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FULL PAPER

A sheathing mycorrhiza between the tropical bolete *Phlebopus spongiosus* and *Citrus maxima*

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Abstract *Phlebopus (Ph.) spongiosus* was recently described from several pomelo orchards (*Citrus maxima*) in southern Vietnam. This fungus was suspected to associate with pomelo plants as an ectomycorrhiza, although members of the genus *Phlebopus* have previously been presumed saprotrophic. To clarify this association, pomelo roots collected from the orchard (in situ roots), and those cultured with *Ph. spongiosus* (in vitro roots) in test tubes for 12 wk, were examined for ectomycorrhizal colonization. Both in vitro and in situ roots were analyzed for colonization using fungal LSU nuclear ribosomal DNA sequencing. The in situ roots exhibited the anatomical features of ectomycorrhizae: a thick fungal mantle, Hartig net, and extramatrical hyphae. The Hartig net, however, was very rare and showed discontinuous development. The in vitro association between *Ph. spongiosus* and *C. maxima* showed ectomycorrhiza-like structures, i.e., mantles and rhizomorphs in the plant roots, but no Hartig net development in the roots. Continuous hyphal penetration was restricted to the exodermis in both in situ and in vitro roots. Although the association between *Ph. spongiosus* and *C. maxima* could be considered ectomycorrhizal, its anatomy matches the unique feature known as sheathing mycorrhiza.

Keywords Black bolete · Ectomycorrhiza · Pomelo · Thick mantle

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Introduction

The pantropical genus *Phlebopus* (R. Heim) Singer includes about 12 species and is thought to be saprotrophic (Kirk et al. 2008), despite several records of mycelial crust formation between *Phlebopus* spp. and plant roots in the field, described as "crypta" (Singer 1986; Watling 2006), and the successes of ectomycorrhization with exotic trees in vitro (Kirk et al. 2008).

In the field, the best known phenomenon initially described by Goncalves and Milanez (1940 in Singer 1986) was a tripartite association among mycelia of *Phlebopus (Ph.) tropicus* (Rick) Heinem. & Rammeloo, roots of Amazonian citrus (*Citrus* sp.), and root aphids (*Pseudococcus comstocki* Kuwana). The vegetative mycelium of *Ph. tropicus* formed a crust around citrus roots and

enveloped colonies of root aphids that are usually "cultured" as "milk cows" by *Solenopsis* ants (Watling 2006). A similar structure was assumed to have evolved in several other *Phlebopus* spp. (Singer 1986; Tedersoo et al. 2010), such as the south Asian and Australian *Ph. marginatus* Watling & N.M. Greg. (Watling and Gregory 1988; Watling 2006). However, a Hartig net has never been observed to be formed by *Phlebopus* spp. (Singer 1986; Watling and Gregory 1988). The association between *Phlebopus* spp. and plant roots, in which Hartig net formation has never been observed, is described as "facultative" ectomycorrhization because of this ambiguity (Watling 2006; Tedersoo et al. 2010). Other associations of *Phlebopus* species with plants have been recorded, such as *Ph. beniensis* with the leguminous *Hymenaea courbaril* (Miller et al. 2000) and *Ph. marginatus* with eucalyptus species (Bougher 1995). Both have been presumed to be ectomycorrhizal without any definitive evidence.

Under in vitro conditions, ectomycorrhization has been morphologically achieved between *Ph. sudanicus* and *Acacia* species (Thoen and Ducouso 1989), and between *Ph. portentosus* and *Pinus kesiya* (Sanmee et al. 2010). However, the fungus associated with the *Acacia* spp. was suspected to be a *Scleroderma* contaminant rather than *Ph. sudanicus* (Tedersoo et al. 2010). In addition, the distribution of *P. kesiya* above 1,000 m elevation in subtropical Southeast Asia (Zonneveld et al. 2009) prevents it from naturally meeting *Ph. portentosus*, a pantropical species. The association between *Ph. portentosus* and *P. kesiya* may thus be merely artificial. Recently, Ji et al. (2011), from anatomical observation of plant roots in pot cocultures, suggested that *Ph. portentosus* is saprotrophic.

Citrus spp. are some of the most popular fruit crops in the world, and their roots normally have arbuscular mycorrhizae (Rayner 1935). According to Smith and Read (2008), *Citrus* is a typical arbuscular-mycorrhizal experimental genus, and the family Rutaceae (in which *Citrus* is placed) is not listed as having any ectomycorrhizal associations.

After the description of Boedijn (1951), almost all *Phlebopus* specimens in Southeast Asia and Australia were recorded as *Ph. portentosus*. Recent, *Ph. portentosus* sensu lato has been reclassified into *Ph. portentosus*, *Ph. marginatus* (Watling and Gregory 1988), and *Ph. spongiosus* (Pham et al. 2012), and several undescribed *Phlebopus* species are suggested (Watling 2001, 2006). *Phlebopus spongiosus* Pham & Har. Takah is an edible mushroom belonging to the Boletinellaceae (Boletales). This species, recently discovered in southern Vietnam, is characterized by the unique spongy tissue in the hymenophores of immature basidiomata (Pham et al. 2012). Most basidiomata have been found in pomelo orchards (*Citrus maxima*), appearing around the bases of the plants. Our aim was to investigate the association of *Ph. spongiosus* with pomelo trees in the field and its ability to form ectomycorrhizae in vitro with a *C. maxima* var. *hirado* host.

Materials and methods

Organisms

A mycelial strain of *Phlebopus spongiosus* was isolated from a paratype basidioma and maintained on modified Melin and Norkrans (MMN) medium (Marx 1969 in Brundrett et al. 1996) at 20° ± 1°C. The paratype basidioma was collected from a pomelo orchard (described below), and stored in the Natural History Museum and Institute, Chiba, Japan (CBM), as voucher specimen FB-38670 (Pham et al. 2012). Plant seeds of *Citrus maxima* var. *hirado* were obtained from the experimental orchard of Chiba University (Atagawa, Shizuoka Prefecture, Japan) and kept at 5°C until use. To attain aseptic seedlings, pomelo seeds were treated with 0.1% (v/v) solution of Joy Ultra dishwashing liquid (Procter & Gamble, Cincinnati, OH, USA) to remove exterior mucilage, washed with tap water, sterilized by shaking for 20 s in a sodium hypochlorite solution (minimum 5% available chlorine; Wako, Tokyo, Japan) with a drop of polyoxyethylene (20) sorbitan monooleate (Wako, Tokyo, Japan), and then rinsed aseptically with distilled water (four times, 2 min each). The seeds were then transferred to Murashige Skoog medium (Murashige and Skoog 1962), without sugar, and incubated at 25° ± 3°C in the dark. After 3–5 wk, 75–90% of the seeds had germinated, and these were cultured in the same medium for 2 wk. Seedlings were always cultured at 25° ± 3°C in a light:dark cycle of 15:9 h with a white fluorescent lamp (Hitachi, Tokyo, Japan), providing about 85 μmol/m^2 s on the substrate surface.

Mycorrhizal colonization

Substrate preparation

The substrate, as modified from Yamada and Katsuya (1995), consisted of 200 g dried vermiculite-sphagnum moss (Iris Ohyama, Sendai, Japan/Lixil Viva Corporation, Ageo, Japan) in a 49:1 (w/w) mixture, with 500 ml modified MMN liquid medium containing 0.1% glucose. Thirty-five milliliters of the substrate was poured into each glass test tube (25 mm diameter, 250 mm length; Pyrex Iwaki, Tokyo, Japan), corked with a Silicosen plug (Shin-Etsu Polymer, Tokyo, Japan), autoclaved at 121°C for 45 min, and cooled to room temperature.

Colonization preparation

Each *C. maxima* seedling was transferred into the prepared substrate and cultured for 1 wk before mycorrhizal inoculation.

A mycelial disk 4 mm in diameter was removed, using a cork borer, from subperipheral regions of colonies grown on MMN agar, and aseptically transferred into 20 ml MMN liquid medium in a 50-ml conical flask (Pyrex Iwaki, Tokyo, Japan), corked with a sterilized Silicosen plug. After 3–4 wk incubation, the whole mycelium in each flask was inoculated into substrate in test tubes containing the plant seedlings.

The combination of seedling, fungal inoculum, and substrate in each test tube is called a 'spawn.' Fifteen spawns were prepared and cultured for 12 wk before observation.

Field sample collection

The occurrence of *Ph. spongiosus* basidiomata was recorded using GPS (global positioning system: eTrex Legend H; Garmin, Olathe, KS, USA), as the area surrounding GPS point UTM 48P 646165mE 1151001mN. These sites were in a pomelo orchard (MyTho City, TienGiang Province, Vietnam) and were visited four times (January 2006, April 2006, January 2010, and October 2010) for collection of pomelo roots. Each time, the soil around the basidiomata was dug up and carefully examined for *C. maxima* root fragments that were connected to mycelia originating from the basidiomata.

In situ root fragments and leaves of *C. maxima* were also collected from different sites of the same orchard to check the identification and grafted status of these plants. The surfaces of all root fragments and leaves were rinsed with tap water (Brundrett et al. 1996; Yamada and Katsuya 1995) immediately after collection, then immersed in 40°C CTAB buffer [2 g hexadecyl trimethyl-ammonium bromide, 10 ml 1 M Tris pH 8.0, 4 ml 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0, 28 ml 5 M NaCl, 40 ml distilled water, 1 g polyvinylpyrrolidone 40 (MW 40,000), with pH adjusted to 5.0 with 1 M HCl and made up to 100 ml with distilled water] (Saghai-Maroof et al. 1984). This buffer was kept at room temperature before use.

Molecular analysis

Total nuclear DNA (nDNA) of dried mycelia and basidiomata was extracted from disintegrated tissue using 200 µl 0.5-mm glass beads (Yasui Kikai, Tokyo, Japan) and 500 µl TES buffer [50 mM Tris–HCl, pH 7.5, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS)] by vigorous shaking (FastPrep System; mp-Biomedicals, Solon, OH, USA) at 6.5 m/s for 45 s. Total nDNA from infected roots was extracted by the same method, but using CTAB buffer instead of TES buffer. The outer layer of the roots of *C. maxima* used for plant identification was peeled off, and their cores were ground using a mortar and pestle; the leaves of *C. maxima* used for plant identification also were ground using a mortar and pestle. Total nDNA was then extracted using the foregoing protocol. Soluble fractions were recovered by centrifugation. DNA was purified using TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and saturated phenol/chloroform/isoamyl alcohol (Nippon Gene, Tokyo, Japan) extraction followed by an isopropyl alcohol precipitation. After desiccation of the DNA pellet, DNA was dissolved in 30 µl TE buffer. For some samples, genomic DNA was further purified using NucleoSpin Extract II (Macherey-Nagel, Duren, Germany), following the manufacturer's recommendations.

For plant identification, the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were polymerase chain reaction (PCR-) amplified from total nDNA extracted from leaves and roots of field-collected *C. maxima* and sequenced using the primers ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCG TAACAAGG-3′) (White et al. 1990), following Kyndt et al. (2010). To verify the *Ph. spongiosus* strain used, 28S rDNA (large subunit, LSU) was amplified from the total nDNA extracted from dried basidiomata and mycelia of *Ph. spongiosus* and sequenced using the primers LR0R (5′-A CCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTGAGG GAAACTTCG-3′) (Vilgalys and Hester 1990).

To identify the fungal symbiont within the ectomycorrhizae, a *Ph. spongiosus*-specific primer pair Phl-LSUF (5′-TTGATGTCAGTCGCGTCGAT-3′) and Phl-LSUR (5′-AAGTCCCGAACCCTCGAAGA-3′) was designed by comparison of the LSU sequences of *Ph. spongiosus* with those of other fungal species, using Primer-BLAST on the NCBI website. nDNA from root fragments was initially used for LSU amplification using primers LR0R and LR5, and then the product was reamplified using the primer pair Phl-LSUF and Phl-LSUR. The amplified fragment (Phl-LSU) was sequenced using the primers Phl-LSUF and Phl-LSUR, to confirm that the PCR product was actually the *Ph. spongiosus*-specific gene fragment.

PCR reactions were carried out using KOD FX (Toyobo, Tokyo, Japan) following the manufacturer's instructions. PCR products were purified using NucleoSpin Extract II, and DNA fragments were directly sequenced using the BigDye Terminator ver3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the protocol provided. Reactions were cleaned up using the Centri Sep (Princeton Separations, Adelphia, NJ, USA), before analyzing by capillary electrophoresis on a 3130x DNA Analyzer (Applied Biosystems). Sequences were

assembled and edited using ATSQ software (Genetyx, Tokyo, Japan).

Observation

The ectomycorrhiza-like fragments of both in situ and in vitro roots were examined and photographed under a stereomicroscope (Stemi 2000; Zeiss, Oberkochen, Germany). Each fragment was then hand-sectioned by razor blade under the stereomicroscope and observed using differential interference contrast (DIC) on a Nomarski microscope (Axio Image X1; Zeiss, or B51; Olympus, Tokyo, Japan), and a fluorescence microscope (BH2; Olympus). Morphological and anatomical descriptions followed Agerer (1994, 2006).

Results

Molecular analysis

In total, two LSU fragments from dried basidiomata (a holotype CBM FB-38014 and a paratype CBM FB-38670 stored in the Natural History Museum and Institute, Chiba, Japan) and one from a mycelium were obtained by PCR using primers LR0R and LR5. All three LSU sequences were analyzed and found to be identical. This LSU sequence, 894 bp in length, was deposited in the DDBJ/EMBL/GenBank nucleotide sequence database, under accession number AB673396. By BLAST (NBCI) searching, our LSU sequence of *Ph. spongiosus* was found to be identical to *Phlebopus* sp. strain REH8795 (FJ153623).

The Phl-LSU fragment was 204 bp in length, as expected. All Phl-LSU sequences from in vitro roots were identical with those from the dried basidiomata and the mycelium. Fragments of in situ roots used for morphological analysis were positive with the primer pair Phl-LSUF–Phl-LSUR, and their Phl-LSU sequences were identical with the LSU sequences from the dried basidiomata and the mycelium.

The ITS sequence from in situ leaves was identical to that from in situ roots prepared for plant identification. By BLAST searching, we found that the ITS sequence from leaves or roots closely resembled the ITS sequence FJ641954 from *C. maxima* (Kyndt et al. 2010) with one per 682-bp difference. Sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession number AB673398 for the leaf specimen and AB673397 for the root specimen; this showed that the pomelo trees in the field belonged to *C. maxima* and were not grafted plants.

Cultured mycelial growth and colonization on *C. maxima* roots

Colonies of *Ph. spongiosus* covered the whole surface of each MMN agar plate about 4 wk after inoculation. Colonies on MMN agar were yellow, close to the substrate surface, somewhat striate at margins, and forming mycelial bundles but not forming rhizomorphs. Mycelia grown in MMN liquid medium (20 ml broth in a 50-ml flask) constituted 43.6 ± 10.9 mg dry biomass after 3–4 wk of inoculation. In both agar plates and liquid medium, culture changed the color of the substrate from pale yellow to dark yellow after 3–4 wk inoculation, and then to pure black depending on the length of the incubation period. The hyphae of *Ph. spongiosus* usually had clamp connections in aseptic culture (Pham et al. 2012).

Mycelia were observed weaving about each other between particles of substrate in the spawn tubes 3 wk after inoculation. *Citrus* roots in spawn tubes were partially colonized by fungal mycelium after 6–7 wk of inoculation (Fig. 1c). After 12 wk of incubation, all *C. maxima* seedlings had an ectomycorrhiza-like morphological characteristic, as described next.

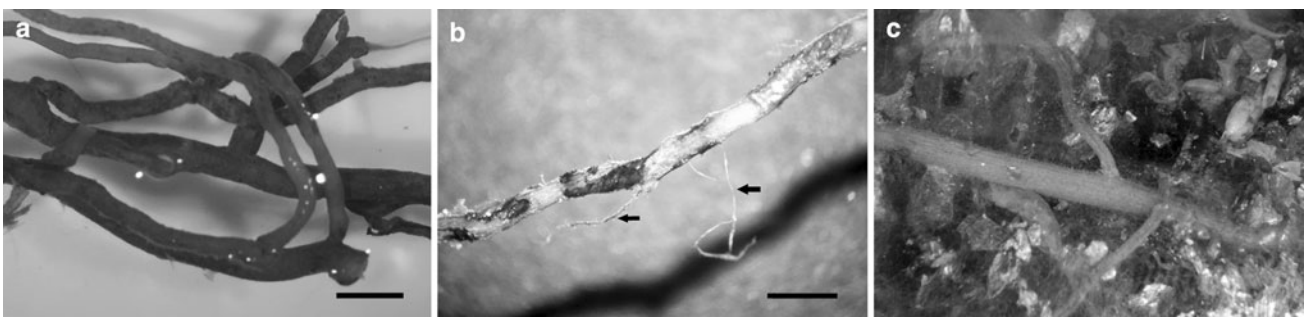

Fig. 1 Macrocharacteristics of ectomycorrhizal association between *Citrus maxima* and *Phlebopus spongiosus*. **a** In situ ectomycorrhiza. **b** In vitro ectomycorrhiza with rhizomorphs (*black arrows*). **c** In vitro root fragments of *C. maxima* var. *hirado* colonized by mycelia of *Ph. spongiosus*. *Bars* **a**, **b** 2 mm

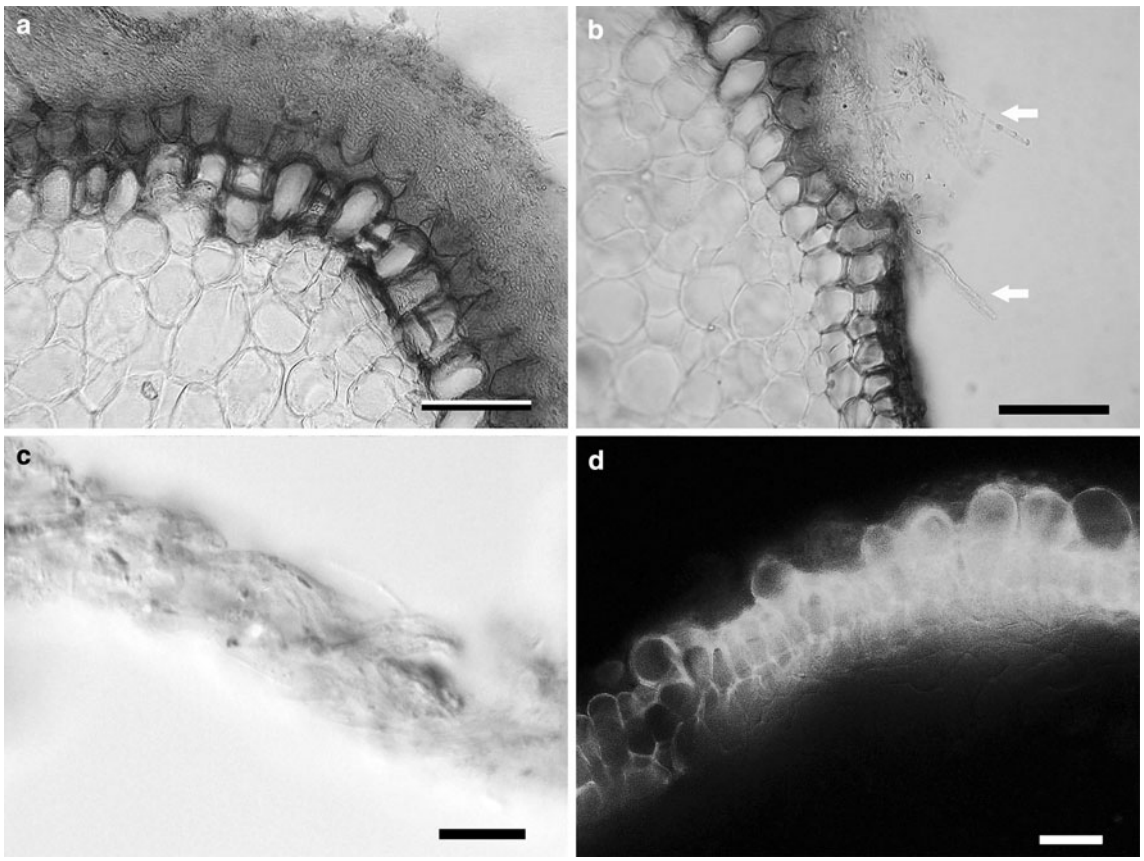

Fig. 2 Micromorphological characteristics of the ectomycorrhizal association between *Citrus maxima* and *Phlebopus spongiosus*. **a** Thick mantle observed in vertical section of an in situ root with fungal hyphae. **b** Thin mantle observed in vertical section of an in situ root with fungal clampless hyphae (*white arrows*). **c** Rhizomorph in an in vitro root. **d** Autofluorescence of a vertical section of an in situ root. *Bars* **a**, **b**, **d** 30 μm; **c** 10 μm

Morphological characteristics of the mycorrhizae

The mycorrhizal tips were yellow to brown, darker in the in situ roots; approximately 0.5–1.0 mm in diameter; branched, partly curved, and somewhat thickened (Fig. 1a). The mycorrhizal mantles in the in vitro specimens were mostly thin (sticking closely to the outermost cell layer; Fig. 2b), but those in the in situ specimens were often more than 20 μm thick (Fig. 2a). Thick mantles were composed of two layers, the outer and inner mantles, both of which could be seen as different mycelial arrangements, respectively, in the transversal (Fig. 2a). The outer mantle was loose with directional arrangement of hyphal bundles. The inner mantle was compact with a differentiated arrangement of hyphal bundles. Inside hyphae were 2–3 μm in diameter; clamp connections were not observed (Fig. 2b). The Hartig net was patchily present at the boundary between root exodermis and inner cortex, with loosely arranged broad fungal cells in a palmate shape (Fig. 3). The root exodermal cells showed strong autofluorescence (whitish yellow) under UV irradiation, indicating cell wall suberization (Peterson et al. 2004); however, cortical cells

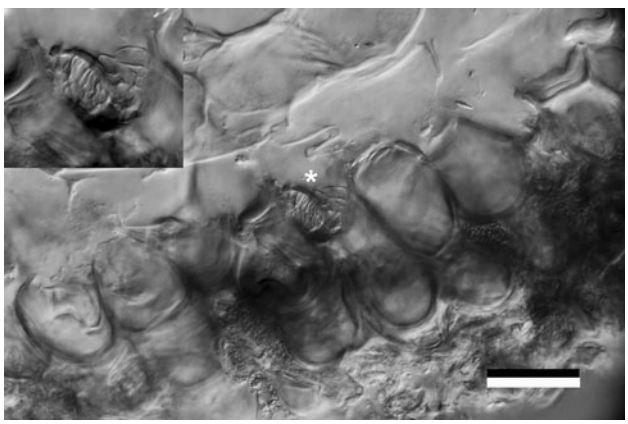

Fig. 3 Hartig net (*white asterisk*) in vertical section of an in situ root (higher magnification of Hartig net in *inset* in *upper left of figure*). *Bar* 10 μm

did not show this (weak yellowish gray autofluorescence) (Fig. 2d). Hyphae were not observed penetrating beyond the exodermis in almost all the roots. Rhizomorphs were only observed in the in vitro roots of *C. maxima* seedlings (Figs. 1b, 2c); these were yellow or pale brown, with

differentiated cell types and twisted structures with randomly distributed thick and thin hyphae.

Discussion

The mantles of *C. maxima* roots colonized by *Ph. spongiosus* were easily observed, but the Hartig net was rarely seen in the in situ roots. Transverse section of the thick mantle (Fig. 2a) suggests its plectenchymatous structure and differentiation into at least two mantle layers. As we did not conduct enough mantle observation to describe the "plan views" (Agerer 1994), further study is necessary to clarify the fungal anatomical characteristic. The lack of Hartig net in the in vitro mycorrhiza might be caused by a difference of fungal strains between in situ and in vitro. Even in a typical ectomycorrhizal association between *Pisolithus* isolates and its host, *Eucalyptus grandis* (Burgess et al. 1994), some fungal strains were unable to form a Hartig net. The similar case was also recorded in a fungal strain of *Paxillus involutus* that was unable to form a Hartig net with a *Betula pendula* host (Feugey et al. 1999). Also, nutrient deficiency or lack of micronutrients in the in vitro substrate might also cause the lack of Hartig net, as has been suggested in the in vitro ectomycorrhizae of the aspen *Populus tremula* (Langer et al. 2008) and Casuarinaceae species (Theodorou and Reddell 1991). The almost complete lack of Hartig net on the mycorrhizae between *Ph. spongiosus* and *C. maxima* in the present study is similar to the "sheathing mycorrhiza" of *Pisonia grandis* roots in Australia, which shows a distinct fungal mantle but lacks a Hartig net (Ashford and Allaway 1982, 1985; Cairney et al. 1994). However, as the field-sampled *Citrus* roots that are colonized by *Ph. spongiosus* rarely had a patchy Hartig net, this mycorrhizal association would be regarded as ectomycorrhizal. To clarify whether *Ph. spongiosus* has the ability to form a distinct Hartig net development on a given potential host may aid the understanding of the mycorrhizal association between this fungus and *C. maxima.*

Ph. spongiosus colonization and the patchy Hartig net formation were mostly limited to the exodermis of the mycorrhizal root tip despite the development of a thick mantle. The limitation could be determined by the physiochemical strength of the plant cell wall. The exodermal cells of *C. maxima* showed a suberization with thick walls and much evidence of intracellular phenolic compounds (Fig. 2d). Most ectomycorrhizal plants show distinct suberization of cell walls only in the endodermal cells; this probably prevents Hartig net development from passing through these cells (Smith and Read 2008). The lack of rhizomorphs in the in situ roots could be explained by being broken during cleaning off their soil cover. The local soil has a high clay content and quickly becomes compact and hard in drought conditions.

Since the discovery of "cryptas" by Goncalves and Milanez (1940: Singer 1986), several researchers have discussed the association of *Phlebopus* spp. with plants. However, some studies involved unproven or confused examples or were not authenticated, such as *Ph. beniensis* (Miller et al. 2000), *Ph. sudanicus* (Thoen and Ducouso 1989), and *Ph. marginatus* (Bougher 1995). Some associations failed, including *Ph. bruchii* (Nouhra et al. 2008) and *Ph. portentosus*, in the study by Ji et al. (2011). The only success has been between *Ph. portentosus* and *Pinus kesiya* (Sanmee et al. 2010), and this was merely artificial association. In nature, the distribution of *P. kesiya* above 1,000 m elevation above sea level in subtropical areas (Zonneveld et al. 2009) prevents its interaction with *Ph. portentosus*, a pantropical species (Boedijn 1951; Kirk et al. 2008). Almost all mycorrhizal researchers of *Citrus* spp. have reported arbuscular-mycorrhizal formation but not ectomycorrhization (Smith and Read 2008). *Citrus* usually appears to form endomycorrhizae (Rayner 1935) and is considered a typical, arbuscular-mycorrhizal experimental genus (Smith and Read 2008). Our study is the first record of ectomycorrhization between *Citrus* and *Phlebopus* species in the field and in vitro.

A further field study is required to clarify the effect of farm management procedures on ectomycorrhization, as well as the interactions between ectomycorrhizae and arbuscular mycorrhizae on *Citrus* spp. In situ *C. maxima* roots have not been surveyed at sites where *Ph. spongiosus* was not found. Comparing symbiotic effects of both types of mycorrhizae could be useful in the development of Vietnamese horticulture. A further study should clarify whether *C. maxima* ectomycorrhizae form any tripartite associations (Goncalves and Milanez in Singer 1986). Although we did not record a tripartite association between *Ph. spongiosus*, *C. maxima*, and root aphids, we did learn from locals about the presence of root aphid-like insects at the citrus orchards we investigated. The effect of *Ph. spongiosus* on the growth of the host should also be examined in other *Citrus* species, to enable understanding of fungal effects on Vietnamese *Citrus* orchards and the implications for management.

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