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FULL PAPER

In vitro cultivation and fruit body formation of the black bolete, *Phlebopus portentosus*, a popular edible ectomycorrhizal fungus in Thailand

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Abstract The tropical black bolete *Phlebopus portentosus* is highly favored in the cuisine of northern Thailand. It is suspected to form ectomycorrhizae with many host trees. Mycelium of *P. portentosus* isolated from a basidiome in Chiang Rai Province in 2003 grew well on modified Gamborg, modified Melin–Norkans, and Murashige and Skoog media at 30°C and at pH 4. In vitro fructification of *P. portentosus* on sorghum grain medium without a host plant is presented for the first time. Basidiomes emerged 3 months after inoculation on the medium, and the produced basidiospores germinated on agar, indicating the completion of its life cycle in vitro without a host. Five putative host plants (*Castanopsis tribuloides*, *Dipterocarpus alatus*, *Dimocarpus longan*, *Pinus kesiya*, and *Syzygium cumini*) were inoculated with mycelium on sorghum grain medium in a greenhouse to confirm its ectomycorrhizal status. Ectomycorrhizal roots were observed only on *Pinus kesiya*, suggesting that *P. portentosus* may be facultatively ectomycorrhizal. Identification of the synthesized ectomycorrhizae was confirmed by PCR amplification of ITS with a designed specific primer (HAR2A).

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Introduction

"Hed har" or "hed tub tao dum" are local Thai names for *Phlebopus portentosus* (Berk. & Broome) Boedijn (syn. *Phaeogyroporus portentosus*; in Index Fungorum, http://www.indexfungorum.org/Names/Names.asp), one of the most popular wild edible poroid mushrooms consumed by local people in northern Thailand (Watling 2001; Sanmee et al. 2003). It develops very large basidiomes, and the texture is similar or even better than that of *Boletus edulis*, which is important in the mushroom market in Europe, estimated consumption being 20000–100000 tons annually (Hall et al. 1998). In Thailand, basidiomes of *P. portentosus* are collected during the rainy season (June–August) in various types of forests and also in some orchards. The retail price is higher than that of cultivated mushrooms because of the better flavor and texture, higher protein content, and lower fat content (Sanmee et al. 2003). Northern Thailand has a monsoon climate with dry (November–May) and wet (June–October) seasons. The mixed forests contain many ectomycorrhizal (ECM) tree species, such as *Quercus*, *Fagus*, *Pinus*, and *Dipterocarpus*, which annually provide many wild edible mushrooms. According to mushroom harvesters, *P. portentosus* can be found associated with many tree species such as *Elaeocarpus hygrophilus*, *Syzygium cumini*, *Mangifera indica*, *Mimosa pigra*, and *Dimocarpus longan*, suggesting that the fungus may not be obligately ECM. Boletinellaceae, which includes *Phlebopus*, is known to contain both saprobic and ECM species (Binder and Bresinsky 2002).

It is uncommon for ECM fungi to produce basidiomes under artificial conditions (Godbout and Fortin 1990). Of

great interest is the potential to produce basidiomes of commercially important edible ECM fungi by growing mycelium on defined substrates, as is the case for saprobic fungi such as *Pleurotus* spp., *Lentinula edodes*, *Hypsizygus marmoreus*, *Flammulina velutipes*, *Agrocybe cylindracea*, and *Hericium erinaceus*. However, to date, only a few ECM fungi are amenable to manipulate sporocarp production, such as *Lyophyllum shimeji* (Ohta 1994b) and *Hebeloma* species (Ohta 1998) on a mixture of sawdust and barley grain supplemented with inorganic nutrients. Only truffles (*Tuber* spp.) have been successfully cultivated in association with their hosts for commercial production in Europe, Australasia, and North America (Paolocci et al. 1995; Lefevre and Hall 2001). *Cantharellus cibarius* basidiomes can be produced with host tree seedlings under laboratory conditions (Danell and Camacho 1997). A few ECM species do not need the host to stimulate the production of fruit bodies (Ohta 1994a, b, 1998; Yamanaka et al. 2000; Ohta and Fujiwara 2003).

The objectives of this study were to define growth conditions of *P. portentosus* for mushroom production and to confirm whether the fungus is obligately ECM.

Materials and methods

Isolation

Phlebopus portentosus was isolated from a natural mature basidiome (CMU2210, lodged in the Chiang Mai University Herbarium) purchased from a roadside stall in Amphur Weing-Par-Pao in Chiang Rai Province. It was originally collected in a Fagaceae forest along a local roadside. The isolation method basically followed Brundrett et al. (1996). The basidiome was briefly brushed to remove adhering soil particles, broken open, and a small piece of tissue was removed with sterilized fine forceps and placed on solid Murashige and Skoog (MS) medium at pH 4 in a Petri dish (Straatsma et al. 1986). The pure culture isolate, designated as *P. portentosus* WPPH2, was used for all experiments. Mycelial cultures were maintained on MS medium at 20°C and were routinely subcultured every 2 months.

Mycelial growth tests

Effect of culture media

Nine solid agar-based media were tested for the growth ability of *P. portentosus* mycelium: Fries medium (Fries 1978), modified Gamborg medium (Gamborg et al. 1968), Hagem medium (Modess 1941; cited in Molina and Palmer 1982), malt extract medium (MA; 2% (w/v) malt extract; Scharlau, Spain), modified Fries medium (MFM; Danell 1994), modified Melin Norkans medium (MMN; Marx 1969), Murashige and Skoog medium (MS; Straatsma et al. 1986), Pachlewski medium (PACH; Pachlewski and Pachlewski 1974), and potato dextrose agar (PDA; using potato). Agar (1.5% w/v) was added to each medium before autoclaving for 15 min at 121°C; then, 20 ml media was poured into a 90-mm-diameter Petri dish. Plates were inoculated with a 6-mm-diameter plug of mycelium from the growing edge of a 20-day-old culture and sealed with Parafilm (Whatman, Maidstone, UK) to prevent water loss and contamination. Cultures were incubated in darkness at 30 ± 0.5°C. Mycelial growth was evaluated by measuring colony size (diameter and thickness) every 2 days. The experiments were repeated three times with five replicate plates per treatment.

Effect of temperature

Phlebopus portentosus was grown on MMN agar medium with different temperature at 20, 25, 30, and 37°C in the dark. This medium was chosen because the good mycelial growth was obtained in the previous experiments. Mycelial growth was evaluated by observing colony appearance every 2 days. The experiments were carried out twice with five replicate plates.

Effect of pH

Phlebopus portentosus was grown in MMN medium as a broth at pH range of 1–9. The pH was adjusted with various buffers before autoclaving (Stoll and Blanchard 1990): pH 2–3 with glycine-HCl (0.05 M glycine and HCl), pH 4–5 with succinic acid (0.05 M succinic acid and NaOH), pH 6–8 with phosphate buffer (0.05 M Na_2HPO_4 and NaH_2PO_4), and pH 9 with glycine-NaOH (0.05 M glycine and NaOH). Buffered liquid medium (25 ml) were transferred into 150-ml plastic flasks (Elmate, Tokyo, Japan), autoclaved as described elsewhere. Two mycelium disks (6 mm diameter) were cut from the growing edge of a 16 days-old culture in MS medium and inoculated into MMN broth, and incubated for 30 days under laboratory conditions (27 ± 2°C) on a reciprocal shaker at 120 rpm. There were triplicate flasks for each treatment. The initial and final pH values of the culture media were recorded. At the 30th day, the cultures were filtered through previously weighed oven-dried Whatman no. 1 filter papers, oven dried (55°C) overnight, and weighed to determine the biomass.

Statistical analysis

Numerical data of mycelial growth were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11. In all analyses, the *P* value was given if it was less than 0.05.

Solid substrate inoculum

The capability to grow mycelium on seed and sawdust was tested. Five types of culture media, i.e., rye grain, barley grain, sorghum grain, rubber wood sawdust, and peat-vermiculite, were tested in glass test tubes (diameter 25 mm, height 245 mm) closed with cotton wool. The rye grain medium was prepared by placing 12 g grain into 14 ml distilled water in each test tube, and the mix was incubated at 4 ± 1°C overnight to allow the grains to absorb water (Ohta 1994a). The barley grain medium was prepared by autoclaving 12 g grain with 9 ml distilled water at 100°C for 10 min. The sorghum grain medium was prepared by boiling grains for 30 min, blotting the excess water, and adding 12 g moist grain into each test tube. The sawdust medium contained 100 g composted rubber sawdust obtained from dry wood, 10 g rice bran, 0.5 g agricultural lime powder, 0.5 g $CaSO_4$, 0.2 g $MgSO_4$, and 1 g sucrose. Sufficient water was added to moisten the mix, and 12 g sawdust medium was added into each test tube. Peat and vermiculite were mixed in the ratio 1:8 (w/w), and sterile water was added to moisten the mix. All media were autoclaved for 15 min at 121°C and cooled down at air temperature before inoculation. There were three replicates for each treatment. Five agar plugs (0.6 mm diameter), which were removed from the edge of 15- to 20-day-old colonies, were placed on the top of the media in each test tube. The cultures were incubated under laboratory conditions (27 ± 2°C). Mycelial growth was visually assessed after 1 month. To ascertain whether the cultures were still alive, the grown mycelium was inoculated on MS agar plates.

Fructification

The fructification method was modified from that of Ohta (1994a, 1998) and Ohta and Fujiwara (2003). A sorghum grain medium was chosen for this test. The solid medium was autoclaved twice for 15 min at 121°C and transferred to 15-cm-diameter plastic Petri dishes before adding 30 ml sterile MS liquid medium. After inoculation with a 6-mm-diameter piece of mycelial disk cut from the growing edge of a 22-day-old culture, each Petri dish was sealed with Parafilm (Whatman). The cultures were incubated at 26 ± 1°C in the dark. After 90 days, the cultures were transferred to 12 h light/12 h dark at 24 ± 1°C and the Parafilm was removed. Light intensity was about 15 μmol m^{-2} s^{-1}. The Petri dishes were set 20 cm from the fluorescent light source.

DNA analysis

To compare the identity of the produced fruiting bodies with the inoculated isolate (WPPH2), a small piece of the hymenium was frozen in liquid nitrogen and ground in the extraction buffer of Raeder and Broda (1985) in a 1.5-ml Eppendorf tube with a glass rod. Other samples used for comparison were basidiomata specimens of *P. portentosus* (CMU46127, CMU 2210, CMUe0100, CMU46168), *Helvella* sp. CMU46106, *Laccaria amethystea* CMU45221, *Amanita hongoi* CMU45216, *Tricholoma fulvocastaneum* CMU4556, *Boletus* sp. CMU 4555, *Lactarius volemus* CMU2195, and *Astraeus hygrometricus* (CMU46158, CMU46145; PHOR5). DNA was extracted (Taylor et al. 2005) and purified using the Ultrabind DNA purification kit according to the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA, USA). The universal primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and designed primer HAR2A of *Phlebopus portentosus* (5′-CAAGTCCAAGCCATGCC TC-3′) (Sanmee 2004) were used to amplify the internal transcribed spacer (ITS) region. Specific polymerase chain reaction (PCR) reactions were performed under the following concentration in a 25-μl reaction mixture: 10 ng genomic DNA, 0.5 μl 10 pmol of each primer, 5.0 μl 5 × PCR polymerization buffer, 2.0 μl 25 mM $MgCl_2$, and 0.1 μl Taq DNA polymerase. The PCR reaction was carried out using the GeneAmp PCR System 2700. The PCR parameters were as follows: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min. A negative control using Ultrapure PCR grade water instead of template DNA was included in each amplification. PCR products were electrophoresed in 1% (w/v) agarose gel at 80 V for 40 min and visualized under a UV transilluminator after staining with 0.5 μg ml^{-1} ethidium bromide.

Mycorrhizal synthesis

Soil preparation

A moistened loamy nursery soil, which was used for seedling production, was pasteurized using steam for 2.5–3 h, and the treatment was repeated twice over the next 2 days. A mixed soil for potting consisted of red loam, river sand, and coconut bark (fuzzed) in the ratio 2:2:1 (w/w/w), which was pasteurized as described previously. The red loam, which was sourced from Mae Hie, Chiang Mai Province, is reputed to have a low nutrient content.

Plant materials

Five tree species were tested: *Castanopsis tribuloides* (Sm.) A.DC. var. *tribuloides*, *Dipterocarpus alatus* Roxb. ex G. Don, *Dimocarpus longan* Lour. ssp. *longan* var. *longan* Leenh, *Pinus kesiya* Royle ex Gordon, and

Syzygium cumini (L.) Skeels. Although *Dimocarpus* is not an ECM genus, it was included in the trial because mushroom collectors have sometimes reported gathering basidiomes of *P. portentosus* under longan. Seeds of *P. kesiya* provided from the Silviculture Research Centre 1, Chiang Mai Province, were originally collected in Huay Luang, Hod District, Chiang Mai Province. Seeds of *C. tribuloides*, *D. altus*, *D. longan*, and *S. cumini* were collected from Huay Kog Ma, Doi Suthep-Pui National Park; the avenue of trees on the old road between Chiang Mai and Lamphun; a longan orchard; and along the Ping River in Chiang Mai, respectively. The seeds of *D. alatus*, *D. longan*, and *S. cumini* were surface sterilized by soaking in 70% ethanol for 3 s, followed by 5% sodium hypochlorite for 10–15 min, then washed with sterile water three times for 5–10 min. The seeds of *C. tribuloides* and *P. kesiya* were surface sterilized using 1% H_2O_2 for 5 days at 5°C, rinsed in sterile water, placed in 30% H_2O_2 for about 35 min, then washed with sterile water 3 times for 5–10 min. The sterilized seeds were planted in pasteurized loamy soil in plastic bags.

Planting and inoculation

After 2.5 months, the seedlings were transplanted into potting mix in plastic pots, lined with a new plastic bag with drainage holes in the bottom. A 25 g (fresh weight) sorghum grain medium containing growing mycelium of *P. portentosus* was inoculated around the roots of host plant after transplanting. There were 10 replicate pots in each treatment. In the control, a 25 g autoclaved sorghum grain medium was used. Pots were placed in a randomized complete block design on raised benches in an open-sided greenhouse at the Chiang Mai University Coffee Center and incubated for 8 months. Water was added by hand as required. No fertilizer was added.

Microscopic observation and DNA analysis

Roots were first observed under a dissection microscope for the confirmation of ectomycorrhization (Brundrett et al. 1996), followed by sectioning, and observed under a compound light microscope. Putative ECM tips were fixed in 0.25 M phosphate buffer (pH 7.0) containing 3% glutaraldehyde overnight at 4°C, washed in several changes of the same buffer, dehydrated in an acetone series, and then infiltrated for at least 2 h in a series of solutions containing increased concentrations of Spurr's resin (Spurr 1966) in acetone. Specimens were polymerized in fresh Spurr's resin at 60°C for 24 h, sectioned in 1-μm thickness using glass knives, and dried on glass slides at 60°C. The resin was removed using a saturated solution of KOH in 100% ethanol, and the sections were stained with a mixture of 1% azur II and 1% methylene blue in 1% sodium tetraborate (Richardson et al. 1960). Single putative mycorrhizal root tips of *P. portentosus* were collected and placed in DNA extraction buffer (Raeder and Broda 1985). The rDNA was extracted, and *P. portentosus*-specific primers (Sanmee 2004) were used for the PCR amplification.

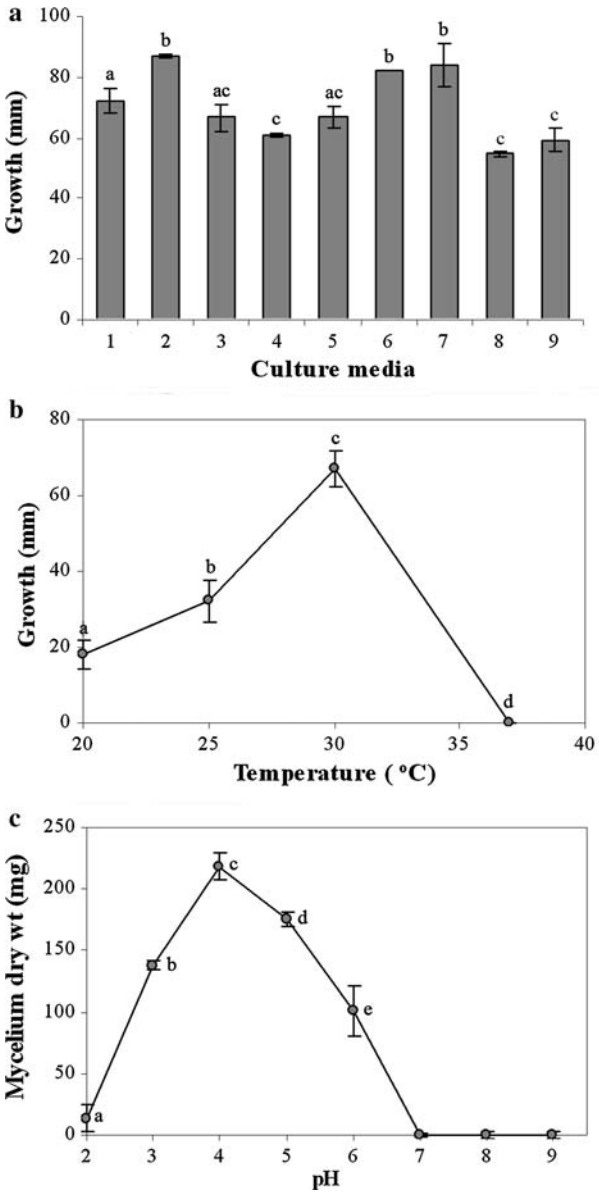

Fig. 1 The effect of culture media, temperature, and pH on the growth of *Phlebopus portentosus* WPPH2. **a** Culture media: *1* fries medium, *2* modified Gamborg medium, *3* Hagem medium, *4* malt extract medium, *5* modified Fries medium, *6* modified Melin Norkans medium, *7* Murashige and Skoog medium, *8* Pachlewski medium, *9* potato dextrose agar. **b** Temperature 20–37°C. **c** pH 2–9. Significant differences ($P < 0.05$) in the growth radiances (**a**, **b**) or mycelium dry weight (**c**) were tested by one-way ANOVA, followed by a Duncan's multiple range test. Statistical differences are indicated by *different letters*

Results

Vegetative growth condition

Phlebopus portentosus WPPH2 grew in all tested culture media (Fig. 1a). Colony expansion of WPPH2 was faster on the modified Gamborg than on the other media, reaching 87 mm diameter after 26 days. However, as the mycelium on this medium was sparse, the medium was not appropriate for this fungus. Radial growth on MMN and MS media was not as fast as on the Gamborg medium ($P < 0.05$), but the colonies were denser and had more primordium-like structures. The young mycelium of WPPH2 was yellow (C 0%, Y 30%, M 0%) and become olive color (C 20%, Y 70%, M 20%) when mature (Brundrett et al. 1996). Small primordium-like structures with brown exudates were formed on the mycelial colony on all media tested. In the MA, MMN, PACH, and PDA media, the cultured plates turned brown or dark brown, in contrast to the Fries, Gamborg, Hagem, MFM, and MS media, which did not discolor.

The optimal temperature for mycelial growth was 30°C, and the mycelial growth was reduced by 50% at 25°C. No mycelial growth occurred at 37°C (Fig. 1b). The optimal pH for mycelial growth was 4. The colony grew very slowly at pH 2 and failed to grow at pH > 7 (Fig. 1c).

Utilization of solid substrates

WPPH2 was able to grow in all types of grain media except for the peat-vermiculite mix. The sorghum grain medium was the best for mycelium production, and primordium-like structures developed only on this medium. WPPH2 grew slowly on rubber sawdust medium, producing only sparse mycelium.

Fructification on solid medium

Four *P. portentosus* basidiomes developed on two of seven plates of sorghum grain medium (two basidiomes per plate) (Fig. 2a, b). Initially, two small basidiomes (0.5 and 1.0 cm in diameter cap size) were found in one plate 17 days after transfer under the light/dark regimen with lower temperature. Three days later, basidiomes (0.4 and 1.5 cm diameter cap size) were observed on another plate. Under the culture conditions, only the larger basidiome on the former plate developed to maturity within 6–7 days and dispersed basidiospores that germinated on MMN agar. The basidiome was not full size, resulting from the limited volume of the container and depletion in nutrient and water supply. The stipe was stunted, and the texture was not firm. The basidiome produced in the laboratory is described as follows.

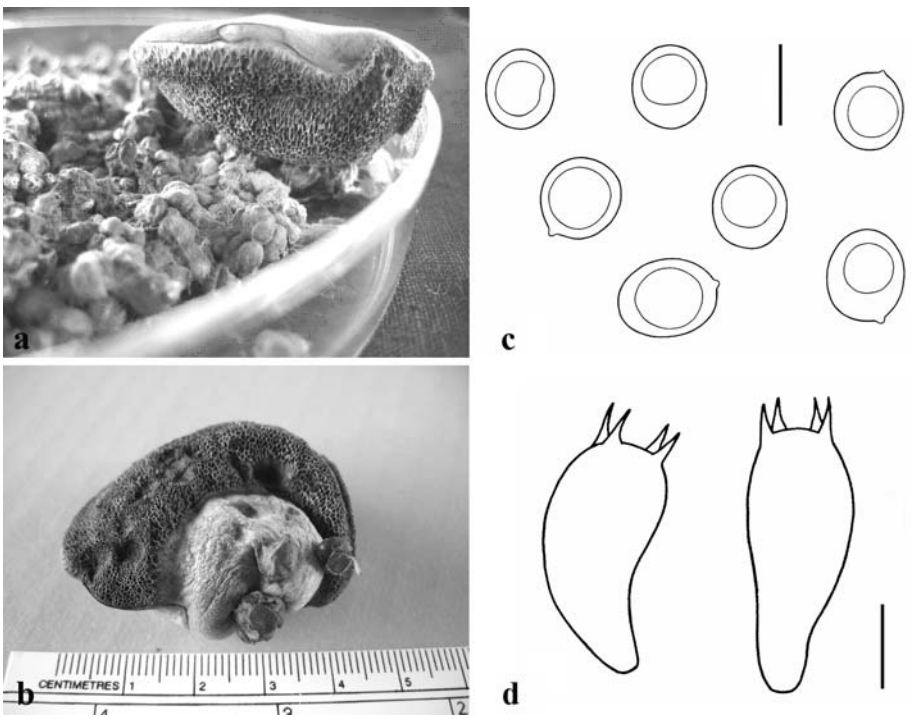

Fig. 2 *Phlebopus portentosus* (CMU46172). **a** Basidiome growing on sorghum grain medium. **b** Basidiome removed from medium showing stipe and hymenium. **c** Basidiospores. **d** Basidia. *Bars* 10 μm

Pileus 4.5 × 4 cm, brown in color, darker at the margin; total basidiome 2 cm in height. Tubes up to 0.6 cm long, brown, concolorous with cap or darker. Trama brownish, not bluish after injury. Spore print olive brown. Basidia 23–39 × 9–13 µm, clavate, bearing 4 spores; sterigma 4–9 µm long (Fig. 2c). Basidiospores (100 measured) (8.5–) 9.0–9.5 (–11.5) × (7.0–) 8.0–9.0 (–9.5) µm [$Q = (1.09–)\ 1.10–1.22\ (–1.32)$, $Q = 1.19 \pm 0.05$], broadly ellipsoid, sometimes subglobose or ellipsoid, smooth (Fig. 2d). Clamp connections present. Basidiome was deposited in the Chiang Mai University Herbarium as CMU 46172.

Molecular analysis confirmed the produced basidiome in the Petri dish (CMU46172) as *P. portentosus* (CMU2210 and WPPH2) as shown in Fig. 3. The ITS sequence of *P. portentosus* WPPH2 was deposited in GenBank with the accession number FJ603112.

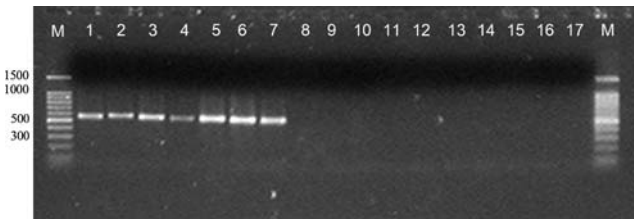

Fig. 3 Polymerase chain reaction (PCR) amplification using ITS1F and designed primer for *Phlebopus portentosus* (HAR2A): *lane 1 P. portentosus* WPPH2 (pure culture), *lane 2 P. portentosus* Hed Tub Tao (pure culture), *lane 3 P. portentosus* CMU46127, *lane 4 P. portentosus* CMU 2210, *lane 5 P. portentosus* CMUe0100, *lane 6 P. portentosus* CMU46168 (pure culture), *lane 7* fruit body (CMU46172) of cultured *P. portentosus* WPPH2, *lane 8 Helvella* sp., *lane 9 Laccaria amethystea*, *lane 10 Amanita hongoi*, *lane 11 Tricholoma fulvocastaneum*, *lane 12 Boletus* sp., *lane 13 Lactarius volemus*, *lane 14 Astraeus hygrometricus 15 A. hygrometricus*, *lane 16 A. hygrometricus* PHOR5, *lane 17* negative control, *M* 100-bp DNA ladder

ECM formation

Root tips of *Pinus kesiya* seedlings inoculated with WPPH2 were brown, 3 mm long, felty, Y-shape, paler at the end (Fig. 4a), and differed from those of uninoculated seedlings, which were simple in shape, thin, and whitish to brownish. ECM had a well-developed mantle, 15 µm across, consisting of 4–6 layers of hyphae, and a Hartig net extending between the epidermal cells and the outer cortex (Fig. 4b). Molecular analysis confirmed the presence of *P. portentosus* in the ECM tip. The PCR product by the primer ITS1F-HAR2A was about 550 bp (100% identity). None of the other plant species tested as hosts formed ECM of *P. portentosus*.

Discussion

This is the first report of the basidiome production of *Phlebopus portentosus* in vitro. The basidiomes did not show a typical bruising reaction, which might result from culture conditions restricting certain metabolic reactions or the absence of some compounds supplied from the host. Although this fungus is ECM, as confirmed by the greenhouse experiment with *Pinus*, it also expressed an ability to grow basidiomata without a host. Ji et al. (2007) obtained *P. portentosus* basidiomes in potted *Coffea arabica* plants. They claimed that the plants were ectomycorrhizal seem uncertain because *C. arabica* is an arbuscular mycorrhizal plant. Further work is required to optimize the conditions necessary to grow the fungus commercially. Very few members of the Boletales have been shown to fruit under axenic culture conditions. The five bolete taxa that are known to have the ability to fruit in pure culture are *P. sulphureus* (syn. *Buchwaldoboletus sphaerocephalus*) and *P. lignicola* (syn. *Buchwaldoboletus lignicola*) (Pantidou 1961, 1962), *Boletus rubinellus* (syn. *Chalciporus rubinellus*) (McLaughlin 1964, 1970), *B. reticulata*

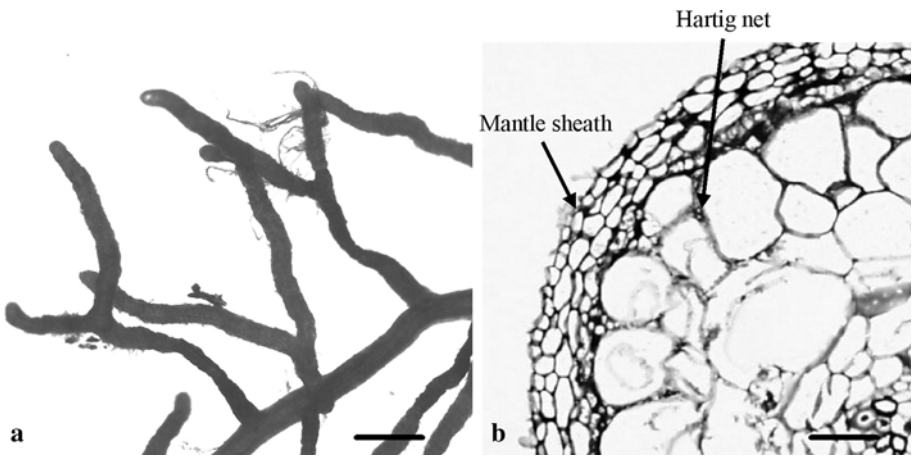

Fig. 4 External morphology (**a** *bar* 0.4 mm) and cross section (**b** *bar* 15 µm) of ectomycorrhizal (ECM) tips of *Phlebopus portentosus* WPPH2 on *Pinus kesiya*

(syn. *Ceriporia reticulata*) (Yamanaka et al. 2000), and a *Boletus* sp. (Ohta and Fujiwara 2003). Other agaric ECM fungi that have produced basidiomes in pure culture include *Lyophyllum shimeji* (Ohta 1994a, b; Kawai 1997), and *Hebeloma radicosum* (Ohta 1998).

Ohta (1994a, b, 1998) and Ohta and Fujiwara (2003) used barley grains and sawdust or barley grains and synthetic liquid medium as substrates to produce basidiomes of ECM fungi. Ohta and Fujiwara (2003) stated that ECM fungi require a large amount of carbohydrates to produce fruit bodies. Some ECM fungi have the ability to utilize starch and related compounds contained in grains (Ohta 1997). In contrast to these polysaccharides, a high concentration of monosaccharides in the media causes osmotic stress (Ohta 1998). Furthermore, it is suggested that changing the environmental conditions, such as temperature or light, can stimulate basidiome induction (Ingold 1979; Sakamoto et al. 2002; Marino et al. 2003). These factors should be studied further for *P. portentosus* as it is not known whether basidiome initiation resulted from the change in temperature, the photoperiod, or a combination of these factors.

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