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Full Paper

Culture condition, inoculum production and host response of a wild mushroom, *Phlebopus portentosus* strain CMUHH121-005

Naritsada Thongklang [1], Kevin D. Hyde [2, 3], Boonsom Bussaban [1] and Saisamorn Lumyong [1,*]

[1] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand
[2] School of Science Mae Fah Luang University, Chiang Rai 57100, Thailand
[3] Botany and Microbiology Department, College of Science, King Saud University, Riyadh, Saudi Arabia

* Corresponding author, e-mail: scboi009@chiangmai.ac.th

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Abstract: The optimal media and growth conditions of an edible wild mushroom, *Phlebopus portentosus* strain CMUHH121-005, were investigated for inoculum production. The optimum temperature for mycelial growth was 30ºC and the optimum pH was 4.0. Malt extract and yeast extract as carbon and nitrogen sources respectively, with a C:N ratio of 10:1, were most suitable. The biomass production in 16 types of cereal grain media was investigated. The fungus was found to grow best in barley grain mixed with Murashige and Skoog solution over 30 days following inoculation. Further incubation at 30°C for 60 days in the dark caused numerous agglomerations of mycelia as if fruiting bodies were being formed. The data presented provide growth requirements that will be useful in a future development of *P. portentosus* as a cultivatable mycorrhizal mushroom.

Keywords: *Phlebopus portentosus*, mushroom cultivation, mycorrhizae, mushroom inoculum

INTRODUCTION

The edible wild mushroom *Phlebopus portentosus* belongs to the order Boletales and in Thailand is commonly known as *hed har* or *hed tubtaodum*. It is a highly sought-after and valued edible mushroom, especially in northern and north-eastern Thailand [1]. It forms ectomycorrhizal associations with host plants such as elaeocarpus (*Elaeocarpus hygrophilus*), jambul (*Syzygium cumini*), longan (*Dimocarpus longan*) and giant mimosa (*Mimosa pigra*) [2] and is sold as a tree inoculum [3].

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Ectomycorrhizal fungi are difficult to cultivate as they need to grow in association with their host. However, *Phlebopus portentosus* is unusual among the Boletales and possibly most mycorrhzal fungi as it produces clamp connections and can be easily isolated in culture. It thus has the potential to produce fruiting bodies in vitro and to be a cultivatable species without a host plant [4]. Because it is easy to isolate and can grow readily in culture it is also possible to inoculate various hosts with this fungus and these associations improve host growth and health. Furthermore, since many of its hosts are fruit trees, the establishment of a symbiosis with *P. portentosus* may increase fruit yield and result in a yearly mushroom yield of considerable value.

Most cultivatable mushrooms have specific requirements for growth in axenic culture [5-10]. The main factors affecting growth are nutrient sources and environmental factors such as temperature and pH [11-14]. The media generally contain a carbon source, nitrogen source and vitamins. The carbon source is especially important and should be in greater quantities than other essential nutrients, and generally in the range of 3-28% [15]. Mushrooms can be grown on different carbon sources such as glucose, galactose, mannose, fructose, sucrose, cellulose, dextrin and starch [15-18]. Nitrogen sources such as ammonium nitrate, calcium nitrate, yeast extract, soya bean, arginine and glutamic acid have been used to promote mycelium growth [10, 15-17]. The optimum temperature and pH of mycelial growth varies with the strain or species of mushroom. For example, *Volvariella volvacea* grows well at 35°C, *Pleurotus eryngii* at 25°C, *Pleurotus ostreatus* and *Pleurotus pulmonarius* at 30°C, *Agrocybe aegerita* at 25°C or 30°C, *Lentinus strigosus* at 35°C and *Lentinula edodes at* 20°C or 30°C [13-20]. Yamanaka [13] reported that the optimum pH of ammonia fungi, saprobic mushrooms and mycorrhiza mushrooms are pH 7, 7-8 and 5 or 6 respectively. *Phlebopus portentosus* CMU 2210 grows well on modified Gamborg medium over 26 days and the optimum condition for growth is 30°C and pH 4 [21]. The work by Sanmee et al. [21] did not establish a suitable strain for cultivation since the fruiting bodies produced in vitro were deformed when compared to natural basidocarps.

Production of many ectomycorrhizal and cultivated mushroom inocula (spawn) frequently involves using cereal grains such as millet, wheat, corn, rice, barley [22-23], sawdust + barley grain and sawdust + rice grain [24] or sorghum grain as the substrate [2]. The advantages of cereal-grain-based media are production in large scale due to their general low cost and ease of handling [25].

In this study, fast-growing strain selection, culture conditions for mycelial growth, and evaluation of suitable solid media for growth of selected *P. portentosus* strains are investigated for better inoculum production.

MATERIALS AND METHODS

Fungal Strain Isolation and Preparation of Starting Culture

Fruiting bodies of *P. portentosus* were collected from different sites in Chiang Mai, Lamphun and Chiang Rai provinces, Thailand. Their morphological structure was recorded. Dried specimens were numbered in sealed plastic bags with silica gel and deposited in the herbarium of the Laboratory of Applied Microbiology, Department of Biology, Chiang Mai University.

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Mycelia were isolated from the fruiting bodies and cultured on potato dextrose agar (PDA) medium (Labscan Asia Co.,Ltd, Thailand). The pure cultures were named as CMUHH in a code series and the colony diameters were measured. CMUHH121-005 grew faster than other isolates and was chosen as a presentative strain for investigating a suitable basal growth medium. It was grown on 12 types of agar media, viz. corn meal agar, glucose peptone yeast extract agar, malt extract agar, oat meal agar, potato carrot agar, potato dextrose agar (PDA), potato sucrose agar [26], Fries agar, Murashige & Skoog (MS) agar medium [27], fungus-host medium [28], modified Gamborg medium [21], and modified Schenk & Hildebrandt medium [29]. All cultures were incubated at 30°C for 21 days. Colony morphology and dry weight were recorded at 3-day intervals during incubation. Stock cultures were kept on PDA slants at 4°C.

A mycelium plug (0.5-mm diameter) of *P. portentosus* CMUHH121-005 grown on PDA for 14 days was inoculated in Murashige & Skoog (MS) agar medium. All cultures were incubated at 30°C in the dark.

Effect of Temperature and pH

MS agar medium plates were centrally inoculated with mycelial plugs of approximately 0.5-mm diameter cut from an actively growing mycelia colony and incubated at 20, 25, 30, 37, 40 and 45°C. The medium was melted and washed away with hot water, leaving the fungal mycelia. Growth of the mycelia was evaluated by determination of dry weight in triplicate every 3 days.

The optimal pH was evaluated in MS medium broth that was adjusted to pH 2, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8 and 10 with 1N HCl or 1N NaOH before autoclaving. The MS broth (50 ml) was inoculated with a mycelial plug of approximately 0.5 mm in diameter from an actively growing mycelia colony. All cultures were incubated at 30°C, followed by incubation on a rotary shaker at 140 rpm in the dark. Mycelial growth was evaluated by dry weight determination every 3 days for 14 days. The experiment was conducted in triplicate.

Effect of Different Carbon and Nitrogen Sources

In the testing of different carbon sources, MS agar media (without glucose) was supplemented separately with a 3% (w/v) carbon source comprising one of the following: glucose, fructose, sucrose, starch or malt extract. The pH of the medium was adjusted to 4 with 1N HCl. All cultures were incubated at 30°C in the dark. The medium was melted and washed away with hot water, leaving the fungal mycelia. Mycelial growth was evaluated by determination of the dry weight every 3 days for 21 days. The experiment was done in triplicate.

In the testing of different nitrogen sources, MS agar media (without NH_4NO_3) was supplemented separately with a 3.55% (w/v) nitrogen source comprising one of the following: NH_4NO_3, KNO_3, NH_4NO_3 + KNO_3, peptone, or yeast extract. The pH of the medium was adjusted to 4 with 1N HCl. All cultures were incubated at 30°C in the dark. The medium was melted and washed away with hot water, leaving the fungal mycelia. Mycelial growth was evaluated by determination of the dry weight every 3 days for 21 day. The experiment was done in triplicate.

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Effect of C:N Ratio

The best carbon and nitrogen sources (malt extract and yeast extract respectively) were mixed up in a ratio of 1:1, 1:2, 1:3, 1:10, 2:1, 3:1 and 10:1. Each medium was adjusted to pH 4 with 1N HCL. Five ml of the MS broth was inoculated with a mycelium plug of approximately 0.5 mm in diameter from the actively growing mycelia colony. All cultures were incubated at 30°C, followed by incubation on a rotary shaker at 140 rpm in the dark. Mycelial growth of was evaluated by dry weight determination after 7 days. The experiment was done in triplicate.

Effect of Cereal Media for Inoculum Production

Sixteen types of cereal media were used as substrates for mycelial growth (Table 1). Tissue culture bottles containing 100 g of each medium were inoculated with 3 mycelial plugs of approximately 0.5 cm in diameter from the actively growing mycelia colony. The cultures were incubated in the dark at room temperature and growth rate was estimated from the linear expansion of mycelia growing through the medium. The experiment was done in triplicate.

Table 1. Growth of *P. portentosus* CMUHH121-005 on various cereal grain media

Cereal grain media	Ratio	Growth of mycelia (days*)
Sorghum grain	-	70.00±1.00
Barley grain	-	55.67±0.58
Corn grain	-	95.00±1.00
Wheat grain	-	89.33±0.58
Sorghum grain: barley grain: wheat grain	1:1:1	89.33±0.58
Green bean peel: water	10: 3	No growth
Green bean	-	74.67±0.58
Ground nut peel: water	10: 3	72.00±0.00
Ground nut peel: MS solution (modified from MS agar)	10: 3	59.67±0.58
Barley grain: MS solution	100: 1	41.00±1.00
Barley grain: MS solution	10: 3	30.33±0.58
Barley grain: sawdust: MS solution	5: 5: 3	60.67±0.58
Barley grain: sawdust: MS solution	5: 1: 1	50.00±1.00
Barley grain: synthetic solution [24]	10: 3	44.67±0.58
Barley grain: sawdust: synthetic solution	5: 5: 3	120.00±1.00
Barley grain: sawdust: synthetic solution	5: 1: 1	120.33±0.58

* no. of days for mycelia to cover all of cereal grain medium in the container

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Host Responce to Fungal Inoculum

Four host plant seedlings (*Elaeocarpus hygrophilus* Kurz, *Adenanthera pavonina* L., *Clausena lansium* (Lour) Skeels, and *Sauropus androgynus* Merr.) were grown in pots filled with sterile soil mixed with sand in the ratio of 2:1 (w/w) and were inoculated with 25 g of *P. portentosus* CMUHH121-005 inoculum and incubated in an open green house. The pots were watered everyday for 6 months. Root infection was determined once every month after staining with trypan blue and examining under light microscope [29].

RESULTS AND DISCUSSION

Strain Isolation and Selection

Twenty two basidiocarp samples associated with different host plants were collected from forests, orchards, roadsides and other locations (Figure 1). They were observed to have different characteristics (Figure 2). Each isolated strain was grown on PDA and incubated in culture for 14 days. Strain CMUHH 121-005, isolated from a basidiocarp sample bought from a roadside market in Wiang Pa Pao district, Chiang Rai province, grew faster than other isolates (data not shown) based on colony diameter. This strain was selected for further study. Among the 12 media tested, the strain grew in all media at different rates (Figure 3). The fastest growth rate was observed when the isolate was grown on MS agar medium, with the colony reaching 8 cm in diameter after 21 days (data not shown). It is unusual amongst mycorrhizal fungi as it can be isolated and cultured in agar media and forms clamp connections in the media (Figure 1d). With the discovery that the taxon can be cultivated, there has been avid interest in trying to cultivate this species at an industrial scale [21]. It is also believed that the taxon can form mycorrhizal association with various trees including some fruit trees and thus has potential to be 'farmed' as a mycorrhizal edible fungus (Figure 1b).

Effect of Temperature and pH on Mycelial Growth

Mycelia of *P. portentosus* CMUHH 121-005 grew in the range of 20–37°C, but did not grow at 40 or 45°C (Table 2). Optimal dry growth weight occurred at 30°C with an average dry growth weight of 0.2050 g in 21 days.

Various cultivated mushrooms have different temperature optima (e.g. *Volvariella volvacea* at 35°C, *Pleurotus eryngii* at 25°C [19]), while the temperature for optimum growth of *P. portentosus* CMUHH121-005 is 30°C. This makes sense as the fruiting bodies of this fungus are produced from May to July when the air temperature ranges between 23-34°C [30].

Strain CMUHH121-005 grew very slowly at pH 2, 6, 6.5, 7, 8 and 10 (Table 2). The optimal pH was 4-5.5, with a maximum average dry weight of 0.0959 g at pH 4 in 14 days. These results agree with reports on other mycorrhizal species. Niitsu et al. [31] reported the optimum pH of 4 for the growth of *Mycena chlorophos* while most other mushrooms produce fruiting bodies in neutral or slightly acidic pH of 6-7 [32-33]. Many ectomycorhizal mushrooms grow at acidic pH [34].

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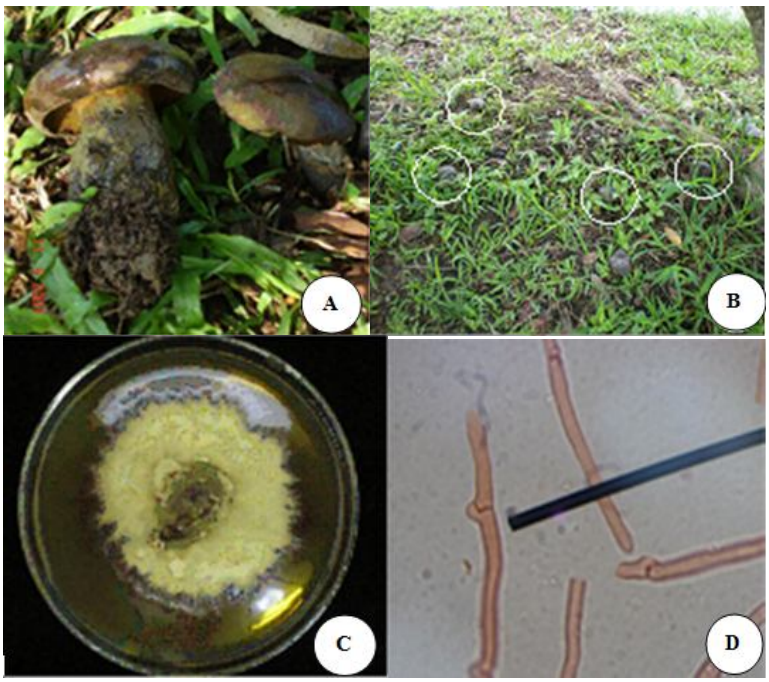

Figure 1. *Phlebopus portentosus*: A) olive brown to dark olive brown fruit body; B) fruit-body forming associate with host plant; C) mycelium growth on medium; D) clamp connection of *P. portentosus* in medium

Figure 2. Different macrocharacteristics of *P. portentosus*

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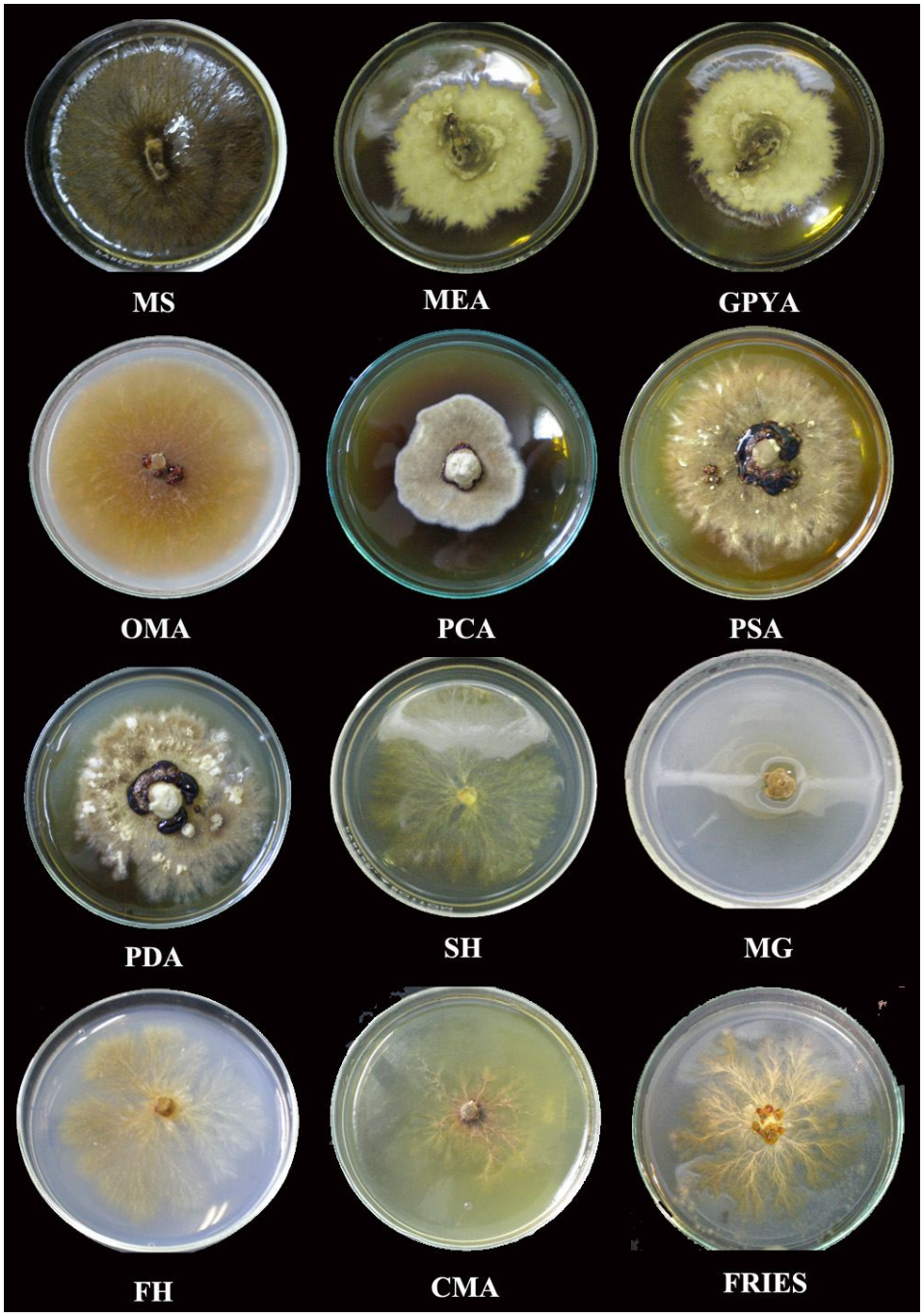

Figure 3. Mycelial growth on various solid media when incubated for 21 days at 30°C (MS = Murashige & Skoog agar, MEA = malt extract agar, GPYA = glucose-peptone-yeast extract agar, OMA = oat meal agar, PCA = potato carrot agar, PSA = potato sucrose agar, PDA = potato dextrose agar, SH = modified SH medium, MG = modified Gamborg, FH = fungus-host medium, CMA = corn meal agar, FRIES = Fries agar)

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Effect of Carbon and Nitrogen Sources on Mycelial Growth

Phlebopus portentosus CMUHH 121-005 was observed to grow in all media at varying rates. Maximum growth occurred on malt extract agar (Table 2). The average dry growth weight was 0.2868 g in 21 days. The strain also grew well in starch with 0.1583 g dry weight of mycelia produced. The lowest growth occurred on media supplemented with glucose, sucrose and fructose (0.0925 g, 0.1004 g and 0.1128 g respectively).

Carbon is an essential nutrient needed by fungi and affects mycelial growth. *Pleurotus ostreatus*, for example, grows well on medium that contains millet extract [10] while broth media that contain dextrose are good for *Pleurotus florida* mycelial growth [17]. Jonathan and Fasidi [15] reported that the edible mushroom *Psathyerella atroumbonata* grows well on glucose. Growth of *P. portentosus* CMUHH121-005, on the other hand, was observed to be significantly enhanced in MS medium that contained 3% malt extract in this study.

Table 2. Dry weight of mycelium grown in axenic culture under various conditions for 21 days

Parameter	Dry weight + Standard error (g)
Temp (°C)	
20	0.0229±0.0008^{c}
25	0.1043±0.0112^{b}
30	**0.2050±0.0104^{a}**
37	0.1241±0.0103^{b}
40	0.0000±0.0000^{c}
45	0.0000±0.0000^{c}
pH	
2	0.0020±0.0001^{c}
4	**0.0959±0.0027^{a}**
4.5	0.0928±0.0016^{ab}
5	0.0915±0.0015^{ab}
5.5	0.0869±0.0018^{b}
6	0.0081±0.0001^{c}
6.5	0.0062±0.0002^{c}
7	0.0027±0.0004^{c}
8	0.0020±0.0002^{c}
10	0.0020±0.0002^{c}
Carbon source	
Glucose	0.0925±0.0038^{c}
Malt extract	**0.2868±0.0165^{a}**
Sucrose	0.1004±0.0046^{c}
Fructose	0.1128±0.0056^{c}
Starch	0.1583±0.0009^{b}

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Table 2. (Continued)

Parameter	Dry weight + Standard error (g)
Nitrogen source	
NH_4NO_3	0.1550±0.0037[b]
KNO_3	0.1413±0.0107[b]
Yeast extract	**0.3273±0.0213[a]**
Peptone	0.2939±0.0085[a]
NH_4NO_3+KNO_3	0.1241±0.0103[b]
C:N ratio	
1:1	0.0108±0.0056[b]
1:2	0.0139±0.0015[b]
1:3	0.0224±0.0081[b]
3:1	0.0188±0.0073[b]
2:1	0.0121±0.0017[b]
1:10	0.0074±0.0009[b]
10:1	**0.1035±0.0197[a]**

Note: Values with the same letter are not significantly different (p=0.05) according to Tukey's multiple range test.

Inorganic nitrogen such as NH_4NO_3, KNO_3 and NH_4NO_3+KNO_3 were poor sources of nitrogen for growth of the CMUHH121-005 strain. The best mycelia growth was found in the medium that contained organic nitrogen, viz. yeast extract and peptone, with an average dry mycelial weight of 0.3273 g and 0.2939 g respectively in 21 days (Table 2). Hatakeyama and Ohmasa [16] reported that ammonium tartrate was the best nitrogen source for *Boletinus* sp. On the other hand, *Pleurotus florida* grew well on a basal medium that contained casein, which was better than the basal medium that contained urea, yeast extract and peptone [17]. The optimal C:N ratio for growth of mycelium also seems to depend on the fungal species. A suitable C:N ratio for *P. florida* was 5:3 [17] while Jonathan and Fasidi [15] reported that a suitable C:N ratio for *Psathyerella atroumbonata* was 2:3. In this study, *Phlebopus portentosus* grew best in MS medium with a C:N ratio of 10:1 (Table 2).

Effect of Cereal Media on Inoculum Production

In the cultivation of many mushrooms, inocula comprising cereal grains such as sawdust + barley grain, sawdust + rice grain, and sorghum grain are frequently used [2, 24]. Cereal-grain-based media are advantageous for large scale production as the cost is low and the media are easy to manage [25]. This study confirms that the fungus can grow in all types of media except green bean peel (Table 1). Optimal growth was found in barley grain mixed with MS solution. After further incubation at 30°C over 60 days in the dark, numerous primordia-like structures were formed (Figure 4), although no fully-formed fruiting bodies developed. Other strains of *P. portentosus* grew

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well on sorghum grain media [2]. Kawagishi et al. [35] reported that *Tricholoma matsutake* grew better when D-isoleucine was added to the media.

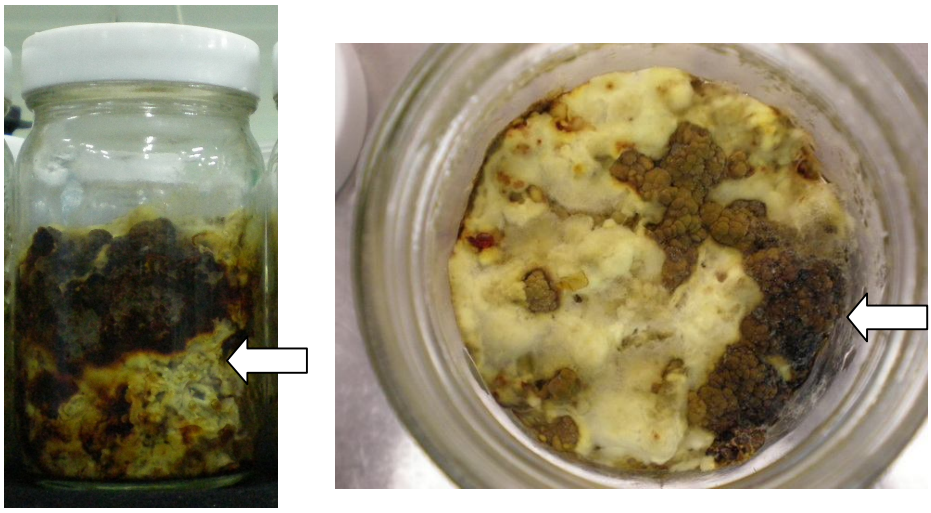

Figure 4. Primordia-like structures of *P. portentosus* (strain CMU121-005) in barley grain mixed with MS solution when incubated in the dark at 30°C for 90 days

Host Response for Fungal Inoculum

Inoculation of four plant species with the fungus resulted in root associations only in *Elaeocarpus hygrophilus* after 6 months. Mycelial infection of the host roots was observed under a stereo microscope, although a mantle sheath was not observed (Figure 5). The effect of inoculating the hosts with *P. portentosus* needs to be better evaluated since it was not clear in this experiment if the host really formed a mycorrhizal association with the fungus. It is also desirable to establish whether inoculating the host plants with this mushroom results in a continuous future production of the fruiting bodies. Some researchers have succeeded in inoculating different hosts in pots with different strains of this mushroom [4, 21].

CONCLUSIONS

Optimal conditions for mycelial growth are important when inducing fruiting body formation in vitro on solid media without the host plant. As yet we have not managed to obtain fruiting bodies of *P. portentosus* CMUHH 121-005 in vitro. However, by providing data on conditions for optimum growth, it is expect that it will eventually be possible to achieve this aim. Growth in culture may lead to commercialisation of the fungus. Pure cultures of the fungus can be established in cereal media that can then be used as inocula for saplings in nurseries or even established orchards so that fruit farmers can reap the reward of a mushroom in addition to the fruit harvest.

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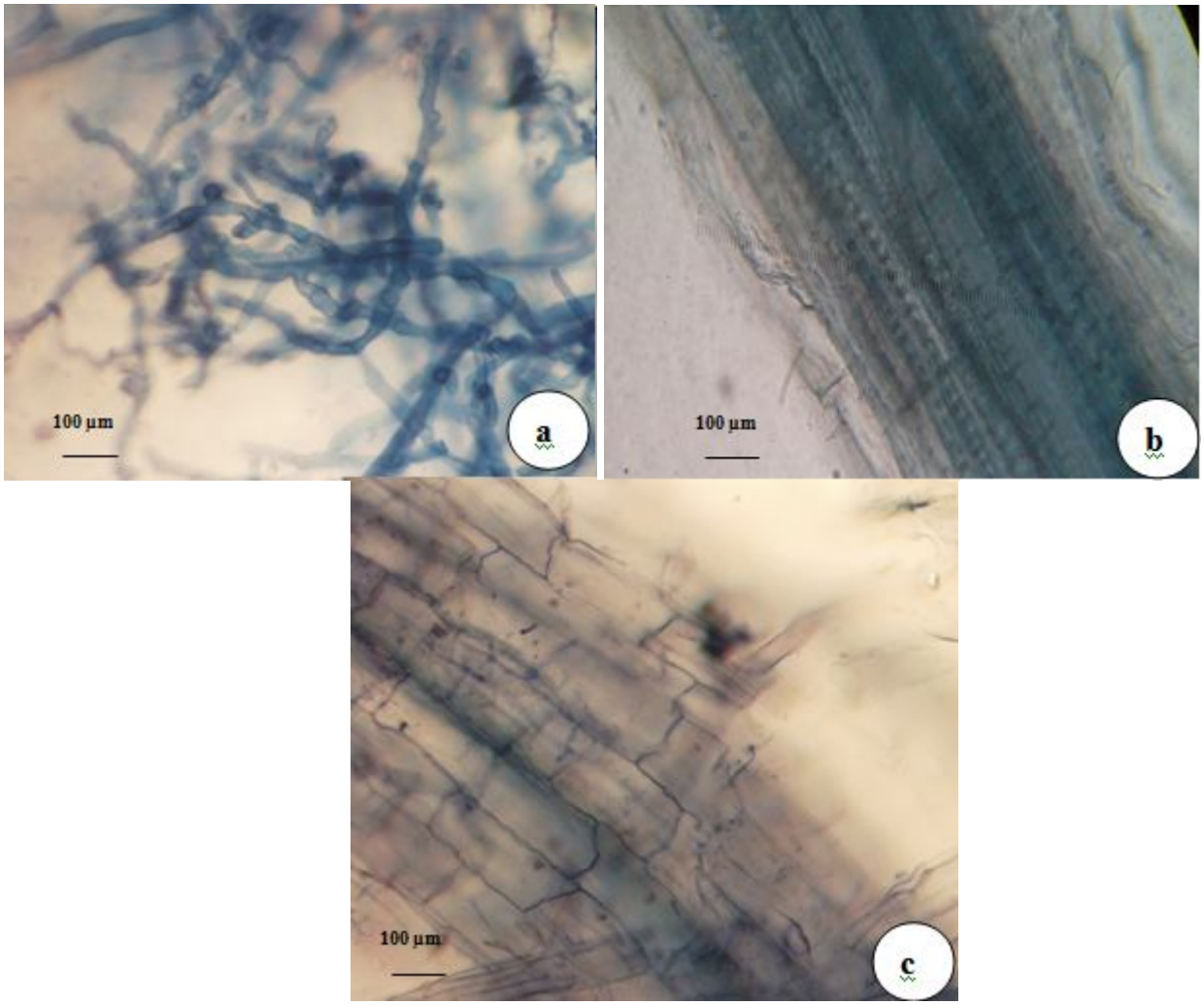

Figure 5. a) *P. portentosus* mycelia; b) and c) root infection, 6 months after inoculation with *P. portentosus* CMUHH121-005 in *E. hygrophilus*

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