MYCELIAL CULTIVATION OF PHLEBOPUS SPONGIOSUS, AN EDIBLE ECTOMYCORRHIZAL MUSHROOM IN SOUTHERN VIETNAM

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(Received: July 06, 2017; Revised: July 23, 2017; Accepted: August 08, 2017)

ABSTRACT

Phlebopus spongiosus is an endemic edible ectomycorrhizal mushroom (EEMM) in Southern Vietnam. Strains of *Phlebopus spongiosus* – collected from Pomelo orchards, Ben Tre Province, Vietnam – were cultured on different media, temperatures, pH and carbon/nitrogen sources to define mycelial growth conditions for spawn mushroom production. *Phlebopus spongiosus* BC-F0075 grew the fastest on the modified MS medium. The optimum temperature for mycelial growth was 30°C and the optimum pH was 4 – 5. BC-F0075 grew well on the MS media containing saccharose, glucose, fructose and maltose, but did not with lactose. Additionally, $NH_4H_2PO_4$ was reported to be the best nitrogen source to the growth of BC-F0075 mycelia, whereas urea ($(NH_2)_2CO$) was not utilized as a nitrogen source.

Keywords: *Phlebopus spongiosus;* mycelial cultivation; edible ectomycorrhizal mushroom (EEMM); chlamydospore.

1. Introduction

Phlebopus spongiosus Pham & Har. Takah is a new tropical bolete in family Boletinellaceae that was recently discovered by Pham et al. (2012a). This mushroom was described to be an ectomycorrhizal species with pomelo roots (Pham et al. 2012b). Most fruit bodies have been found in pomelo orchards, thereby *P. spongious* is also named as "nấm bưởi" in Vietnamese. *P. spongiosus* is an endemic edible ectomycorrhizal mushroom (EEMM) in Southern Vietnam. EEMMs are considered delicacies, beneficial nutraceutical and medicinal value, therefore, they are sold at a relatively high price compared to other foods. These mushroom could potentially be commercially measured in US$ billions (Hall et al. 2003, Sitta and Davoli 2012). In recent years, interest was shown in many EEMMs for cultivation, such as *Tuber magnatum*, *Tricholoma matsutake*, *Boletus edulis* (Giomaro et al. 2005) and *Phlebopus portentosus* (Thongklang et al. 2010, Sanmee et al. 2010). *P. spongiosus* has a close relationship with *P. portentosus* in the genus *Phlebopus*. *P. portentosus* is also one of the most popular edible ectomycorrhizal mushrooms in northern Thailand and China, because it has a large fruiting body and prized flavor. *P. portentosus* has been successfully cultivated without a host plant (Ji et al. 2011, Kumla et al. 2014), and thus has the potential to produce fruiting bodies of EEMM in the absence of a host plant.

This study aims to define mycelial growth conditions and suitable solid media for spawn

mushroom production of *Phlebopus spongious*.

2. Materials and Methods

Isolation

Mushroom samples were collected in pomelo orchards, Ben Tre Province, Vietnam, based on *Phlebopus spongiosus* described by Pham et al. (2012a). Two samples were obtained named BC-F0075 and BC-F0076. Mycelia were isolated from the fruiting bodies and cultured on potato dextrose agar (PDA) medium (Himedia, India).

DNA analysis and phylogeny

DNA was extracted from colonies on media or from fruiting bodies for PCR amplification (Izumitsu, 2012). The set primer of LR0R and LR5 was used to amplify the LSU sequence (Vilgalys and Hester 1990). PCR products were purified and sequenced by Macrogen, Inc. (Korea). The sequences were analyzed using ATGC ver 7.0.1 (Genetyx, Japan). Phylogenetic relationships based on the LSU sequence (Pham et al. 2012a) were analyzed using the Neighbor joining method in MEGA ver 6.0 with a Bootstrap analysis involving 1000 replication rounds (Tamura et al. 2013).

Effect of culture media

Seven different solid media were used: Melin Norkans medium (MMN; Marx 1969), modified Murashige and Skoog medium (MS; Straatsma et al. 1986), Fungus-host medium (FH; Vaario 2000), Ohta medium (Ohta 1990), malt yeast extract agar (MYA 2%; Merck, Germany), potato dextrose agar (PDA; Himedia, India) and modified potato dextrose agar (PDA*; Ji et al. 2011).

For standardized tests, 20 ml of media were dispensed into 90-mm-diameter petri dish. Plates were inoculated with a 5-mm-diameter plug of mycelium from the periphery of the growing colony on PDA after 25-days. Cultures were incubated in darkness at 30 ± 0.5°C. The experiment was done in triplicate. Colony diameters at 30 days after inoculation were measured.

Effect of temperature

Phlebopus spongiosus BC-F0075 was grown on MS agar medium with different temperatures at 20, 25, 30 or 35°C in the dark. MS medium and strain BC-F0075 was chosen because the good mycelial growth was obtained in the previous experiments. The experiment was done in triplicate. Colony diameters at 15 days after inoculation were measured.

Effect of pH

Phlebopus spongiosus was grown in Ohta medium as a broth at pH range of 2 - 9. Two mycelium plugs (5 mm diameter) were cut from the growing edge of a 15 days-old culture in MS medium and inoculated into Ohta broth, and incubated for 15 days under laboratory conditions (30 ± 2°C) on a reciprocal shaker at 150 rpm. There were triplicate flasks for each treatment. At the 15th day, the cultures were filtered with Whatman no. 1 filter papers, oven dried (60°C), and weighed to determine the dry-biomass.

Effect of different carbon and nitrogen sources

In the testing of different carbon sources, MS agar media (without saccharose) was supplemented separately with carbon sources comprising one of the followings: saccharose, glucose, fructose, maltose or lactose.

In the testing of different nitrogen sources, MS agar media (without NH_4NO_3 and KNO_3) was supplemented separately with nitrogen sources comprising one of the followings: $(NH_4)_2SO_4$, NH_4OH, NH_4Cl, NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, KNO_3 or $(NH_2)_2CO$ (urea).

All cultures were incubated at 30°C in the dark. The experiment was done in triplicate. Colony diameters at 15 days after inoculation were measured.

3. Results and discussion

Isolation

Mushroom samples were collected in pomelo orchards (Fig 1a). Mycelia were cultured on potato dextrose agar (PDA)

medium and the colony covered the whole Petri dish after 45 days (Fig 2A). Mushroom samples grew on MS medium with abundant clamp connections and chlamydospores. Chlamydospores were light yellowish to orange yellow in colour, thick-walled and had variable forms (Fig 2B, 2C). Chlamydospores formed in intercalary or terminal mycelia. The chlamydospore is an important type of asexual spore, each spore is capable of germinating and forming mycelia. Formation of chlamydospores usually indicates good spawn production of *Volvariella*, therefore selection of strains is normally done based on the chlamydospores' production (Nannapaneni and Subbiah 2016).

Figure 1. Mushroom samples were collected in pomelo orchards

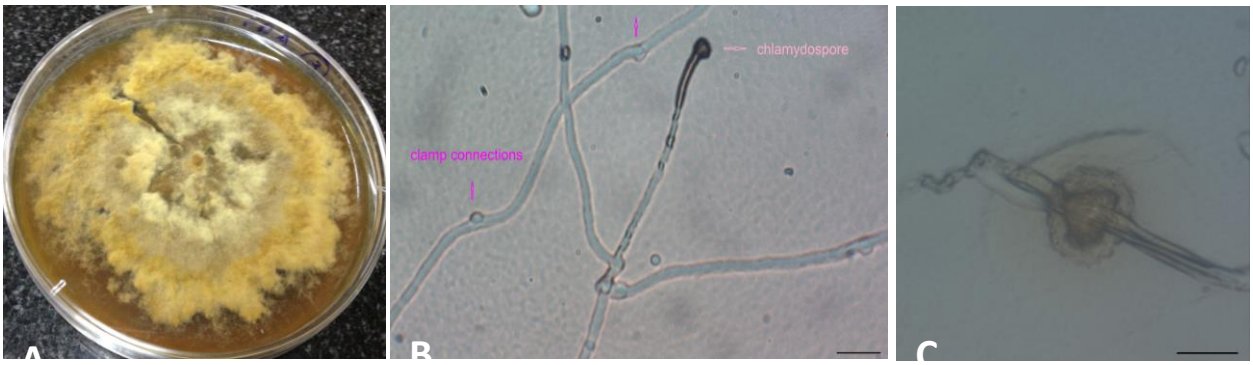

Figure 2. Isolate BC-F0075. (A) mycelium growth on PDA after 45 days, (B) clamp connections and chlamydospore, bar 10 µm, (C) chlamydospore, bar 10 µm

DNA analysis and phylogeny

Phylogram base on the LSU sequence showed that BC-F0075 and BC-F0076 clustered with *Phlebopus spongiosus* (AB673396) into a group with 100% of sequence similarity. This group is a clade which is separated with other *Phlebopus* species in the genus *Phlebopus* (Fig 3). *P. spongiosus* is a new tropical bolete in family Boletinellaceae that was recently described by Pham et al. (2012a).

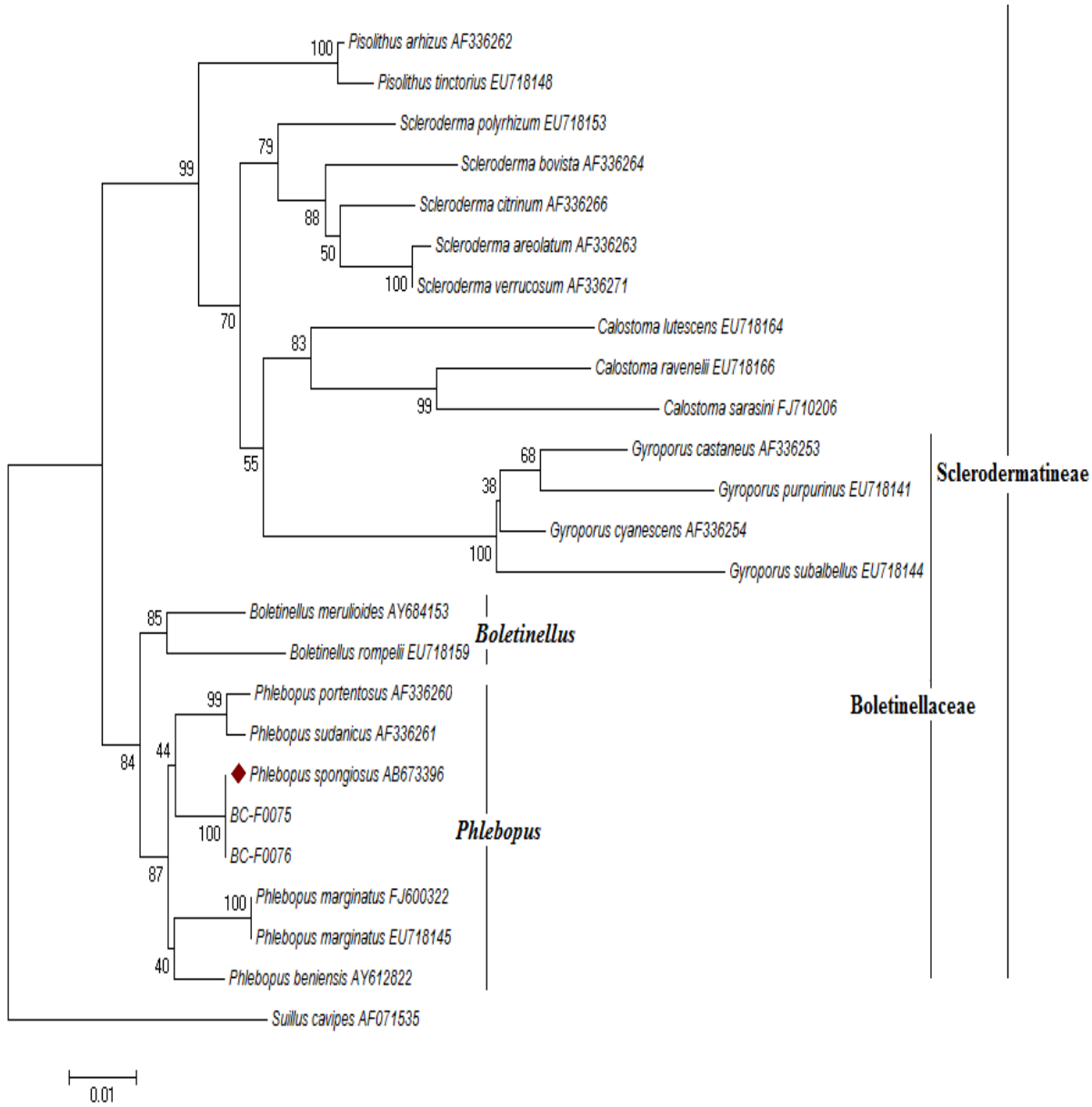

Figure 3. Phylogram of *Phlebopus spongiosus* in suborder Sclerodermatineae based on the LSU sequence. The outgroup was *Suillus cavipes*

Effect of culture media

Phlebopus spongiosus BC-F0075 and BC-F0076 grew in all tested culture media (Fig 4). Mycelial growth of BC-F0075 was faster than BC-F0076. Additionally, BC-F0075 grew the fastest on the modified MS medium, with the colony reaching 85.343 mm in diameter after 30 days (Table 1). The cultured plates turned brown in the PDA, PDA*, MYA and Ohta media, in contrast to the MNM, MS and FH media, which did not discolor. Sanmee et al. (2010) reported that *Phlebopus portentosus* WPPH2 turned brown or dark brown in the PDA and MNM media, but did not in the MS medium. Small primordium-like structures with brown exudates were formed on modified MS medium (Fig 5). *Phlebopus portentosus* CMUHH 121-005 grew the fastest and formed primordium-like structures on MS medium (Thongklang et al. 2010) that look similar to *P. spongiosus* BC-F0075.

Table 1

Mycelial growth of isolates on various solid media when incubated for 30 days

Medium	PDA	PDA*	MYA	MNM	Ohta	MS	FH
Mycelial growth of BC-F0075 (mm)	68.540^{c}	74.663^{b}	51.646^{i}	56.330^{g}	49.333^{j}	85.343^{a}	63.456^{e}
Mycelial growth of BC-F0076 (mm)	51.420^{i}	55.566^{h}	45.093^{l}	47.183^{k}	51.143^{i}	66.636^{d}	60.450^{f}

Values with the same letter are not significantly different (p=0.05).

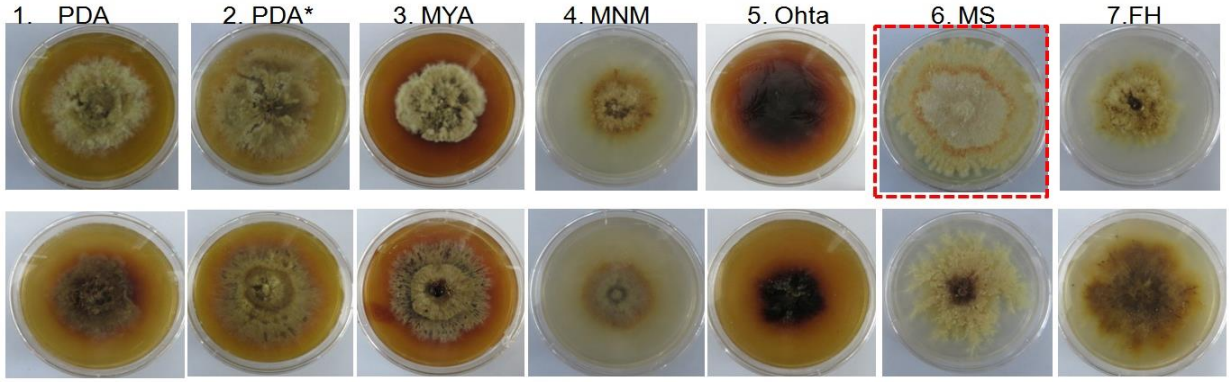

Figure 4. Mycelial growth of isolates on various solid media when incubated for 30 days. Row 1 is colony of BC-F0075, row 2 is colony of BC-F0076

Figure 5. Primordia-like structure on MS medium

Effect of temperature

Mycelia of BC-F0075 grew within a temperature range of 20 - 35°C (Fig 6), but not at 40°C (data not shown). The optimal for mycelial growth of BC-F0075 was 30°C with colony reaching 77.05 mm in diameter after 15 days (Table 2). Sanmee et al. (2010) reported that the optimal temperature for mycelial growth of *P. portentosus* WPPH2 was 30°C. *Phlebopus* is a genus of tropical boletes in the family Boletinellaceae, so *P. spongiosus* BC-F0075 is the potential species for mushroom cultivation industry in southern Vietnam with a hot and humid climate.

Table 2

Mycelial growth of BC-F0075 on MS medium with different temperatures

Temperature	20°C	25°C	30°C	35°C
Mycelial growth (mm)	15.448[d]	50.594[c]	77.050[a]	75.204[b]

Values with the same letter are not significantly different (p=0.05).

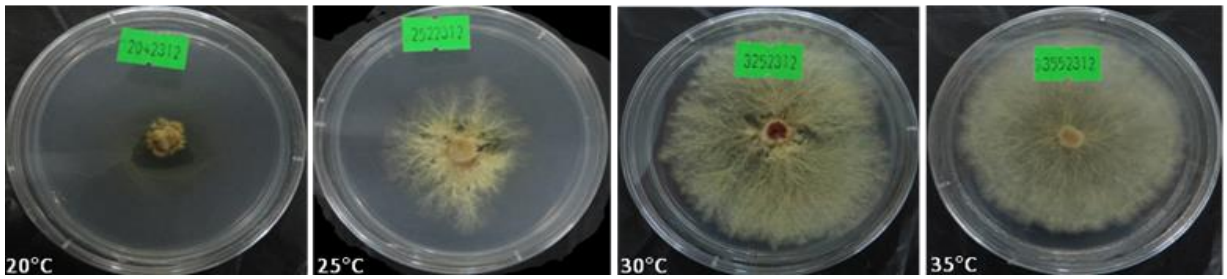

Figure 6. Mycelial growth of BC-F0075 on MS medium with different temperatures

Effect of pH

The optimal pH was 4 - 5 (Table 3). Mycelia of BC-F0075 grew well within a pH range of 3 - 6, but grew very slowly at pH 2, 7, 8 and 9. The optimal pH for mycelial growth of *Phlebopus spongiosus* BC-F0075 is similar to that for mycelial growth of *Phlebopus portentosus* MUHH121-005 by Thongklang et al. (2010). The optimum pH of ammonia fungi, saprobic mushrooms are pH 7, 7-8 and mycorrhiza mushrooms are 5 or 6 (Yamanaka 2003). Smith and Bonito (2012) reported that many EM basidiomycetes grow well in acidic soils.

Table 3

Mycelial growth of BC-F0075 on Ohta medium with different pHs

pH	2	3	4	5	6	7	8	9
Dry weight (g/100 ml)	0.083[cd]	0.138[b]	0.172[a]	0.173[a]	0.157[b]	0.094[c]	0.069[d]	0.043[e]

Values with the same letter are not significantly different (p=0.05)

Effect of different carbon and nitrogen sources

Carbon is an essential nutrient. Mycelia of BC-F0075 will not grow, if the medium does not have a carbon sources (data not shown). Colony diameters of BC-F0075 that grew on the MS media containing saccharose, glucose, fructose and maltose were not significantly different. BC-F0075 grew well on the MS media containing saccharose, glucose, fructose and maltose, but not with lactose (Fig 7).

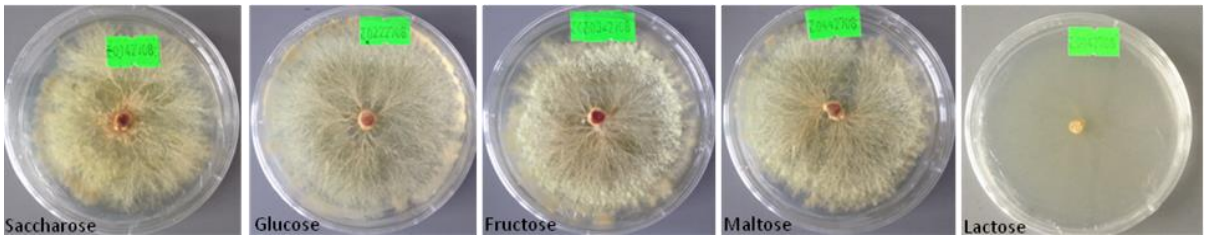

Figure 7. Mycelial growth of BC-F0075 on MS medium with different carbon sources

$NH_4H_2PO_4$ was the best nitrogen source to mycelia growth of BC-F0075 with colony reaching 79.07 mm in diameter (Table 4). Mycelial growth of BC-F0075 did not grow on the MS medium containing urea ($(NH_2)_2CO$). Kumla et al. (2011) reported that no strain of *Phlebopus portentosus* could use urea as a nitrogen source.

Table 4

Mycelial growth of BC-F0075 on MS medium with different nitrogen sources

Nitrogen sources	$(NH_4)_2SO_4$	NH_4OH	NH_4Cl	NH_4NO_3	$NH_4H_2PO_4$	$(NH_4)_2HPO_4$	KNO_3	$(NH_2)_2CO$
Mycelial growth (mm)	77.93^b	60.00^f	67.70^e	74.00^c	79.07^a	77.77^b	69.67^d	0^g

Values with the same letter are not significantly different (p=0.05).

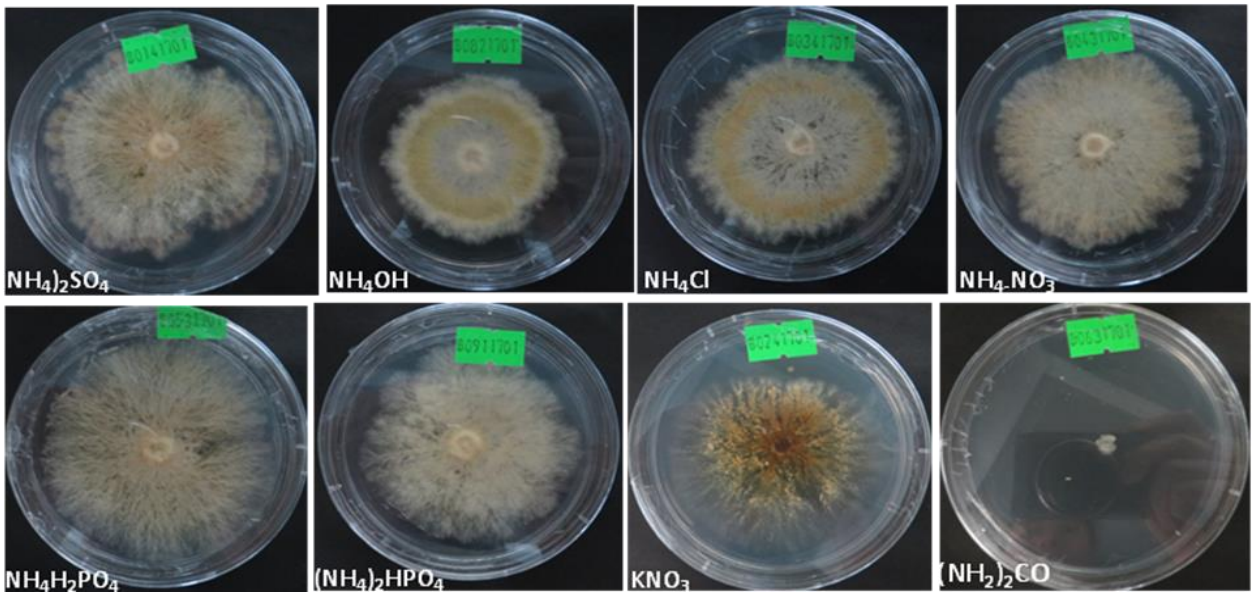

Figure 8. Mycelial growth of BC-F0075 on MS medium with different nitrogen sources

4. Conclusions

Mycelial growth of BC-F0075 was faster than BC-F0076. *Phlebopus spongiosus* BC-F0075 grew the fastest on the modified MS medium and formed small primordium-like structures on this medium. 30°C was the best temperature for mycelial growth and the pH 4 - 5 was the optimum pH value. BC-F0075 grew well on the MS media containing saccharose, glucose, fructose and maltose. $NH_4H_2PO_4$ are the suitable nitrogen sources for mycelial growth of BC-F0075. *P. spongiosus* is an endemic EEMM in southern Viet Nam. It's necessary to study and cultivate this EEMM■

Acknowledgement

This research was supported by the Grant VS04/15-16 of Biotechnology Center of Ho Chi Minh City, Vietnam.

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