, temperature, and illumination. The processes of basidiospore germination and hypha and mycelial development of the two evaluated Lentinus spp. discussed in the preceding section are very similar to those of L. tigrinus, which, to our knowledge, is the first studied Lentinus species for basidiospore germination and morphogenesis. This peculiar germination process could be very valuable in the confirmation of identity of certain species if belonging to genus Lentinus. It is noteworthy to mention that the two Lentinus spp. have unique cultivation characteristics in the formation of basidiocarp as indicated by the nonappearance of popcorn stage in L. swartzii and browning and hardening stage in L. strigosus. This study on morphogenesis and cultivation phases established that L. strigosus is a fast-growing species.

**Conclusion**

In conclusion, this study demonstrated the effects of culture media, pH, and physical factors such temperature and illumination on the basidiospore germination of L. swartzii and L. strigosus. Liquid culture using coconut water and potato broth with a pH of 5 to 7 at 30°C and dark condition is an effective technique to activate and stimulate germination of the basidiospores. It has also been established that the vegetative phase occurs in four significant stages while the reproductive phase has six distinct stages in both mushrooms. Popcorn stage and browning stage appeared individually but not concurrently in each life cycle. The obtained data provide basic information on the developmental biology of the two Lentinus species, which is highly utilizable in the development of practical, innovative, and effective technologies for the management of cultures, commercial production of mushroom biomass, and hybridization and selection of strains. For consistency of the germination rate and process, it is worthy to further investigate the influence of different nutrient sources in order to design a chemically defined medium. Elucidation of chemical compositions and biological activities of basidiospores, germinating basidiospores, basidiospore-derived mycelia, and spent liquid culture medium is of scientific and practical interest.

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Contribution of Authors

Dulay RMR: Contributed in conceptualization of the study, data collection, literature review search, experimental design and data interpretation and manuscript writing
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