Investigating the Effect of Tissue Size on Mycelial Growth of Seven Mushroom Species by Using a Novel Device for Precise Tissue Isolation

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Abstract Tissue isolation from mushrooms is frequently practiced by both researchers and growers to isolate new and improved strains. In the present study, we designed a simple and convenient device for precise tissue isolation and therefore investigated the effect of tissue size on mycelial growth of seven mushroom species. The developed device consists of a cutting needle and a transfer needle. The cutting needle was used to obtain circular tissue plugs having a height up to 3 mm and variable diameters (2–5 mm) from mushroom fruit bodies. The transfer needle was a stainless steel round rod (1.5 mm in diameter) with a blade-like end. It can be used for collecting mushroom tissue when the cutting needle fails to extract it. With the aid of these devices, precise tissue isolation was achieved. Plate cultures demonstrated that tissue size had little effect on mycelium extension for *Lentinula edodes* (the winter shiitake), *Hypsizygus marmoreus*, and *Agrocybe aegerita*, but influenced the aerobic mycelium density. For *Pleurotus ostreatus*, *Pleurotus eryngii*, and *Volvariella volvacea*, large tissue plugs produced faster mycelial growth and higher aerobic mycelium density compared with small ones. On the contrary, small plugs from the tissue of the flower shiitake and *Agaricus bisporus* favored mycelial growth. The present study revealed that the preferable tissue size for mycelial growth varies among mushroom species, and the developed device is expected to greatly facilitate the isolation of new and improved mushroom strains.

Keywords Edible mushroom · Tissue isolation · Mycelial growth · Mycelial colony · Aerobic mycelium density

Mushrooms have been utilized by humans as edible and medical provisions for millennia. Chemical studies have demonstrated that mushrooms contain high levels of various nutrients such as proteins, carbohydrates, essential amino acids, vitamins, and minerals [1], and have therefore been considered as an excellent food source. Many mushroom species such as *Lentinula edodes*, *Ganoderma lucidum*, and *Trametes versicolor* are known to demonstrate multiple biological activities, exhibiting anticancer, immunomodulatory, antidiabetic, and hepatoprotective properties [2]. Recently, a variety of pharmaceutically active compounds such as polysaccharides and triterpenoids have been isolated from mushrooms [3]. Owing to their high nutritional and medicinal value, there is a growing consumer demand for mushrooms worldwide.

Currently, a huge amount of lignocellulosic waste is generated annually worldwide from the agricultural, forest, and food industries, requiring appropriate disposal methods. Although multiple forms of physical, chemical, and biological strategies for treating this waste have been proposed and attempted [4], mushroom cultivation is still considered the only economically viable option for efficient waste utilization [5, 6]. Given that mushroom cultivation not only provides humans with foods and medicinal materials but can also reduce the deleterious impact of industrial waste products on the environment, it has been welcomed by governments and farmers, and is now prospering in China, Japan, South Korea, and Thailand, as well as in many African countries.

Mushroom researchers and cultivators commonly isolate mushroom strains using three methods: tissue isolation,

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spore isolation, and substrate mycelium isolation. Tissue isolation involves isolating a small portion of the inner tissue of fruit bodies for re-growth to obtain strains of interest, and has been employed for the isolation of the basidiomycetes, *G. lucidum* [7], *Tricholoma matsutake* [8], and *Pleurotus* spp. [9]. Spore isolation is mainly used to obtain single-spore cultures, which can be employed as units for hybridization breeding of mushroom species, including the successful breeding of improved *Pleurotus ostreatus* and *L. edodes* strains [10, 11]. Single-spore cultures can also be used for mating system analysis of mushrooms [12, 13]. As for substrate mycelium isolation, strains can be obtained from a substrate in which mushrooms have been grown previously [14]. Of the three methods, tissue isolation is practiced most frequently because of the low contamination rate, high reliability, and ease of manipulation.

The cultivation techniques, physiology, and breeding of mushrooms have been extensively investigated. With regards to tissue isolation, Asghar et al. [15] revealed that the portion that joins the cap and stipe of *Pleurotus sajor-caju* gave excellent mycelial growth. Zhang et al. [16] reported that desirable parts of the fruit bodies of *L. edodes*, *Pleurotus sapidus*, and *Flammulina velutipes* had different effects on mycelial growth. The effect of the size of tissue plugs of *L. edodes* on mycelial growth was also demonstrated [17]. Nevertheless, the optimal tissue size for the tissue isolation of most mushroom species remains unclear. Moreover, there is no specific tool for tissue isolation currently available, making it an extremely inconvenient and difficult process. In the present study, a simple and convenient device was designed and manufactured for precise tissue isolation. As shown in Fig. 1, the developed device consisted of two separate parts: a 4-piece cutting needle (Fig. 1a) and a transfer needle (Fig. 1b). The cutting needle was made of a round hollow stainless steel rod with an outer diameter ranging from 2–5 mm. A cutting hole was specially created at one end of the needle, which was used for tissue isolation. The transfer needle was made from a solid stainless steel round rod (12 cm in length) with a blade-like end. It was used in some cases to completely separate the cut tissue from the original.

To test the suitability of the developed device for mushroom tissue isolation and to determine the effect of tissue size on mycelial growth, fresh, healthy, and mature fruiting bodies of *L. edodes* (including both the winter shiitake and flower shiitake), *Agaricus bisporus*, *P. ostreatus*, *Pleurotus eryngii*, *Volvariella volvacea*, *Hypsizygus marmoreus*, and *Agrocybe aegerita* were used as test mushroom species. They were thoroughly washed with sterile water, wiped clean with sterile gauze to remove any dirt and damaged external tissue, and swabbed with 75 % ethanol to remove any surface contaminant. Under sterile

Fig. 1 Schematic diagram of the device developed for mushroom tissue isolation. **a** Cutting needle: 1, hollow round stainless steel rod; 2, circular cap; 3, spring; 4, solid round stainless steel rod; 5, circular cap; 6, cutting hole; **b** Transfer needle: 1, solid, round, stainless steel rod; 2, blade-like end

conditions, these washed and disinfected fruiting bodies were torn open by hand along the center, from the bottom of the stalk to the pileus. The surface of the open inner tissue from the two halves was kept facing upwards.

For the isolation of mushroom tissue, a sterile cutting needle with variable diameters was directly placed on the surface of the open inner tissue and driven down carefully about 3 mm deep, using a reference line in the device that indicates the depth. The needle was then carefully removed. In most cases, cut tissues remained in the cutting hole, so that they could be released by simply pressing the cap of the movable needle, and were then seeded on Petri dishes containing potato dextrose agar (PDA) medium. In some cases, cut tissue remained on the original site and would be extracted using the transfer needle, before being placed on the surface of PDA medium. To germinate the mycelium of the isolated tissues for *L. edodes*, *P. eryngii*, *P. ostreatus*, *H. marmoreus*, and *A. aegerita*, all inoculated PDA plates were grown in an incubator at 25 °C for 7 d. Culture temperature was the same for *A. bisporus*, but the incubation time was 10 d due to its slow growth. As *V. volvacea* requires a higher temperature for growth and grows quickly, the inoculated PDA plates of this species were grown at 32 °C for 3 d. The diameter of each colony was measured along two perpendicular directions, and the average was calculated. Growth was calculated by

subtracting the original diameter of cut tissue from the measured value of the mycelial colony after cultivation. The density of the aerobic mycelium of a colony was assessed visually.

The appearance of the fruiting bodies of the mushroom species after tissue isolation is shown in Fig. 2, demonstrating that the device worked well for tissue isolation. During the manipulation, we found that cutting needles 2–3 mm in diameter could effectively retain the tissue sample in the cutting hole. The sample could be released directly by pushing the cap, resulting in a very convenient method of tissue isolation. By contrast, cutting needles with diameters of 4–5 mm sometimes failed to extract the tissue, which then had to be collected using the transfer needle or an inoculation hook. These observations demonstrated that the developed device facilitates mushroom tissue isolation. An additional benefit is that quantitative tissue isolation can be achieved by using cutting needles of different sizes.

The mycelial colonies of the seven mushroom species originating from different amounts of cut tissue, growing

in Petri dishes, are shown in Fig. 3. Detailed growth data are summarized in Table 1. These show that the initial tissue size of the winter shiitake, *H. marmoreus*, and *A. aegerita* did not significantly influence mycelium extension, but considerably affected aerobic mycelium density. Specifically, an initial large tissue sample produced a denser mycelium compared with a smaller tissue sample. Lin et al. [17] also found that tissue size influenced mycelium density in *L. edodes*, in which a large tissue sample resulted in more dense mycelial growth. It is worth noting that *H. marmoreus* and *A. aegerita* formed a uniform aerobic mycelium on the entire colony, whereas *L. edodes* (the winter shiitake) showed a gradual decrease in mycelium density from the center to the periphery of the mycelial colony. According to the literature [18], this difference may result from the difference in the branching type of growing hyphae among these mushroom species.

For *P. ostreatus*, *P. eryngii*, and *V. volvacea*, large initial tissues supported higher mycelium extensions than small tissue plugs. In the case of *P. ostreatus* and *P.*

Fig. 2 Photographs of fruiting bodies of representative edible mushroom species after tissue isolation using the developed device

Fig. 3 Mycelial colonies of seven mushroom species originating from differently sized tissue plugs. The number at the bottom of each picture indicates the diameter of the cutting needle employed

eryngii, a statistical difference in mycelium extension rate between 2 and 3 mm or above ($P < 0.01$) was observed, while for *V. volvacea* it occurred between tissue plugs of 2 and 4 mm or above. This implies that initial tissue size of the former had a more pronounced effect on growth rate. All three species showed a higher density of colonies in the portion of aerobic mycelium closer to the inoculated tissue. We reason that, these three species may have a similar hyphal branching type. In contrast to the strains investigated above, *A. bisporus* produced better mycelial growth from small tissue samples than from larger ones. A significant difference ($P < 0.01$) in mycelial extension rate was observed between 2 and 5 mm tissue samples. In addition, the

aerobic mycelium from smaller tissue appeared to be whiter compared with that from large tissue plugs. Regarding the flower shiitake, small initial tissue (2 mm) yielded vigorous mycelial growth, while growth from larger tissue plugs was poor and considerably delayed. This growth pattern differed markedly from that of the winter shiitake. The differences in moisture content, maturity, and physiological status between the two mushrooms may be responsible for the observed growth variation. The poor growth resulting from larger tissue samples in both the flower shiitake and *A. bisporus* likely resulted from the toxicity of quinones formed during enzymatic browning [17, 19, 20], since the browning was much more pronounced on these large tissues.

Table 1 Mycelial growth originating from initial tissue plugs with varying size, isolated from seven mushroom species

Mushroom species	Isolation site	Tissue size ^a (mm)	Diameter of colony (mm)	Aerobic mycelial density (Visual)
<i>Lentinula edodes</i> (the flower shiitake)	Pileus	2	16.54 ± 2.32a	+++++
		3	15.46 ± 1.47a	++++
		4	2.14 ± 0.31b	++
		5	3.12 ± 0.26c	++
<i>Lentinula edodes</i> (the winter shiitake)	Pileus	2	27.26 ± 2.30a	++
		3	28.82 ± 1.10a	+++
		4	28.47 ± 2.67a	++++
		5	36.36 ± 2.19b	+++++
<i>Agaricus bisporus</i>	Pileus	2	9.34 ± 1.21a	+++++
		3	9.10 ± 0.84a	++++
		4	8.72 ± 0.41a	++++
		5	8.01 ± 0.54b	++++
<i>Pleurotus eryngii</i>	Stalk	2	29.46 ± 2.18a	+++
		3	32.14 ± 4.06b	++++
		4	43.56 ± 2.13c	+++++
		5	53.10 ± 3.19d	+++++
<i>Pleurotus ostreatus</i>	Pileus	2	46.20 ± 4.31a	+++
		3	52.26 ± 6.45b	+++
		4	61.11 ± 4.23c	++++
		5	69.27 ± 3.74d	+++++
<i>Hypsizigus marmoreus</i>	Stalk	2	26.32 ± 1.20a	+++
		3	26.45 ± 1.34a	++++
		4	25.64 ± 0.78a	+++++
		5	24.67 ± 2.12b	+++++
<i>Agrocybe aegerita</i>	Stalk	2	16.54 ± 0.47a	+++
		3	16.20 ± 0.36a	++++
		4	16.42 ± 0.54a	+++++
<i>Volvariella volvacea</i>	Pileus	2	67.34 ± 0.87a	+++
		3	71.26 ± 1.72a	+++
		4	82.34 ± 1.34b	++++
		5	83.64 ± 2.47b	++++

Each tissue isolation was performed ten times in triplicates, and data were analyzed using SAS software (version 8.1). The results were expressed as the mean ± standard deviation (SD). Significant differences between groups were determined by Student's t test

Values of each of the strains in the same column are not significantly different ($P > 0.01$) if followed by the same letter

^a Tissue size indicates the diameter of cutting needle employed; ++ to ++++ - Very thin to highly dense mycelial growth

In conclusion, a simple and convenient device that exhibited excellent suitability for precise mushroom tissue isolation was present in this work. The device is expected to greatly facilitate the isolation of new and improved mushroom strains. By employing this device we proved that the mycelial growth varied significantly between mushroom species, with a clear difference in preferable size for tissue isolation. These data are valuable since they can be used as a reference for mushroom tissue isolation in the future.

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