Article

Cultivation of Edible Tropical Boletus, *Phlebopus spongiosus*, in Thailand and Yield Improvement by High-Voltage Pulsed Stimulation

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Abstract: Tropical boletus, *Phlebopus spongiosus*, is an edible ectomycorrhizal mushroom indigenous to northern Thailand. This mushroom has the ability to produce fruiting bodies without the need for a host plant. In this study, the technological cultivation of *P. spongiosus* was developed. Cultivation experiments indicated that fungal mycelia could completely colonize the cultivation substrate over a period of 85–90 days following inoculation of liquid inoculum. Primordia were induced under lower temperatures, high humidity and a 12-h photoperiod. Mature fruiting bodies were developed from young fruiting bodies within a period of one week. Consequently, yield improvement of *P. spongiosus* cultivation was determined by high-voltage pulsed stimulation. The results indicated that the highest degree of primordial formation, number of mature fruiting bodies and total weight values were obtained in cultivation experiments involving a high voltage of 40 kV. The total weight of the mushrooms increased by 1.4 times after applying high-voltage pulses when compared with the control. Additionally, the results revealed that the size of the fruiting body and the proximate composition of the fruiting bodies from high-voltage stimulation treatments were not different from the control. This research provides valuable information concerning successful cultivation techniques and yield improvement by high-voltage pulsed stimulation for the large-scale commercial fruiting body production of *P. spongiosus*.

Keywords: boletus; cultivation technique; edible mushroom; high-voltage pulsed stimulation; yield improvement

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1. Introduction

Edible ectomycorrhizal (ECM) mushrooms have long been consumed as a functional food but have also been used for medicinal purposes [1–4]. These mushrooms have been defined as a seasonal and highly priced crop for which the production yield is based on the climatic conditions of each year. A significant increase in the consumer demand of edible ECM mushrooms has led to their elevated value on the global market [1,5]. *Botetis edulis* (king boletes), *Cantharellus* spp. (chanterelles), *Morchella* spp. (morels), *Tricholoma matsutake* (matsutake) and *Tuber* spp. (truffles) are known to be the most expensive edible ECM mushrooms in the world [2,5–7]. The requirements involving essential nutrients, host plant associations and the particular conditions for fruiting body production are the primary problems associated with the successful cultivation of ECM mushrooms. Additionally, some of these mushrooms may rely on a specific species of host plant [2,6–9]. Cultivation of effective fruiting body production for some ECM mushrooms (chanterelles, king boletes, matsutake and truffles) have been established with these host plants [1,2,6]. However, only a few ECM mushroom species have the ability to produce fruiting bodies without

the need for a host plant when cultivated in a suitable substrate under specific growth conditions [10–13].

The tropical bolete known as *Phlebopus spongiosus* is an edible ECM mushroom that is indigenous to northern Thailand and southern Vietnam [14–17]. This bolete is a highly priced crop (5 to 10 USD/kg) in northern Thailand. *Phlebopus spongiosus* produces fruiting bodies in natural habitats during the months of May to July (the end of the hot season to the onset of the early rainy season) and sometimes in October (the end of the rainy season) of each year [14]. Thus, this mushroom can typically be collected for consumption from natural habitats during one or two specific periods of time each year. It produces large fruiting bodies and a texture that is similar to the king bolete (*Boletus edulis*), which is also a popular edible marketable bolete in Europe. In nature, certain fruit trees namely, *Citrus maxima* (pomelo), *Dimocarpus longan* (longan) and *Mangifera indica* (mango), are known to form an ectomycorrhizal association with this mushroom [14,16,17]. Most *Phlebopus* species are considered delicacies with potentially high commercial value in many countries in Asia and South America [2,4,14,16–20]. The *Phlebopus* species can be isolated and cultured in artificial media; thus, their potential to produce fruiting bodies without a host plant is reliant upon their saprophytic lifestyle [17,21–23]. To date, only *P. portentosus* have been successfully produced on a commercial scale without host plants in China [19]. Recently, our previous study has demonstrated that *P. spongiosus* is a second *Phlebopus* species that it has the potential to produce mature fruiting bodies without a host plant in greenhouses [14]. However, the relevant mushroom cultivation technology for high-production yields would still require improvement. Several previous studies have demonstrated that the suitable cultivation method and substrate, as well as the appropriate nutritional supplementation, electric stimulation, and application of different wavelengths of light could improve yield of various mushrooms, e.g., *Agaricus* spp. (button mushrooms), *Flammulina velutipes* (golden needle mushroom), *Pholiota nameko* (nameko), *Pleurotus* spp. (oyster mushrooms) and *Lentinula edodes* (shitake) [24–28]. Therefore, this study developed effective cultivation techniques and protocols of *P. spongiosus* in the absence of host plants. Moreover, the electrical stimulation delivered by a high-voltage pulsed power generator was applied to modify the cultivation techniques for improved yields of this mushroom. The results of this study can provide valuable information for the commercial cultivation of this mushroom in the absence of a host plant. Moreover, our findings could further enhance relevant strategies for yield improvement of this mushroom using high-voltage pulsed stimulation.

2. Materials and Methods

2.1. Fungal Strains

A pure culture of *P. spongiosus* strain SDBR-CMU0517 [14] was selected and used in this study. This fungus was obtained from the Culture Collection of the Sustainable Development of Biological Resources Laboratory (SDBR), Department of Biology, Faculty of Science, Chiang Mai University, Thailand. Fungal culture was maintained in 20% glycerol at −20 °C. From this stock culture, a new culture was reactivated on L-MMN agar [29] and incubated at 30 °C for two weeks in the dark.

2.2. Fungal Inoculum Preparation

Liquid inoculum was used in this study. Fungal inoculum was conducted in 500-mL Erlenmeyer flasks containing 200 mL of L-MMN liquid medium (pH 5.0) [29]. Ten fungal mycelial plugs (5 mm in diameter) obtained from a colony growing on L-MMN agar at 30 °C for two weeks were transferred into Erlenmeyer flasks after being autoclaved at 121 °C for 15 min. Cultivation was performed on an orbital shaker with shaking at 120 rpm at 30 °C in the dark. After three weeks of incubation, liquid culture was used as an inoculum (Figure 1A).

Figure 1. Mycelium liquid inoculum of *P. spongiosus* SDBR-CMU0517 (A) and mycelia grow on substrate after 60 days (B) and 90 days (C) at 28 ± 2 °C in the dark after inoculation of liquid inoculum. Scale bar A = 1 cm, B and C = 5 cm.

2.3. Substrate Preparation and Fungal Inoculation

Bag cultivation was employed in this study. The sawdust of a rubber tree (*Hevea brasiliensis*) mixed with rice seed (*Oryza sativa*) at a ratio of 2:1 (*w/w*) was used as a cultivation substrate. Subsequently, 0.5% calcium carbonate (CaCO_3), 0.25% epsom salts (MgSO_4) and 0.05% potassium dihydrogen orthophosphate (KH_2PO_4) were added to the substrate mixture on a dry weight basis. The moisture content of the substrate mixture was adjusted with water at 70 ± 2 % on a wet basis. After adjusting for moisture content, 800 g of the substrate mixture was deposited in polypropylene bags (16.50 cm wide and 31.75 cm long). Bags were then plugged with polyvinyl chloride pipe rings and sealed with cotton. Bags were sterilized by being autoclaved at 121 °C for 1 h and allowed to cool at room temperature (28 ± 2 °C) for 24 h before mycelial inoculation.

Fifteen milliliters of liquid inoculum were added to each sterilized bag containing the cultivation substrate by aseptic inoculation. The inoculated bags were then incubated at 28 ± 2 °C in the dark. The fungal mycelia completely covered the substrate after 85–90 days of incubation (Figure 1B,C). Bags were subsequently transferred into the cropping room for fruiting body production.

2.4. Fruiting Body Production

Bags were incubated in a cropping room at 25 ± 2 °C and a 12 h photoperiod by fluorescent lamp (light intensity of 1500–2000 lux). The relative humidity was set at 80% using a humidifier (CAREL HumiDisk 65, Turin, Italy), while a table fan allowed for cross-ventilation. The room was disinfected with 10% formaldehyde prior to initiating the experiment. Bags were cased with 300 g of sterilized soil (pH 6.5–6.8, saturated with water) at approximately 4–5 cm height and placed on an aluminum shelf. The relevant periods of time for both primordial formation and fruiting body formation were observed. The average numbers of primordia of each bag were counted and recorded. Mature fruiting bodies were harvested and immediately weighed. The size of the mature fruiting bodies was also measured.

2.5. Yield Improvement by High-Voltage Pulsed Stimulation

2.5.1. Pulsed Power Generator

A high-voltage pulsed power generator (Figure 2A) was used in this study according to the method described by Takahashi et al. [25] with a Cockcroft-Walton circuit (GM100; Green techno, Kanagawa, Japan). The pulsed power generator was employed to generate high voltage DC from AC 100 V plug power.

Figure 2. A high-voltage pulsed power generator (A) and the application of high-voltage pulses on the mushroom bag (B).

2.5.2. High-Voltage Pulsed Stimulation

Bags that had the substrate completely covered with fungal mycelia were used in this experiment. The application of high-voltage pulsed stimulation on mushroom production in this study was accomplished according to the procedure described by Takahashi et al. [25] with some modifications. Briefly, each mushroom bag was placed in a laminar cabinet. An aluminum needle that was 3 mm in diameter was driven into the mushroom bag to a depth of 8 cm. The aluminum needle was connected to an earth lead power supply as is shown in Figure 2B. This experiment was divided into a total of six treatments, as indicated in Table 1. Five treatments were stimulated by pulsed voltage at 10 s with different amounts of high-voltage pulses. The remaining treatment was considered a control. After application of high-voltage pulses, bags were cased with soil and placed on a shelf in the cropping room under the conditions described above. The numbers of primordia and mature fruiting bodies of each bag were counted and recorded. The weight and size of the mature fruiting bodies were measured. The average numbers of primordia and mature fruiting bodies per bag, and the average weight of each fresh fruiting body per bag, were then calculated. Subsequently, the total weight of the fresh fruiting bodies in each treatment was calculated. Thirty replications were carried out in each treatment with two independent runs.

Table 1. Treatment details of this study.

Treatment Name	Treatment Details
0 kV	Control (non-high-voltage pulsed application)
20 kV	Application of 20 kV high-voltage pulse with 10 s
40 kV	Application of 40 kV high-voltage pulse with 10 s
60 kV	Application of 60 kV high-voltage pulse with 10 s
80 kV	Application of 80 kV high-voltage pulse with 10 s
100 kV	Application of 100 kV high-voltage pulse with 10 s

2.6. Proximate Composition Analysis

The proximate composition (ash, carbohydrate, fat, fiber, and protein) of the mushroom samples from each treatment was investigated according to the method established by the Association of Official Analytical Chemists (AOAC) [30] at the Central Laboratory

(Chiang Mai, Thailand) Company Limited (Chiang Mai, Thailand). Five replications were performed for each treatment.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) was applied to compare mean values using the statistical program SPSS program version 23.0 for Microsoft Windows 10. Significant differences between treatments were indicated by Tukey's test at the $p \leq 0.05$ level.

3. Results and Discussion

3.1. Fruiting Body Production and Yield Improvement by High-Voltage Pulsed Stimulation

After 85–90 days of incubation, fungal mycelia completely covered the substrate, and bags were transferred to the cropping room (Figure 3). Use of the casing method, in combination with conditions of low temperature and high humidity, were necessary to induce the primordia formation of *P. spongiosus* [14]. Similarly, previous studies have reported that primordia formation of *Agaricus bisporus* [31], *A. subufescens* [32], *P. portentosus* [13,22,33,34] and *Stropharia rugosoannulata* [35] required a casing step. Under specified conditions in the cropping room, yellow to yellow-brown primordia formed after 10–15 days of incubation and subsequently developed to young fruiting bodies (Figure 4A–C). Young fruiting bodies then completely developed into mature fruiting bodies within one week (Figure 4D,E). However, only one or two young fruiting bodies developed to a fully mature stage. From each bag, we obtained one or two mature fruiting bodies. This outcome was similar to the findings of previous studies, which reported that the number of mature fruiting bodies obtained from the cultivation of *P. portentosus* under the absence of host plant conditions ranged from one to two fruiting bodies per bag [13,33,34]. In our study, caps of mature fruiting bodies were 6.5–10.5 cm in diameter, and the stipes were 6.00–9.50 cm high and 3.00–4.50 cm wide. The weight of each mature fruiting body was observed to be within the range of 30.28 to 60.45 g.

Figure 3. Cultivation of *P. spongiosus* in the cropping room.

Figure 4. Fruiting body production of *P. spongiosus* in cropping room. Primordial formation after 10–15 days of incubation (A,B). Primordia develop to young fruiting bodies (C). Young fruiting bodies develop to mature fruiting bodies within one week (D,E). Scale bar = 1 cm.

In this study, five different voltage values of 20, 40, 60, 80 and 100 kV with 10 s for each voltage amount were administered to improve *P. spongiosus* cultivation. The results indicated that the application of high-voltage pulses did effectively influence the cultivation of this mushroom. It was found that the average number of primordial formations of each bag increased when high-voltage pulse values of 20 and 40 kV were applied, but decreased after values of 60, 80 and 100 kV were applied. Remarkably, the application of 40 kV was the optimum amount of voltage that resulted in the highest average number of mature fruiting bodies per bag, highest average weight of each fresh fruiting body per bag and the highest total fresh weight of the fruiting bodies (Table 2). Interestingly, the total fresh weight of the mushrooms increased by 1.4 times after the application of 40 kV. It was found that the application of high-voltage pulses had no effect on the size of the fruiting body when compared with the control (Table 3). Our results were similar to those of previous studies which had reported that the application of high-voltage pulses could improve mushroom cultivation yields [25,26,36]. However, the optimal value of stimulated voltage was dependent upon mushroom species and time. For example, Takaki et al. [25] found that applying a voltage of 50 kV 5 times and 300 kV 500 times could improve the cultivation yields of shiitake mushrooms (*L. edodes*) via log cultivation and the cultivation yields of a fried chicken mushrooms (*Lyophyllum decastes*) via bed cultivation, respectively. The highest yield of nameko (*Pholiota nameko*) and chestnut (*Hypholoma sublateritium*) mushrooms by log cultivation was observed when a high-voltage pulse at 100 kV was applied a single time [37]. Additionally, Norarat et al. [38] found that the application of high-voltage pulse values of 20, 30, 40 and 50 kV for 10 s could improve cultivation yields of shiitake mushrooms by bag cultivation, while the production yields at each voltage value were not found to be significantly different. However, these cultivation yields were significantly higher than for the control (non-voltage stimulation). Furthermore, Isalam and Ohga [39] found that the application of high-voltage pulses on field plots in pine forests could increase the quality and quantity of matsutake (*T. matsutake*) mushroom production.

Table 2. Cultivation of *P. spongiosus* and high-voltage pulsed stimulation.

Treatment Name	Parameter *			
	Average Number of Primordia per Bag	Average Number of Mature Fruiting Bodies per Bag	Average Weight of Each Fresh Fruiting Body per Bag (g)	Total Weight of Fresh Fruiting Bodies (g)
0 kV	17.13 ± 2.89 c	1.28 ± 0.41 b	38.98 ± 6.71 b	1364.24
20 kV	21.87 ± 2.93 ab	1.27 ± 0.52 ab	41.73 ± 7.28 ab	1585.75
40 kV	24.50 ± 3.02 a	1.47 ± 0.63 a	44.14 ± 6.58 a	1898.21
60 kV	18.63 ± 2.29 b	1.17 ± 0.38 b	39.57 ± 6.39 b	1385.09
80 kV	16.53 ± 1.80 d	1.10 ± 0.40 b	38.62 ± 6.39 b	1274.30
100 kV	15.50 ± 2.11 d	1.06 ± 0.25 b	38.78 ± 5.29 b	1241.05

* The results are mean ± standard deviation. Different letters in the same column are considered significantly different according to Tukey's test ($p \leq 0.05$).

Table 3. Fruiting body size of *P. spongiosus* resulting from the cultivation process and high-voltage pulsed stimulation.

Treatment Name	Size of Cultivated Fruiting Body *		
	Cap Diameter (cm)	Stipe	
		Height (cm)	Width (cm)
0 kV	8.07 ± 2.43 a	7.89 ± 1.19 a	3.63 ± 0.42 a
20 kV	8.06 ± 2.27 a	7.75 ± 1.09 a	3.64 ± 0.54 a
40 kV	8.11 ± 2.44 a	7.83 ± 1.23 a	3.66 ± 0.61 a
60 kV	8.00 ± 2.57 a	7.73 ± 1.18 a	3.70 ± 0.56 a
80 kV	7.96 ± 2.64 a	7.79 ± 1.68 a	3.66 ± 0.52 a
100 kV	8.03 ± 2.42 a	7.74 ± 1.05 a	3.67 ± 0.41 a

* The results are mean ± standard deviation. Different letters in the same column are considered significantly different according to Tukey's test ($p \leq 0.05$).

The mechanisms of high-voltage pulsed stimulation that drive increases in fruiting body formation are not yet fully understood. However, two possible mechanisms have been explained in some previous studies [40–44]. One is that the mushroom mycelia were ruptured by the application of high-voltage pulses, and that physical damage to the mycelia could stimulate fruiting body formation [37,41–43]. Another viable explanation would involve the act of enzyme activation. Some enzymes are activated by applying a high-voltage pulse, and consequently, mushroom fruiting bodies may develop abundantly [40,43,44].

3.2. Proximate Composition Analysis

The amounts of ash, carbohydrate, protein, fat, and fiber of the fruiting bodies obtained from each treatment are shown in Table 4. The results indicate that applications of high-voltage pulses did not cause significant changes in the ash, carbohydrate, protein, fat, and fiber contents when compared with the control. Thus, the application of high-voltage pulsed stimulation had no effect on the proximate composition of the obtained fruiting bodies. Importantly, this is the first report on the nutrition value of the fruiting body of *P. spongiosus* after cultivation in the absence of host plant conditions and application of high-voltage pulse. Additionally, the amounts of the proximate composition in the cultivated fruiting body were compared with those of the fruiting body collected from natural habitats. It was found that protein and fiber contents observed in the fruiting bodies obtained from the cultivation process were significantly lower than in the fruiting bodies collected from the natural habitat (Table 4). Carbohydrate content in cultivated mushrooms was significantly higher than in natural mushrooms. This could be explained by the fact that *P. spongiosus* grown in natural habitats is known to be an ECM fungus that receives carbon and nitrogen from plants and soil, respectively. However, *P. spongiosus* cultivated with use of the bag process has been identified as a saprotrophic-like fungus. The protein and carbohydrate content in the fruiting bodies of *P. spongiosus* grown in

natural habitats may be linked to the high amounts of available nitrogen (ammonium, nitrate and organic nitrogen) obtained from sources in the soil and low amounts of carbon received from plant sources when compared with the cultivation substrate. This would likely have occurred because the cultivation substrate may have contained low amounts of nitrogen and high amounts of carbon. These results are supported by the findings of previous studies, which had reported that the amount and type of available nitrogen and carbon minerals in the growing substrate, and the ability to take up nitrogen and carbon minerals of the mushrooms, were significantly affected in terms of the nutrient composition of the mushrooms, especially in terms of the protein and carbohydrate contents [35,45–47]. Moreover, Kumla et al. [48] found that cultivated *P. portentosus* possessed lower protein and higher carbohydrate contents than *P. portentosus* collected from natural habitats.

Table 4. Proximate composition on a dry basis (% dry weight) of different mushroom samples.

Sources of Fruiting Bodies		% Dry Weight *				
		Ash	Protein	Fat	Fiber	Carbohydrate
Natural sample		9.59 ± 0.30 a	28.03 ± 0.93 a	2.15 ± 0.09 b	6.67 ± 0.38 a	50.86 ± 1.19 b
Cultivated sample	0 kV	9.55 ± 0.22 a	18.80 ± 0.53 b	2.69 ± 0.10 a	5.88 ± 0.15 b	57.21 ± 0.97 a
	20 kV	9.52 ± 0.20 a	18.76 ± 0.47 b	2.66 ± 0.13 a	5.92 ± 0.30 b	56.86 ± 0.33 a
	40 kV	9.53 ± 0.35 a	18.58 ± 0.54 b	2.67 ± 0.09 a	5.87 ± 0.13 b	56.96 ± 0.89 a
	60 kV	9.50 ± 0.13 a	18.25 ± 0.51 b	2.63 ± 0.15 a	5.84 ± 0.17 b	57.14 ± 0.73 a
	80 kV	9.48 ± 0.36 a	18.40 ± 0.54 b	2.61 ± 0.12 a	5.89 ± 0.14 b	56.73 ± 0.56 a
	100 kV	9.53 ± 0.18 a	18.60 ± 0.49 b	2.70 ± 0.10 a	5.90 ± 0.10 b	56.76 ± 0.65 a

* The results are mean ± standard deviation. Different letters in the same column are considered significantly different according to Tukey's test ($p \leq 0.05$).

Additionally, the amounts of ash, carbohydrate, protein, fat, and fiber in cultivated fruiting bodies obtained from control and application of high-voltage pulse treatments were within the ranges described in previous reports. Accordingly, ash (6.7–50.29%), carbohydrate (33.3–65.1%), protein (14.0–36.3%), fat (0.4–21.94%), and fiber (3.1–38.11%) contents were found in several edible ECM mushrooms obtained from natural habitats and in several other cultivated mushrooms [4,45,48–53]. Therefore, the *P. spongiosus* obtained from our cultivation process can be considered one of the cultivated mushrooms that would be highly suitable for the human diet.

4. Conclusions

Phlebopus spongiosus is a well-known edible ECM mushroom with high commercial value. In this study, the technological cultivation of *P. spongiosus* without a host plant was successfully achieved. Notably, it was determined that this mushroom is the second species within the genus *Phlebopus* that could be successfully cultivated on a commercial scale without a host plant. Accordingly, the application of high-voltage pulsed stimulation could improve the cultivation of this mushroom. An optimal voltage of 40 kV was confirmed for efficient fruiting body induction and yield. Moreover, high-voltage pulsed stimulation had no effect on the proximate composition of the obtained fruiting bodies. The outcomes of this study will provide researchers with valuable information with regard to the large-scale industrial production of this mushroom and cultivation yield improvement through high-voltage pulsed stimulation. However, a suitable nutrient composition of the substrate would be required in the future to achieve high nutritional content in the fruiting bodies and certain advantageous biological properties (antioxidant, antimicrobial and anticancer activities) of those cultivated fruiting bodies.

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