MYCOBIOLOGY
2020, VOL. 48, NO. 4, 263–275
https://doi.org/10.1080/12298093.2020.1784592

RESEARCH ARTICLE

OPEN ACCESS Check for updates

A New Report on Edible Tropical Bolete, *Phlebopus spongiosus* in Thailand and Its Fruiting Body Formation without the Need for a Host Plant

Jaturong Kumla[a,b], Nakarin Suwannarach[a,b] and Saisamorn Lumyong[a,b,c]

[a]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand; [b]Research Center of Microbial Diversity and Sustainable Utilization, Chiang Mai University, Chiang Mai, Thailand; [c]Academy of Science, The Royal Society of Thailand, Bangkok, Thailand

ABSTRACT

Phlebopus spongiosus is a well-known edible ectomycorrhizal mushroom indigenous to southern Vietnam. The mushroom specimens collected from northern Thailand in this study were identified as *P. spongiosus*. This identification was based on morphological characteristics and the multi-gene phylogenetic analyses. Pure cultures were isolated and the relevant suitable mycelial growth conditions were investigated. The results indicated that the fungal mycelia grew well on L-modified Melin-Norkans, and Murashige and Skoog agar all of which were adjusted to a pH of 5.0 at 30 °C. Sclerotia-like structures were observed on cultures. The ability of this mushroom to produce fruiting bodies in the absence of a host plant was determined by employing a bag cultivation method. Fungal mycelia completely covered the cultivation substrate after 90–95 days following inoculation of mushroom spawn. Under the mushroom house conditions, the highest amount of primordial formation was observed after 10–15 days at a casing with soil:vermiculite (1:1, v/v). The primordia developed into a mature stage within one week. Moreover, identification of the cultivated fruiting bodies was confirmed by both morphological and molecular methods. This is the first record of *P. spongiosus* found in Thailand and its ability to form fruiting bodies without a host plant.

ARTICLE HISTORY
Received 6 April 2020
Revised 13 June 2020
Accepted 15 June 2020

KEYWORDS
Ectomycorrhizal fungi; edible bolete; fruting body production; mycelial growth condition

1. Introduction

The genus *Phlebopus* was identified by Singer [1] with *P. colossus* being noted as the type species. *Phlebopus* belongs to the family Boletinellaceae of the order Boletales [2,3]. It is characterized by fruiting bodies that are of the boletoid type, producing an olivaceous brown spore print with ellipsoidal smooth olivaceous brown basidiospores and abundant clamp connections on the hyphae of the fruiting body [4,5]. *Phlebopus* is distributed in tropical and subtropical areas of South America, Africa, Asia, and Australia [6–12]. At present, *Phlebopus* is comprised of 17 epithets in the Index Fungorum (http://www.indexfungorum.org/Names/Names.asp). Eight species (*P. braunii*, *P. bruchii*, *P. colossus*, *P. cystidiosus*, *P. harleyi*, *P. latiporus*, *P. silvaticus,* and *P. sudanicus*) are known to be from Africa; four species (*P. marginatus*, *P. spongiosus*, *P. viperinus,* and *P. xanthopus*) are known to be from Asia and Australasia; three species (*P. brasiliensis*, *P. maxicanus,* and *P. tropicus*) are known to be from the neotropics; *P. poretentosus* is known to be from Asia, Africa and neotropics, and the last species; *P. beniensis*, is commonly found in Africa and the neotropics [6,9–17]. Generally, *Phlebopus* species are found around naturally growing or planted plants in natural habitats where mycorrhizae are expected to be found [10,18–21]. Notably, the successful cultivation of ectomycorrhization of *P. portentosus*, *P. spongiosus,* and *P. sudanicus* with seedlings *in vitro* has been reported [8,16,22,23]. However, *P. tropicus* and *P. portentosus* revealed the presence of a tripartite association with both a parasitic aphid (*Pseudococcus comstocki*) and a mealybug (*Paraputo banzigeri*), respectively by forming a special insect gall (crust) on the plant's roots identified through observations in the field [4,8,24,25]. The *Phlebopus* species can be easily isolated in culture; thus, it has the potential to produce fruiting bodies *in vitro* via its saprophytic lifestyle [8,26–28]. To date, *P. portentosus* is the only species in the genus *Phlebopus* that can be successfully cultivated without a host plant [27,29].

A tropical bolete known as *P. spongiosus*, is an endemic edible mushroom that is indigenous to the southern part of Vietnam. Notably, it is priced higher than most other wild edible mushroom

CONTACT Saisamorn Lumyong saisamorn.l@cmu.ac.th, scboi009@gmail.com

Supplemental data for this article can be accessed here.

species [10,28]. It has been reported that this fungus forms a mutualistic relationship with the roots of the pomelo plant (*Citrus maxima*) [10,23]. In Thailand, only four species of *Phlebopus* (*P. braunii*, *P. colossus*, *P. portentosus,* and *P. silvaticus*) have been identified [8,30]. During an investigation of macrofungi in northern Thailand, we found a number of specimens that corresponded to the description of *P. spongiosus* [10] that had not been previously reported in Thailand. In this study, we have described and illustrated the morphological characteristics of this Thai fungus and have provided the results of the molecular phylogenetic analyses. Suitable culture conditions for the mycelial growth of this fungus were investigated. Moreover, the suitable solid media composition and casing type for the formation of the fruiting body without a host plant were determined.

2. Materials and methods

2.1. Sample collection

Fruiting bodies of mushroom were collected in Chiang Mai and Lampung Provinces, northern Thailand during 2018–2019. Fruiting bodies were kept in plastic specimen boxes to be transported to the laboratory. Notes on the macromorphological features and photographs were obtained within 24 h of collection. The specimens were dried at 40–45 °C and deposited at the Herbarium of the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University (SDBR-CMU), Thailand.

2.2. Pure culture isolation

The mycelia were isolated from fresh fruiting bodies by aseptically removing a small piece of mycelium from inside and transferring it to modified Melin-Norkans (MMN) agar (0.05 g $CaCl_2$, 0.025 g NaCl, 0.15 g $MgSO_4{\cdot}7H_2O$, 0.012 g $FeCl_3{\cdot}6H_2O$, 0.001 g thiamin HCl, 10.0 g malt extract, 0.25 g $NH_4H_2PO_4$, 0.5 g KH_2PO_4, 10.0 g glucose, and 15.0 g agar per liter of distilled water, pH 6.0). The isolation plates were incubated at 30 °C in the dark. The mycelia emerging from tissue were picked and transferred to MMN agar. The pure cultures were kept on MMN slants at 4 °C for further use and in 20% glycerol at −20 °C for long-term preservation.

2.3. Morphological studies

Macromorphological data were recorded from fresh specimens. The color names and codes of basidiocaps were followed Kornerup and Wanscher [31]. Micromorphological data were recorded from dry specimens rehydrated in 95% ethanol followed by distilled water, 3% KOH or Melzer's reagent. Anatomical features were based on at least 50 measurements of each structure as seen under a light microscope (Olympus CX51, Tokyo, Japan). For spore statistics, Q is the ratio of spore length divided by spore width.

2.4. Molecular phylogenetic studies

Genomic DNA of fresh specimens and pure mycelia (1–5 mg) was extracted using a Genomic DNA Extraction Mini-Kit (FAVORGEN, Ping-Tung, Taiwan). The LSU of rDNA gene were amplified with LROR and LRO5 primers [32], *tef1* gene was amplified with the EF1-983F and EF1-1567R primers [33], and *rbp2* gene was amplified with the bRBP2-6F and bRBR2-7.1R primers [34]. The amplification program for these three domains was performed in separated PCR reaction and consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s; annealing at 52 °C for 45 s (LSU), 56 °C for 1 min (*tef1*), or 54 °C for 1 min (*rbp2*); and extension at 72 °C for 1 min on a peqSTAR thermal cycler (PEQLAB Ltd., Fareham, UK). PCR products were checked on 1% agarose gels stained with ethidium bromide under UV light. PCR products were purified using a PCR clean up Gel Extraction NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced. Sequencing reactions were performed, and the sequences were automatically determined in the genetic analyzer at 1ST Base company (Kembangan, Malaysia) using the PCR primers mentioned above. Sequences were used to query GenBank *via* BLAST (http://blast.ddbj.nig.ac.jp/top-e.html).

For phylogenetic analyses, the sequences from this study, previous studies, and the GenBank database were used and provided in Supplementary Table S1. The multiple sequence alignment was carried out using MUSCLE [35]. Phylogenetic tree was constructed using maximum likelihood (ML) and Bayesian inference (BI) algorithms, implemented by RAxML v7.0.3 [36] and MrBayes v3.2.6 [37], respectively. The best-fit substitution model for BI and ML analyses were estimated by jModeltest 2.1.10 [38] using Akaike information criterion (AIC). For ML analysis, the bootstrap (BS) replicates were set as 1000 and used to test phylogeny [39]. Clades with bootstrap values (BS) of ≥70% were considered significantly supported [40]. For the BI analysis, the Markov chains were run for one million generations, with six chains and random

starting trees. The chains were sampled every 100 generations. Among these, the first 2,000 trees were discarded as burn-in, while the postburn-in trees were used to construct the 50% majority-rule consensus phylogram with calculated Bayesian posterior probabilities. Bayesian posterior probabilities (PP) $\geq$ 0.95 were considered significant support [41].

2.5. Mycelial growth tests

Three strains of *P. spongiosus* SDBR-CMU0517, SDBR-CMU0518 and SDBR-CMU0519 were used in this study. Twenty-five milliliters of culture medium for each experiment were poured into Petri-dishes (9 cm in diameter) after autoclaving for 15 min at 121 °C. A sterilized cellophane disk (9 cm in diameter) was placed on the surface of the tested culture media. A mycelial plug (5 mm in diameter) was obtained from two-week-old culture at 30 °C on MMN agar and transferred to the tested media. After three weeks of inoculation, the colony diameter was measured, and growth rate was calculated. The mycelial dry weight was obtained after the cellophane disks were dried at 60 °C overnight and maintained in desiccators for 30 min. Each treatment was performed in five replicates.

2.5.1. Effects of different culture media

The following nine different culture media were tested for the mycelial growth of *P. spongiosus*; asparagines (AS) agar [42], fungal-host (FH) agar [43], Heli agar [42], Gamborg agar [44], MMN agar [45], L-MMN agar [45], Murashige and Skoog (MS) agar [46], malt extract agar (MEA; Difco, Franklin Lakes, NJ, USA) and potato dextrose agar (PDA; Condalab, Madrid, Spain). All tested media were adjusted to pH of 6.0 with 1 N HCl or 1 N NaOH before autoclaving. The inoculated plates were incubated at 30 °C in darkness for three weeks. The medium that presented the highest mycelial growth rate and biomass yield was selected for further experiments.

2.5.2. Effect of temperature

Pure cultures of *P. spongiosus* were inculcated in the selected culture medium that had been obtained from previous experiments. The culture media was adjusted to pH of 6.0 before autoclaving. The inoculated plates were incubated in darkness at 10, 15, 20, 25, 30, 35 and 40 °C. The mycelial growth rate and dry weights were measured after three weeks of inoculation. The temperature that presented both highest mycelial growth rate and biomass yield was selected for further experiments.

2.5.3. Effect of initial pH value of culture medium

Pure cultures were inoculated on selected culture medium with initial pH values ranging from 4.0–10.0 before autoclaving. The mycelial growth rate and dry weights were measured after three weeks of incubation in the darkness at the suitable temperature for mycelial growth that was obtained from the previous experiment.

2.6. Determination of the suitable solid substrate for cultivation

Twelve different solid substrates were used in this experiment (Table 1). Corn grain (*Zea mays*), rice seed (*Oryza sativa*), sawdust of rubber tree (*Hevea brasiliensis*), rice straw, and sorghum grain (*Sorghum bicolor*) were used as basal substrates. The grains were prepared by boiling 10–20 min. All tested substrate was mixed with 0.05% KH_2PO_4 and 0.15% $MgSO_4$ on dry weight basis. The moisture content was adjusted to 65% (w/w) on a wet basis. The solid substrate was placed into test tubes (18 mm in diameter × 180 mm long) approximately 10 cm deep and autoclaved at 121 °C for 30 min [47]. After cooling, mycelial plug (5 mm in diameter) from the periphery of the growing colony on L-MMN agar was transferred to tubes and incubated at 30 °C in darkness. Linear mycelial growth was measured, and the growth rate was determined after three weeks. The suitable solid substrate for mycelial

Table 1. Mycelial growth of three strains of *Phlebopus spongiosus* in test tubes.

	Mycelial growth rate (mm/day)*		
Substrate composition	SDBR-CMU0517	SDBR-CMU0518	SDBR-CMU0519
Corn grain	0.87 ± 0.04^{f}	0.77 ± 0.03^{e}	0.74 ± 0.05^{d}
Rice seed	1.29 ± 0.04^{bc}	1.23 ± 0.03^{b}	1.14 ± 0.09^{b}
Sorghum grain	1.31 ± 0.03^{ab}	1.20 ± 0.06^{c}	1.15 ± 0.06^{b}
Rice straw	0.00^{g}	0.00^{f}	0.00^{e}
Sawdust	0.00^{g}	0.00^{f}	0.00^{e}
Corn grain mixed rice straw (1:2, w/w)	0.86 ± 0.07^{ef}	0.72 ± 0.04^{e}	0.84 ± 0.08^{c}
Rice seed mixed rice straw (1:2, w/w)	1.17 ± 0.11^{b}	1.12 ± 0.07^{c}	1.13 ± 0.04^{b}
Sorghum grain mixed rice straw (1:2, w/w)	1.21 ± 0.03^{c}	1.18 ± 0.02^{c}	1.14 ± 0.02^{b}
Corn grain mixed sawdust (1:2, w/w)	0.93 ± 0.05^{e}	0.91 ± 0.04^{d}	0.90 ± 0.03^{c}
Rice seed mixed sawdust (1:2, w/w)	1.39 ± 0.07^{a}	1.36 ± 0.03^{a}	1.33 ± 0.04^{a}
Sorghum grain mixed sawdust (1:2, w/w)	1.37 ± 0.05^{a}	1.34 ± 0.07^{ab}	1.31 ± 0.02^{a}

*Results are means ± SD of five replicates. Data with different letters within the same column indicate a significant difference at $p \leq 0.05$ according to Duncan's multiple range test.

growth was selected for the further experiment. Each treatment was performed in five replications.

2.7. Cultivation for fruiting body production without host plant

2.7.1. Fungal strain

Pure culture of *P. spongiosus* SDBR-CMU0517 was selected and used in this experiment based on the fastest mycelial growth rate than other strains from the previous experiment.

2.7.2. Spawn preparation

Sorghum grain was prepared by boiling for 10–15 min and excess water was drained off. Then, $CaCO_3$, KH_2PO_4, and $MgSO_4$ (1.0% of each) were added into boiled grains and mixed thoroughly. Two hundred grams of mixed grains were filled into 350 mL glass bottles which were then plugged with cotton wool and autoclaved at 121 °C for 30 min. After cooling, three mycelial plugs (5 mm in diameter) from the periphery of the growing colony were transferred to glass bottles and incubated at 30 °C in the darkness. Two-month-old sorghum grain completely covered by fungal mycelia was used as mushroom spawn.

2.7.3. Bag cultivation

Sawdust of rubber tree mixed with rice seed (1:2, w/w) was used as main substrate for cultivation. Substrate was mixed with 0.1% $CaCO_3$, 0.05% KH_2PO_4, and 0.15% $MgSO_4$ on dry weight basis. The mixed substrate had reached a humidity of 65–70% on a wet basic with water and 1.0 kg of mixed substrate was filled in polypropylene bags (16.50 cm wide and 31.75 cm long). The bags were sealed using cotton plugged polyvinyl chloride pipe ring and covered by piece of paper. The bags were autoclaved at 121 °C for 60 min and were cooled for 24 h. The sterilized bags were immediately inoculated with 5 g of mushroom spawn and kept at room temperature (30 ± 2 °C) in the darkness. After 90–95 days, the fungal mycelia covered the substrate. The primordia were induced by casing with substrates on surface. The sterilized vermiculite, soil, sand, perlite, peat–vermiculite (1:1, v/v), soil–vermiculite (1:1, v/v) saturated with water were used for the surface casing. All treatments were transferred to mushroom house (25 °C and 70 ± 5% relative humidity). The number of primordia and mature fruiting body per bag was recorded. Fruiting body production and the entire process were repeated twice with fifteen replicates. Moreover, the identification of the cultivated fruiting bodies was confirmed by both morphological and molecular methods as mention above.

2.8. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using the SPSS program version 23.0 for Windows, and Duncan's multiple range test was used for significant differences ($p \leq 0.05$) between treatments.

3. Results

3.1. Morphological studies

Fruiting bodies of mushrooms SDBR-CMU0517 and SDBR-CMU0518 were collected in 2018 under a longan tree (*Dimocarpus longan*) and a mango tree (*Mangifera indica*), respectively, in Lumphun Province, whereas SDBR-CMU0519 was collected in 2019 under a Longan tree on the campus of Chiang Mai University, Chiang Mai Province. The fruiting bodies were found to be medium to large in size. Pileus were 50–120 mm broad at the first hemispherical section with an incurved margin, then becoming plano-convex to convex with a decurved margin and appearing centrally depressed. The surface was dry, and the specimens were brown to yellowish brown (5D8–5E78) in color (Figure 1(A–C)). The context were 2.0 – 5.7 cm thick and soft, pale yellow (3A3) to light yellow (3A5) in color, sometime slowly bruised blue after injury. Hymenophores were decurrent to subdecurrent at first, then more or less depressed around the stipe; tubes were up to 10 mm long and yellowish orange (5A7 – 5B8) to yellowish brown (5D7) in color. Sponge-like tissues projected from the sides of the tube walls, but these were absent in older specimens (Figure 1(D,E)). Stipes were 60–120 × 15–50 mm, central, subequal or clavate and solid with a dry surface and were yellowish brown (5E4 – 5E8) to brown (5F6 – 5F8) in color, while becoming darker where handled. The basal mycelium was brownish yellow. The spore print was dark brown to reddish brown in color (8F5 – 8F6).

Basidiospores were 8.0–9.0 × 5.5–7.5 μm, $Q = 1.30$, subglobose to shortly ellipsoid, displaying a smooth surface under a light microscope, brown, inamyloid and thick-walled (Figure 1(F)). Basidia were 20–25 × 8–10 μm (without sterigmata), clavate, bearing 4 spores, and sterigmata were 2.5–4 μm long (Figure 1(G)). Hymenophoral trama was divergent-bilateral of the boletus subtype, hyaline, with gelatinized hyphae 5–10 μm in diameter. Sponge-like tissues were composed of heteromorphous terminal cells occasionally protruding into the tube lacunae from the sides of the tube walls; displayed numerous constituent elements, 30–53 × 4.5–7.5 μm, that were broadly clavate or fusoidventricose to ventricose-rostrate, thin-walled, with a yellow intracellular pigment, converging toward the center of the tube chamber at least in young

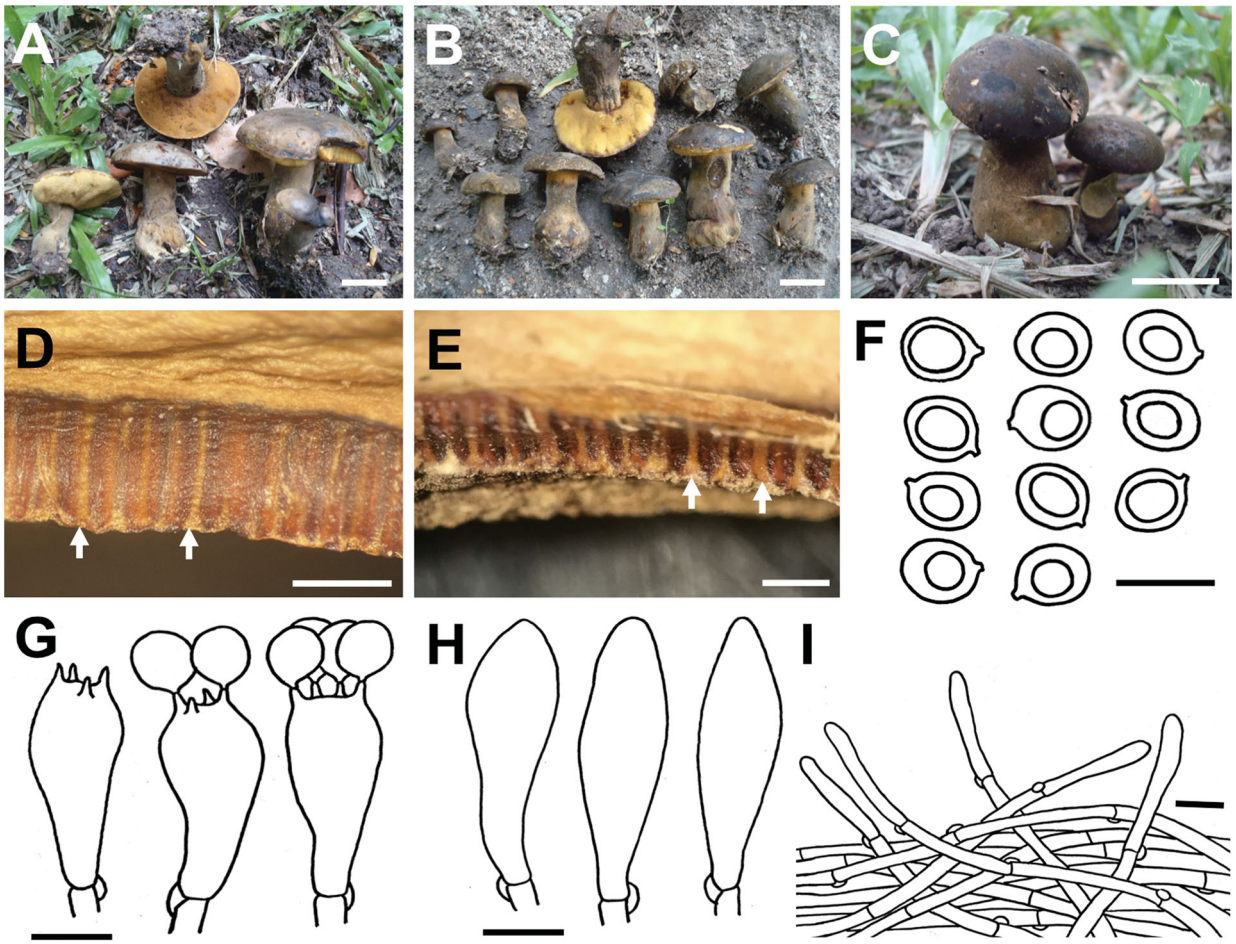

Figure 1. *Phlebopus spongiosus*. Fruiting bodies in natural habitat SDBR-CMU0517 (A), SDBR-CMU0518 (B), and SDBR-CMU0519 (C). Vertical section of the hymenophore in dried specimens of SDBR-CMU0517 (D) and SDBR-CMU0519 (E) showing the sponge-like tissue (arrows). (F) Basidiospores; (G) Basidia and basidiospores; (H) Heteromorphous terminal cells protruding into the lacunae of the tubes from the sides of the tube walls; (I) Pileipellis. Scale bars: (A–C) 50 mm; (D, E) 10 mm; (F–H) 10 μm; (I) 5 μm.

specimens but that were gradually disrupted (Figure 1(H)). Dissepiments resembling cheilocystidia, remarkably projected beyond the hymenium, 25–40 × 4–6 μm, subcylindrical to cylindrical-clavate, thin-walled, distinctly clamped at the base, with a light-yellow intracellular pigment and a membranous thin layer forming on the immature pore surface but then gradually collapsed. Pileipellis consisted of repent, appressed, interwoven hyphal elements; terminal cells were 20–30 × 3–5 μm, cylindrical, thin-walled, with yellow intracellular pigment, with a golden yellow to brownish color in the wall mass (Figure 1(I)). Stipe trama was composed of longitudinally arranged, cylindrical hyphae, 2–4.5 μm in diameter, and were thin-walled and hyaline to brownish. Clamp connections were present in all tissue types. Both macro- and micromorphological characteristics of the samples agreed well with the recorded morphological taxonomic concept of *P. spongiosus* [10].

3.2. Molecular phylogenetic studies

The combined LSU, *tef1,* and *rbp2* sequence dataset consisted of 49 taxa and the aligned dataset was comprised of 2317 characters including gaps (LSU: 1–936, *tef1*: 937–1528, and *rbp2*: 1529–2317). The GTR model with gamma rate heterogeneity and invariant sites (GTR+I+G) was the best-fit model used for both ML and BI analyses that were selected by AIC. The average standard deviation of the split frequencies fell to 0.017859 in the BI analysis of the combined sequences following one million generations. This was observed after a 50% majority consensus phylogram was constructed. The ML analysis of the combined LSU, *tef1* and *rbp2* sequences, was based on the parameters estimated by the GTR + I+G model, and the proportion of the invariable sites and the gamma shape parameters were 0.497 and 0.896, respectively. Additionally, the tree with a log likelihood (−9165.289) was built after 1000 bootstrap replications.

The phylogram of the combined LSU, *tef1,* and *rbp2* sequences is shown in Figure 2. Four main clades of the families Boletaceae, Bolentinellaceae, Gyroporaceae, and Paxillaceae were assigned according the findings of previous phylogenetic studies [2,48–50]. The results indicated that Thai specimens

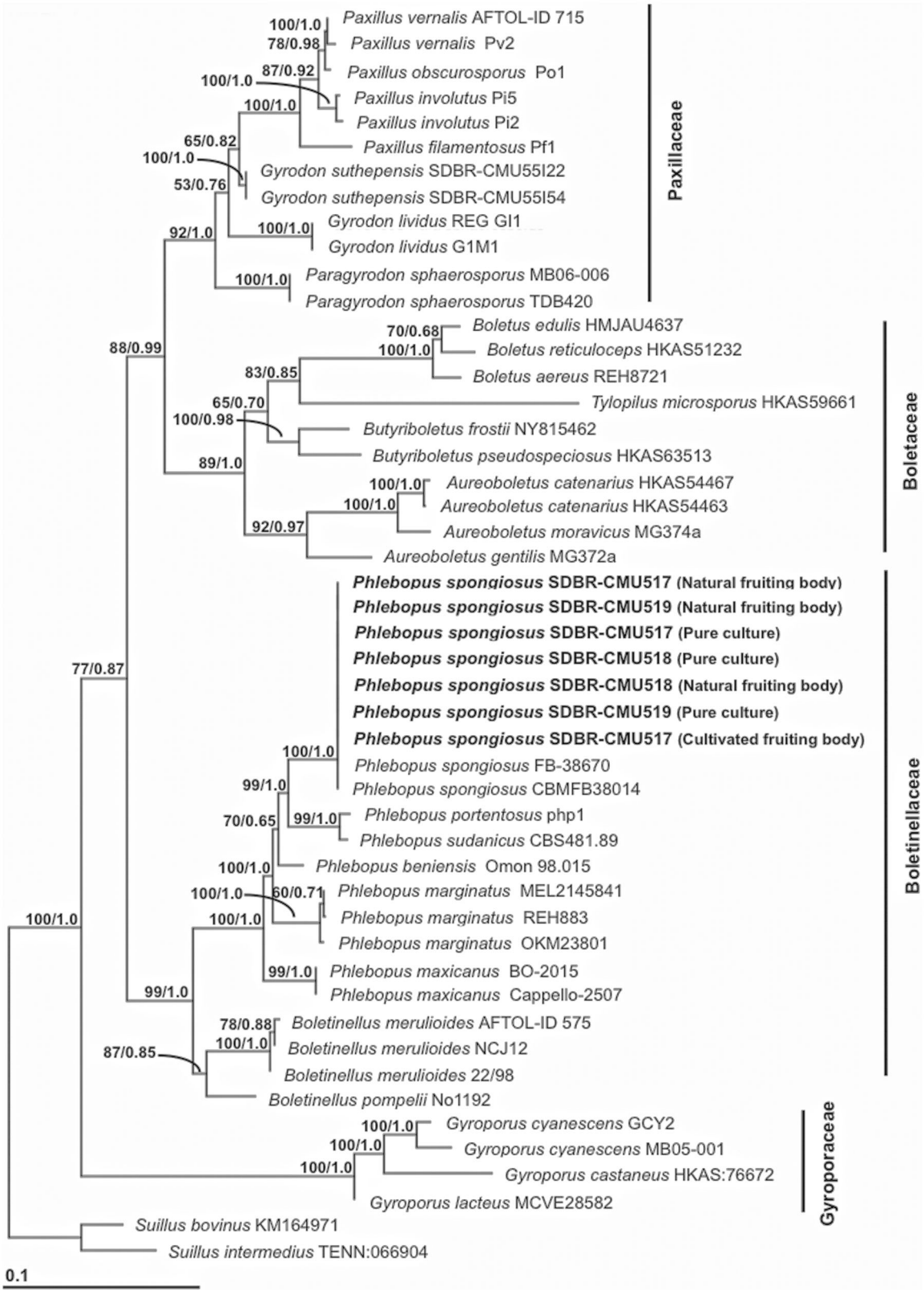

Figure 2. Phylogenetic tree derived from maximum likelihood analysis of combined LSU, *tef1*, and *rpb2* genes of 49 sequences. *Sullius bovinus* and *S. intermedius* were used as outgroup. Numbers above branches are the bootstrap statistics percentages (left) and Bayesian posterior probabilities (right). Branches with bootstrap values ≥50% are shown at each branch and the bar represent 0.1 substitutions per nucleotide position. The fungal isolates from this study are in bold.

placed in the monophyletic clade of *P. spongiosus* were within the family Bolentinellaceae with high BS (100%) and BP (1.0) supporting values. It was found that *P. spongiosus* formed a sister taxon to *P. portentosus* and *P. sudanicus.*

3.3. Mycelial growth tests

3.3.1. Effects of different culture media

After three weeks of incubation, the mycelial growth rate of all fungal strains was significantly affected by

the cultivation media. The highest growth rate value was observed on L-MMN agar. However, the statistical analysis indicated that the growth rate values on L-MMN and MS agar were not significantly different (Figure 3(A)). The growth rate value was found to be the lowest on the AS agar. L-MMN and MS agar exhibited high values in terms of biomass yield (Figure 3(B)). The yellow-brown to dark-brown sclerotia-like structures (1–6 mm in diameter) with yellow to brown exudates were observed on FH, L-MMN, MEA, GAM, MMN, PDA and MS agar (Figure 3(G)).

3.3.2. Effect of temperature

The results showed that all three fungal strains grew at tested temperatures ranging from 10 – 35 °C. The statistical analysis of the data revealed that 30 °C was the best temperature for mycelial growth through observations of both the highest mycelial growth rate and the highest biomass yield (Figure 3(C,D)). However, all strains did not grow at 40 °C.

3.3.3. Effect of initial pH value of culture medium

The effects of pH variations on *P. spongiosus* mycelia growth are shown in Figure 3(E,F). It was found that all fungal strains had the ability to grow at pH values in a range of 4.0 – 10.0. A pH of 5.0 was the optimum initial pH value of the medium at which all isolates produced the highest mycelial growth rate and biomass yield.

3.4. Determination of the suitable solid substrate for cultivation

Values of the linear mycelial growth of *P. spongiosus* SDBR-CMU0517, SDBR-CMU0518, and SDBR-CMU0519 on various solid substrates are shown in Table 1. It was found that all fungal strains did not grow on rice straw and sawdust. The highest growth rate value of all fungal strains was observed on rice seed mixed with sawdust (1:2, w/w). However, the statistical analysis indicated that growth rate values of fungal strains on rice seed mixed with sawdust (1:2, w/w) and sorghum grain mixed with sawdust (1:2, w/w) were not significantly different. Thus, rice seed mixed with sawdust (1:2, w/w) was chosen as a suitable solid substrate for bag cultivation based on its availability to facilitate the thickest degree of mycelial growth by visual assessment.

3.5. Cultivation for fruiting body production without host plant

The fungal mycelia covered the substrate after 90–95 days of cultivation. The yellow brown primordia of *P. spongiosus* SDBR-CMU0517 were found after 10–15 days of incubation in the mushroom house (Figure 4(A,B)). The significantly highest degree of primordial production was observed in the bags containing the soil : vermiculite (1:1, v/v) treatment, followed by treatments of peat : vermiculite (1:1, v/v), soil and vermiculites, respectively (Table 2). Notably, primordia were not formed in the non-casing (control) treatment. The primordia were developed to mature fruiting bodies within one week (Figure 4(C)). The highest number of total mature fruiting bodies was found in the bags containing the soil : vermiculite (1:1, v/v) treatment (Table 2). After harvesting mature fruiting bodies, identification was primarily confirmed using morphological characteristics and molecular data. The pileus of the cultivated fruiting bodies was found to be 4.0–8.5 cm in diameter, yellowish brown to brown in color, convex to subconvex and centrally depressed when mature. Sponge-like tissues were also observed. Stipes were 3.0–7.0 × 2.5–5.0 cm, clavate or tapered to the apex, and concolorous with the pileus. Basidia were 20–25 × 8–10 μm (without sterigmata), clavate, bearing 4 spores, while sterigmata were 2.5–4 μm long. Spores were deposit dark brown and deposited in mass. Clamp connections were also presented. These characteristicss agreed well with the morphological taxonomic concept of *P. spongiosus*. In addition, the molecular phylogeny indicated that the cultivated fruiting bodies were clustered with sequences of *P. spongiosus* (Figure 2).

4. Discussion

The three mushroom specimens collected in northern Thailand were boletoid fruit bodies with dark brown to reddish brown spore prints. They all had brown subglobose to shortly ellipsoid basidiospores with abundant clamp connections on the hyphae of the fruiting body. Thus, these morphological characteristics support the placement of this fungus into the genus *Phlebopus* [4,5]. All specimens were initially identified as *P. spongiosus* after consulting the descriptions presented by Pham et al. [10]. Morphologically, *P. spongiosus* is easily distinguishable from other *Phlebopus* species by its sponge-like tissue that is composed of numerous fusoid-ventricose to ventricose-rostrate pleurocystidioid elements along the subcylindrical to subclavate dissepiments and by its dark brown spore prints [10]. However, *P. portentosus* clearly differs from *P. spongiosus* by its olivaceous brown spore print and the absence of hymenial cystidia [5,6,51]. Our phylogenetic analysis has confirmed that all specimens in this study were identified as *P. spongiosus* within the family Boletinellaceae of the order Boletales, which was in

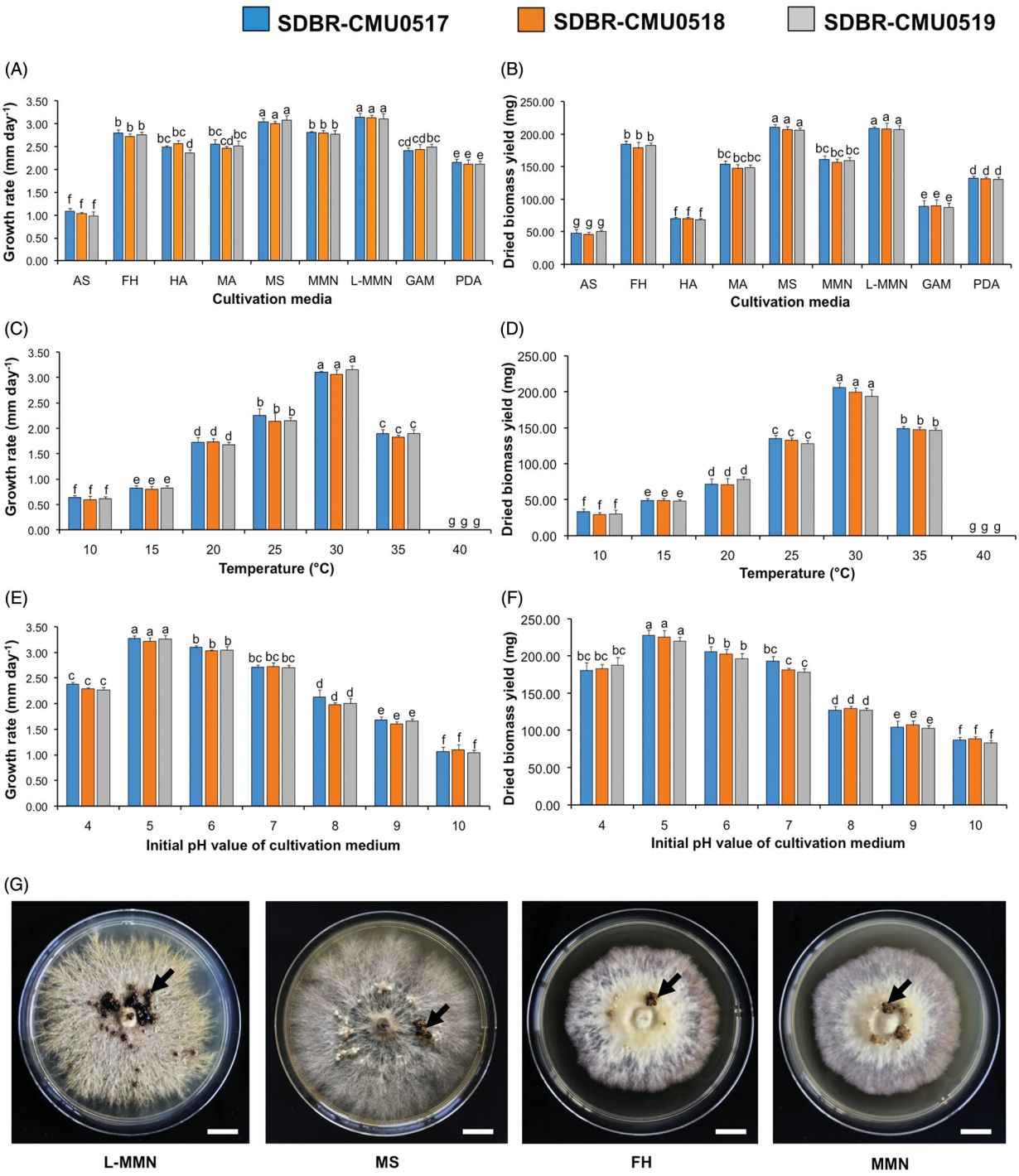

Figure 3. Mycelial growth of *Phlebopus spongiosus* SDBR-CMU0517, SDBR-CMU0518 and SDBR-CMU0519. Effect of cultivation media on mycelial growth rate (A) and biomass yield (B). Effect of temperature on mycelial growth rate (C) and biomass yield (D). Effect of initial pH value of cultivation medium on mycelial growth rate (E) and biomass yield (F). Sclerotia-like structures on cultures of SDBR-CMU0517 (G). AS: asparagines (AS) agar; FH: fungal-host agar; HA: Heli agar; MA: malt extract agar; MS: Murashige and Skoog agar; MMN: modified Melin-Norkans agar; L-MMN: L-MMN agar; GAM: Gamborg agar; PDA: potato dextrose agar. Scale bar = 10 mm. Data are presented as means and the error bar at each graph indicated the ± standard deviation. The different letter of each graph for the same factors indicates a significant difference ($p \leq 0.05$).

accordance with the findings of previous taxonomic studies [2,3,28,48].

The host range of ectomycorrhizal fungi has been determined in the past by plant-sporocarp association in the field and by inoculation of potential hosts under experimental conditions. Many previous studies have described the host range of ectomycorrhizal fungi as possessing a spectrum to a narrow range (typically genus restricted), an intermediate range (often restricted to a plant family) and a broad range (typically extending across diverse plant families and orders), all of which depended upon the fungal species and natural conditions that were present [3,52–55]. In terms of a

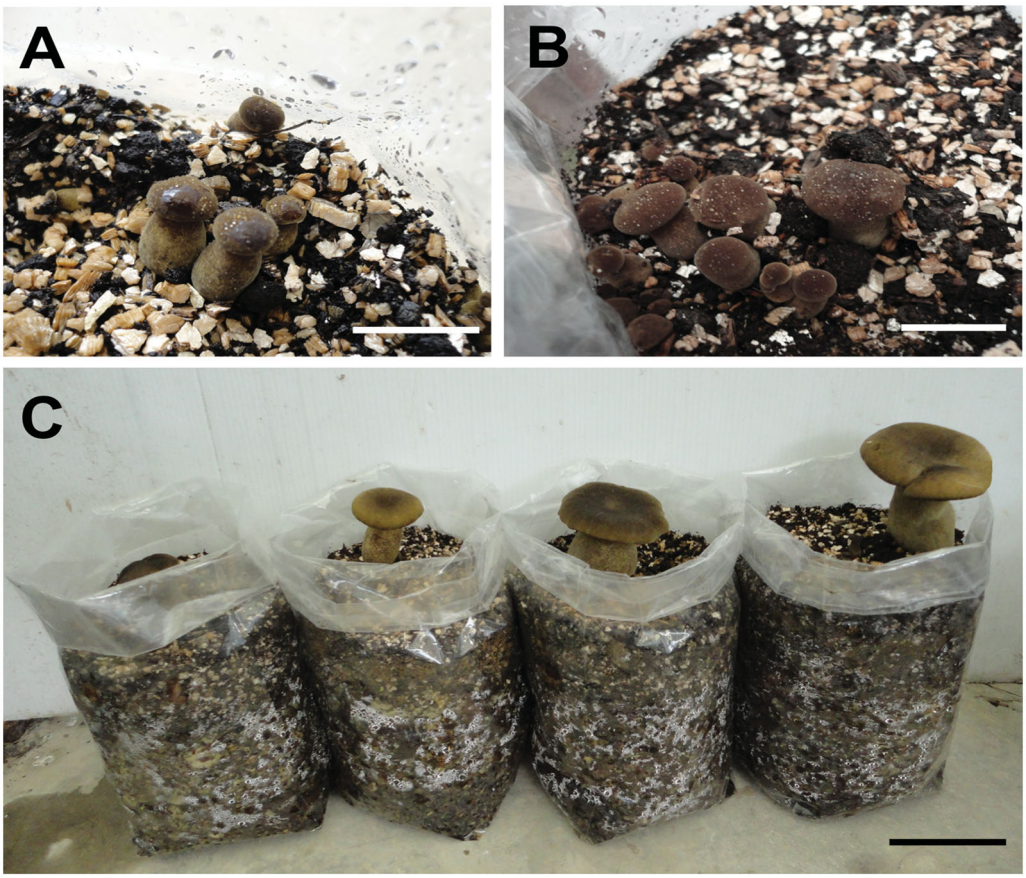

Figure 4. Cultivation of *Phlebopus spongiosus* SDBR-CMU0517 in absence of a host plant. Primordia (A and B). Young and mature fruiting bodies (C). Scale bars: (A, B) 5 mm; (C) 50 mm.

Table 2. Number of primordia formation of *Phlebopus spongiosus* SDBR-CMU-0517 in bag culture on different casings.

Casing	Number of primodia per bag*	Total number of mature fruiting body
Control	0.00^{f}	0
Vermiculite	4.53 ± 2.13^{d}	12
Soil	9.80 ± 2.11^{c}	10
Perlite	3.60 ± 1.40^{de}	8
Peat moss mixed vermiculite (1:1, v/v)	10.47 ± 2.45^{b}	12
Soil mixed vermiculite (1:1, v/v)	16.07 ± 3.88^{a}	14
Sand	2.07 ± 2.96^{e}	9

*Results are means ± SD of 15 replicates. Data with different letters within the same column indicate a significant difference at $p \leq 0.05$ according to Duncan's multiple range test.

specific example, *Boletus edulis* has a broad host range and the known host trees belong to the families Pinaceae, Taxodiaceae, Betulaceae, Fagaceae, Fabaceae, Dipterocarpaceae and Myrtaceae [21,56]. *Suillus pictus* has been known to be restricted to only the pine species of the subgenus Strobus of the genus *Pinus* [54]. *Astraeus asiaticus*, *A. odoratus* and *A. sinrindhorniae* were found to display an association only with plants in the family Dipterocapceae [21,57,58]. In this study, *P. spongiosus* was found to be growing together with longan (Family Sapindaceae) and mango (Family Anacardiaceae) trees in field observations, whereas previous studies reported that this fungus grew under pomelo trees (Family Rutaceae) in natural habitats [10] and could form ectomycoorhizal structures both *in situ* and *in vitro* in the roots of pomelo seedlings [23]. Thus, *P. spongiosus* has a broad host range consisting of fruit trees. Similarly, a closely related species, *P. portentosus*, has reportedly been found to be growing among various kinds of trees including fruit trees [8,9,16,21].

In this study, the mycelial growth of *P. spongiosus* varied in different cultivation media, for which L-MMN and MS agar exhibited both the highest degree of mycelial growth rate and biomass production. This result was supported by the findings of several previous studies which reported that the mycelial growth of ectomycorrhizal fungi in pure cultures is influenced by changes to the components of the cultivation medium. This is because the suitable cultivation medium is dependent upon the fungal species being studied [42,59,60]. Our results were similar to those of Le et al. [28] who showed that the pure cultures of the *P. spongiosus* strains BC-F0075 and BC-F0076 grew the fastest on MS agar. Moreover, MS and FH agar were determined to be a suitable medium for the mycelial growth of *P. portentosus* [8,26,61]. All strains of *P. spongiosus* in this study could form sclerotia-like structures on FH, L-MMN, MEA, GAM, MMN, PDA, and MS

agar. This result is similar to that of Le et al. [28] who found that pure cultures of *P. spongiosus* successfully formed sclerotia-like structures and prinodia on MS agar. The sclerotia-like structures were observed in pure cultures of *P. portentosus* and *P. sudanicus* [22,26,61]. In addition, the sclerotia-like structures in both the culture and those occurring in nature were also found in *Boletinellus merulioides*, a member of the family Boletinellaceae [62].

Temperature and pH value had a significant effect on the mycelial growth of *P. spongiosus*. The optimum temperature and pH value used to produce the highest mycelial growth and biomass production of this fungus were 30 °C and a pH of 5.0, respectively. These results were in accordance with the findings of previous studies which had reported that the optimal temperature for mycelial growth of most cultures of ectomycorrhizal fungi ranged from 20 to 30 °C, and that the optimal pH of the medium ranged from 4.0–7.0 depending on both the fungal species and on the fungal strain [42,60,63]. Similarly, the findings of Le et al. [28] revealed that the optimal temperature and pH value for the mycelial growth of *P. spongiosus* BC-F0075 were observed at 30 °C and pH 4.0–5.0, respectively. Moreover, the optimal temperature and pH value of the medium of *P. portentosus* were investigated. It was revealed that a temperature of 30 °C and pH values in a range of 4.0–5.0 resulted in the highest degree of mycelia growth and biomass production [8,26,61]. Suitable temperature and pH of the medium for the mycelial growth of other bolete species have been reported. Remarkably, the highest degree of mycelail growth of *B. edulis* was obtained at 28 °C and at a pH value of 5.0 [42], while a temperature of 30 °C and a pH of 7.0 exhibited the highest degree of mycelial growth of *Boletus colossus* and *Heimioporus retisporus* [64]. Notably, both *Suillus collinitus* and *S. granulatus* were characterized by faster rates of growth at 22 °C. However, *S. collinitus* grew well at a pH value of 5.8, while *S. granulatus* grew well at a pH value of 5.2 [65].

To date, edible ectomycorrhizal mushrooms are always cultivated with their host plants. However, some strains of edible ectomycorrhizal mushrooms e.g., *Boletus reticulatus* [66], *Phlebopus portentosus* [8,9,27,67,68] and *Tylopilus castaneiceps* [69] are known to form fruiting bodies without a host plant. A large amount of carbohydrates is required for the production of fruiting bodies of ectomycorrhizal fungi without the need for a host plant [47]. In this study, poor cultivation results in terms of the degree of fruiting body production of *P. spongiosus*, such as saprobic fungi, were revealed. Our study found that the mycelial of *P. spongiosus* yielded faster growth rates and produced the thickest mycelia on rice seed mixed with sawdust (1:2, w/w), which was similar to the conditions for the cultivation of *P. portentisus* in China [9]. In addition, sorghum grain and FH solution (10:1, w/v) mixed with sawdust (1:1, v/v) were found to be a suitable medium for the cultivation of *P. portentisus* in northern Thailand [27]. Casing with a layer of saturated soil mixed vermiculite (1:1, v/v) with water and incubation at lower temperatures were the conditions necessary to induce the primordia formation of *P. spongiosus;* however, primordia were not formed in the non-casing experiments. This result was similar to those of previous studies which reported that a casing layer or casing soil overlaying the substrate was a necessary step in the primordia formation of *P. portentosus* [9,27,68]. Notably, a casing layer was a crucial step in the formation of a fruiting body and could effectively increase the productivity yield of some saprobic mushrooms, e.g., *Agaricus bisporus*, *Agaricus subufescens*, *Coprinus comatus*, *Grifola frondosa* and *Stropharia rugosoannulata* [70–72]. In this study, the primordia of *P. spongiosus* were successfully developed to mature fruiting bodies. Thus, this study is the first report on the ability of *P. spongiosus* to form fruiting bodies without the need for a host plant.

5. Conclusions

In this study, a combination of the morphological and molecular characteristics has confirmed *P. spongiosus* as a newly recorded edible bolete for Thailand. Valuable information on *P. spongiosus* mycelial cultivation has been provided. Moreover, fungus is known to be able to form fruiting bodies without the need for a host plant. Importantly, this outcome can provide valuable information to researchers and relevant agencies with regard to the large-scale industrial production of this mushroom.

Acknowledgements

The authors are grateful to Mr Russell Kirk Hollis for kind help in the English correction.

Disclosure statement

The authors declare no competing or financial interests.

Funding

The authors gratefully acknowledge the financial support provided from Thailand Science Research and Innovation [RGU6280009], and partially supported by Chiang Mai University, Thailand.

ORCID

Jaturong Kumla http://orcid.org/0000-0002-3673-6541
Nakarin Suwannarach http://orcid.org/0000-0002-2653-1913
Saisamorn Lumyong http://orcid.org/0000-0002-6485-414X

References

[1] Singer R. The Agaricales in modern taxonomy. Koenigstein (Germany): Koeltz Scientific Books; 1986.
[2] Binder M, Bresinsky A. Derivation of polymorphic lineage of gasteromycetes from boletoid ancestors. Mycologia. 2002;94:85–98.
[3] Wilson AW, Binder M, Hibbett DS. Diversity and evolution of ectomycorrhizal host associations in the Sclerodermatineae (Boletales, Basidiomycota). New Phytol. 2012;194:1079–1095.
[4] Singer R, Araujo I, Ivory MH. The ectotrophically mycorrhizal fungi of the neotropical lowlands, especially central Amazonia. Beih Nova Hedwig. 1983;77:1–352.
[5] Watling R, Li TH. Australian boletes: a preliminary survey. Edinburgh (UK): Royal Botanic Garden; 1999.
[6] Heinemann P, Rammeloo J. Observations sur le genre *Phlebopus* (Boletineae). Mycotaxon. 1982;15:384–404.
[7] Li TH, Watling R. New taxa and combinations of Australian boletes. Edinburgh J Bot. 1999;56:143–148.
[8] Sanmee R, Lumyong R, Dell B, et al. *In vitro* cultivation and fruit body formation of the black bolete, *Phlebopus portentosus*, a popular edible ectomycorrhizal fungus in Thailand. Mycoscience. 2010;51:15–22.
[9] Ji K-P, Cao Y, Zhang C-X, et al. Cultivation of *Phlebopus portentosus* in southern China. Mycol Progress. 2011;10:293–300.
[10] Pham NDH, Takahashi H, Fukiharu T, et al. *Phlebopus spongiosus* sp. nov. (Boletales, Boletinellaceae) with a sponge-like tissue. Mycotaxon. 2012a;119:27–34.
[11] Baroni TJ, Cifuentes J, Santana BO, et al. A new species of *Phlebopus* (Boletales, Basidiomycota) from Mexico. North Amer Fungi. 2010;10:1–13.
[12] Pegler DN. A preliminary agaric flora of East Africa. Kew Bulletin Additional Series VI. Edinburgh (UK): Royal Botanic Garden Kew; 1977.
[13] Pegler DN. Agaric flora of Sri Lanka. Kew Bulletin Additional Series XII. Edinburgh (UK): Royal Botanic Garden Kew; 1986.
[14] Halling RE. A synopsis of Colombian boletes. Mycotaxon. 1989;34:93–113.
[15] Palacio M, Gutiérrez Y, Franco-Molano AE, et al. Nuevos registros de macrohongos (Basidiomycota) para Colombia procedentes de unbosque seco tropical. Actualidades Biológicas. 2014;37:319–339.
[16] Kumla J, Hobbie EA, Suwannarach N, et al. The ectomycorrhizal status of a tropical black bolete, *Phlebopus portentosus*, assessed using mycorrhizal synthesis and isotopic analysis. Mycorrhiza. 2016;26:333–343.
[17] Calaça FJS, Magnago AC, Alvarenga RLM, et al. *Phlebopus beniensis* (Boletinellaceae, Boletales) in the Brazilian Cerrado biome. Rodriguésia. 2018;69:939–944.
[18] Bougher NL, et al. Diversity of ectomycorrhizal fungi associated with eucalypts in Australia. In: Brundrett MC, Dell B, Malajczuk N, editors. Mycorrhizal for plantation forestry in Asia. Canberra (Australia): Australian Centre for International Agricultural Research; 1995. p. 8–15.
[19] Watling R. The relationships and possible distributional patterns of boletes in south-east Asia. Mycol Res. 2001;105:1440–1448.
[20] Watling R. The sclerodermatoid fungi. Mycoscience. 2006;47:18–24.
[21] Mortimer PE, Karunarathna SC, Li Q, et al. Prized edible Asian mushrooms: ecology, conservation and sustainability. Fungal Divers. 2012;56:31–47.
[22] Thoen D, Ducousso M. Mycorrhizal habit and sclerogenesis of *Phlebopus sudanicus* (Gyrodontaceae) in Senegal. Agric Ecosyst Environ. 1990;28:519–523.
[23] Pham NDH, Yamada A, Shimizu K, et al. A sheathing mycorrhiza between the tropical bolete *Phlebopus spongiosus* and *Citrus maxima*. Mycoscience. 2012b;53:347–353.
[24] Williams DJ. Mealybugs of southern Asia. Kuala Lumpur (Malaysia): The Natural History Museum, London, and Southdene; 2004.
[25] Zhang CX, He MX, Cao Y, et al. Fungus-insect gall of *Phlebopus portentosus*. Mycologia. 2015;107:12–20.
[26] Thongklang N, Hyde DK, Bussaban B, et al. Culture condition, inoculum production and host response of a wild mushroom, *Phlebopus portentosus* strain CMUHH121-005. Maejo Int J Sci Technol. 2010;5:413–425.
[27] Kumla J, Danell E, Lumyong S. Improvement of yield for a tropical black bolete, *Phlebopus portentosus*, cultivation in northern Thailand. Mycoscience. 2015;56:114–117.
[28] Le TTN, Tran CH, Phan MH, et al. Mycelial cultivation of *Phlepobus spongiosus*, an edible ectomycorrhizal mushroom in southern Vietnam. J Sci Ho Chi Minh City Open University. 2017;7:14–21.
[29] Zhang C, He M, Liu J, et al. Brief introduction to a unique edible bolete–*Phlebopus portentosus* in southern China. J Agri Sci Tech B. 2017;7:386–394.
[30] Chandrasrikul A, Suwanarit P, Sangwanit U, et al. Mushroom (Basidiomycetes) in Thailand. Bangkok (Thailand): Office of Natural Resources and Environmental Policy and Planning; 2011.
[31] Kornerup A, Wanscher JH. Methuen Handbook of Colour. 3rd ed. London (UK): Methuen; 1978.
[32] Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol. 1990;172:4238–4246.
[33] Rehner SA, Buckley EP. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-alpha sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia. 2005;97:84–98.
[34] Matheny PB. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide

sequences (Inocybe; Agaricales). Mol Phylogenet Evol. 2005;35:1–20.
[35] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32:1792–1797.
[36] Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006; 22:2688–2690.
[37] Ronquist F, Teslenko M, Van der Mark P, et al. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–542.
[38] Darriba D, Taboada GL, Doallo R, et al. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772.
[39] Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985;39: 783–791.
[40] Hillis DM, Bull JJ. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol. 1993;42:182–192.
[41] Alfaro ME, Zoller S, Lutzoni F. Bayes or bootstrap? A simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Mol Biol Evol. 2003;20:255–266.
[42] Xu M, Zhu J, Kang H, et al. Optimum conditions for pure culture of major ectomycorrhizal fungi obtained from *Pinus sylvestris* var. *mongolica* plantations in southeastern Keerqin sandy lands. China. J Forest Res. 2008;19:113–118.
[43] Vaario L-M, Gill WM, Tanaka M, et al. Aseptic ectomycorrhizal synthesis between *Abies firma* and *Cenococcum geophilum* in artificial cuture. Mycoscience. 2000;41:395–399.
[44] Brundrett MC, Bougher N, Dell B, et al. Working with mycorrhizas in forestry and agriculture. Canberra (Australia): Australian Centre for International Agricultural Research; 1996.
[45] Langer I, Krpata D, Peintner U, et al. Media formulation influences in vitro ectomycorrhizal synthesis on the European aspen *Populus tremula L*. Mycorrhiza. 2008;18:297–307.
[46] Danell E. Cantharellus. In: Cairney JWG, Chambers SM, editors. Ectomycorrhizal fungi: key genera in profile. Berlin (Germany): Springer-Verlag; 1985. p. 253–267.
[47] Ohta A, Fujiwara N. Fruit-body production of an ectomycorrhizal fungus in genus *Boletus* in pure culture. Mycoscience. 2003;44:295–300.
[48] Binder M, Hibbett DS. Molecular systematics and biological diversification of Boletales. Mycologia. 2006;98:971–981.
[49] Wu G, Feng B, Xu J, et al. Molecular phylogenetic analyses redefine seven major clades and reveal 22 new generic clades in the fungal family *Boletaceae*. Fungal Divers. 2014;69:93–115.
[50] Wu G, Li YC, Zhu XT, et al. One hundred noteworthy boletes from China. Fungal Divers. 2016; 81:25–188.
[51] Boedijn KB. Some mycological notes. Sydowia. 1951;5:211–229.
[52] Bruns TD, Bidartondo MI, Taylor DL. Host specificity in ectomycorrhizal communities: what do the exceptions tell us? Integr Comp Biol. 2002;42: 352–359.
[53] Martin F, Díez J, Dell B, et al. Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from nuclear ribosomal DNA ITS sequences. New Phytol. 2002;153:345–357.
[54] Hirose D, Shirouzu T, Tokumasu S. Host range and potential distribution of ectomycorrhizal basidiomycete *Suillus pictus* in Japan. Fungal Ecol. 2010;3:255–260.
[55] Bahram M, Põlme S, Kõljalg U, et al. A single European aspen (*Populus tremula*) tree individual may potentially harbour dozens of *Cenococcum geophilum* ITS genotypes and hundreds of species of ectomycorrhizal fungi. FEMS Microbiol Ecol. 2011;75:313–320.
[56] Zang M. Taxonomy, mycogeography and ectomycorrhizal association with the Boletales from China. I. Family Strobilomycetaceae. Mycosystema. 1997;16:264–269.
[57] Phosri C, Martin MP, Sihanonth P, et al. Molecular study of the genus *Astraeus*. Mycol Res. 2007;111:275–286.
[58] Phosri C, Watling R, Suwannasai N, et al. A new representative of star-shaped fungi: *Astraeus sirindhorniae* sp. nov. from Thailand. PLoS One. 2014;9: e71160.
[59] Rossi MJ, Oliveira VL. Growth of the ectomycorrhizal fungus *Pisolithus microcarpus* in different nutritional conditions. Braz J Microbiol. 2011;42: 624–632.
[60] Islam F, Ohga S. Effects of media formulation on the growth and morphology of ectomycorrhizae and their association with host plant. ISRN Agronomy. 2013; 2013:1–12.
[61] Kumla J, Danell E, Bussaban B, et al. Suitable growth conditions and nutrition factors on in vitro culture of *Phlebopus portentosus* (Boletales). Chiang Mai J Sci. 2011;38:156–159.
[62] Cotter HVT, Miller O. Sclerotia of *Boletinellus meruloides* in nature. Mycologia. 1985;77:927–931.
[63] Daza A, Manjón JL, Camacho M, et al. Effect of carbon and nitrogen sources, pH and temperature on *in vitro* culture of several isolates of *Amanita caesarea* (Scop.:Fr.) Pers. Mycorrhiza. 2006;16: 133–136.
[64] Saensuk T, Suntararak S. Mycelia cultivation 4 edible mushroom from Khao Kra-Dong volcano forest park, Thailand. NU J Sci Tech. 2018;2: 38–45.
[65] Lazarević J, Stojičić D, Keča N. Effects of temperature, pH and carbon and nitrogen sources on growth of *in vitro* cultures of ectomycorrhizal isolates from *Pinus heldreichii* forest. Forest Syst. 2016;25:e048.
[66] Yamanaka K, Namba K, Tajiri A. Fruit body formation of *Boletus reticulatus* in pure culture. Mycoscience. 2000;41:189–191.
[67] Lumyong S, Sanmee R, Lumyong P. Is large scale cultivation of boletes possible? Opera Mycol. 2007; 1:34–37.
[68] Kumla J, Bussaban B, Suwannarach N, et al. Basidiome formation of an edible wild, putatively ectomycorrhizal fungus, *Phlebopus portentosus* without host plant. Mycologia. 2012;104:597–603.
[69] Kikuchi K, Matsushita N, Suzuki K. Fruit body formation of *Tylopilus castaneiceps* in pure culture. Mycoscience. 2009;50:313–316.

[70] Martos ET, Zied DC, Junqueira PPG, et al. Casing layer and effect of primordia induction in the production of *Agaricus subrufescens* mushroom. Agric Nat Resour. 2017;51:231–234.

[71] Salmones D, Gaitan-Hernandez R, Mata G. Cultivation of Mexican wild strains of *Agaricus bisporus*, the button mushroom, under different growth conditions *in vitro* and determination of their productivity. Biotechnol Agron Soc Environ. 2018;22:45–53.

[72] Carrasco J, Tello ML, Perez M, et al. Biotechnological requirements for the commercial cultivation of macrofungi: substrate and casing layer. In: Singh B, Lallawmsanga PA, editros. Biology of macrofungi. fungal biology. Cham (Switzerland): Springer; 2018. p. 159–175.