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FULL PAPER

In vitro mycorrhization of edible *Astraeus* mushrooms and their morphological characterization

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Abstract *Astraeus* is the most popular and the most expensive edible ectomycorrhizal mushroom group consumed in Thailand, although it is not commonly consumed in other countries. The purpose of this work was to test the ability of *Astraeus* to form ectomycorrhizae with seedlings of *Pinus densiflora* in vitro. A single 1-week-old *P. densiflora* seedling was transplanted into a glass bottle containing autoclaved forest soil, and suspensions of more than 10^6 basidiospores of *Astraeus odoratus* or *Astraeus hygrometricus* were inoculated onto the seedling. Cultured mycelium was also inoculated onto an axenic pine seedling planted on sterilized vermiculite–sphagnum moss mixture in a glass bottle. Mycorrhization of *A. hygrometricus* was observed a few months after inoculation in both treatments. Fungal species identity of both the inoculum and ectomycorrhizae was assessed by PCR–RFLP analysis of the ITS region of rDNA. At 5–6 months after inoculation, *A. hygrometricus* ectomycorrhizae developed a sheath, rhizomorphs, and Hartig net on pine lateral roots. No ectomycorrhizal formation was found after *A. odoratus* inoculation. These results suggest that *A. hygrometricus* can form mycorrhizae on pine seedlings. The external morphological characteristics of *A. hygrometricus* ectomycorrhizae were distinct from those of other Boletales ectomycorrhizae. This article provides the first morphological and anatomical description of *A. hygrometricus* ectomycorrhizae.

Keywords Ectomycorrhizas · In vitro synthesis · Astraeus · Spore inoculation · Mycelium

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Introduction

Astraeus belongs to the family Diplocystidiaceae, in Boletales, Agaricomycetes, Basidiomycota (Binder and Bresinsky 2002; Kirk et al. 2008). The young basidiomata are commercially harvested as wild edible mushrooms in northern and northeastern Thailand. Although no valid data on the annual harvest of these mushrooms are available, the mushrooms are collected in large quantities, and many are preserved in cans for domestic consumption or exported to other Asian countries. Currently, this is one of the most valuable ectomycorrhizal mushrooms in Thailand, and prices of fresh basidiomata range from 300 to 400 Baht ($7–10 US)/kg (Sanmee et al. 2003). Even in Japan, local harvesting of this fungal group as wild edible mushrooms is known (Ogawa 1992).

Astraeus species are common ectomycorrhizal fungi and are found throughout the world. In Asia, *Astraeus* consists of three species and one variety: *A. odoratus* Phosri, Watling, M. P. Martín & Whalley (syn. *A. thailandicus*; Phosri et al. 2004); *A. asiaticus* Phosri, M. P. Martín & Watling (Phosri et al. 2007); *A. hygrometricus* (Pers.) Morgan (Morgan 1889; Ito 1959), and *A. hygrometricus*

var. *koreanus* V. J. Staněk (Staněk 1958; Kreisel 1976; Imazeki and Hongo 1989). In other geographic regions, *A. hygrometricus* (North America, South America, Europe, Africa, and Australia) and *A. pteridis* (North America and the Canary Islands) are known (Robert 2003; Baseia and Galvão 2001; De Roman et al. 2005; Phosri et al. 2007). *Astraeus hygrometricus* and *A. pteridis* form ectomycorrhizal associations with several host tree species including *Pinus*, *Pseudotsuga*, *Alnus*, *Eucalyptus*, and *Castanea* but *A. odoratus* and *A. asiaticus* have been reported only in dipterocarp forests (Trappe 1967; Malajczuk et al. 1982; Phosri et al. 2007).

To exploit ectomycorrhiza, experiments on the interaction between the symbionts and screening of potential host–fungus combinations are required. This investigation can only be accomplished by synthesizing ectomycorrhiza between known symbionts under controlled conditions. For this, it is desirable to approximate the conditions of natural ecosystems (Peterson and Chakravarty 1991; Yamada et al. 2006). Although establishment of mycorrhizal synthesis in vitro in *Astraeus* has been studied in *A. hygrometricus* and *A. pteridis* (Table 1), available information on their morphology and anatomy is scant. Nothing is known about in vitro mycorrhizal synthesis of *A. odoratus* and *A. asiaticus*. Trappe (1967) described ectomycorrhizae synthesized between *A. pteridis* and Douglas fir (*Pseudotsuga menziesii* var. *menziesii*) as pale brown mycorrhizae, with sparingly tomentose surfaces with numerous pale cinnamon to blackish rhizomorphs appressed against and emanating from the mantle, cylindrical to irregularly branched individual mycorrhiza, 1–12 mm in length and 0.4–0.7 mm in diameter. Malajczuk et al. (1982) determined the mycorrhization ability of *A. pteridis* with many species of eucalypts and *Pinus radiata,* finding it to have a broad host range. The *A. pteridis* mycorrhizae on eucalypts were unbranched with lateral roots often encased within a fungal mantle. Danielson (1984) synthesized ectomycorrhizae between *A. hygrometricus* and *Pinus banksiana* and found that they had a compact dichotomous to coralloid form, vinaceous tint, and abundant vinaceous tinted rhizomorphs.

Although *A. hygrometricus* and *A. pteridis* have a wide host range in their ectomycorrhization, *A. odoratus* shows specific association with dipterocarp forests in nature. At present, ectomycorrhizal synthesis in vitro between *A. odoratus* and a host plant species is undocumented. To establish a cultivation method for edible ectomycorrhizal mushrooms, mycorrhizal synthesis with commonly used plants such as pine is important. As Japanese *A. hygrometricus* is known to be associated with *Pinus densiflora* in nature, a comparative mycorrhization study between *A. odoratus* and *A. hygrometricus* with pine as a common host is ideal. In addition, *P. densiflora* is one of the most important timber species in Japanese forests. Our objectives in this study were (a) to characterize cultured mycelia of *Astraeus* from Thailand and Japan on nutrient medium; (b) to evaluate their ability to form ectomycorrhiza on *P. densiflora* in vitro; and (c) to describe the morphology and anatomy of the synthesized mycorrhizae.

Materials and methods

Fungal collection and pure culture establishment

Specimens of *Astraeus* basidiomata were collected from Thailand and Japan. Samples were freeze-dried and kept in the laboratory. Young basidiomata were surface sterilized with 70% ethanol, and inner tissues were inoculated on modified Norkrans's "C" agar medium (MNC; Yamada and Katsuya 1995). *Astraeus* mycelia that grew from inocula within 4 to 7 days were transferred to another MNC plate for pure culture establishment. Established mycelium strains (Table 2) were subcultured monthly for the subsequent experiments. Cultured mycelia on MNC agar plates that were maintained for a few months at 25°C were observed in terms of colony color and hyphal morphology. A piece of mycelium from each strain was transferred onto a glass slide with a drop of lactic acid and observed under a DIC Nomarski microscope with high magnification (Olympus BX51TF). Several strains were deposited at the NITE Biological Resource Center (NBRC). In addition, several fresh and mature basidiomata were preserved at 4°C until used for the following spore inoculation experiment.

Mycorrhizal synthesis

Both spore suspensions and pure cultured mycelia of all *Astraeus* strains from Thailand and Japan were used in this experiment. A spore suspension from a few basidiomata was prepared according to Castellano and Molina (1989). A drop of Tween 80 was added to the suspension to ensure homogeneous spore dispersion. Spore density was checked via hematocytometry and was adjusted to $>10^6$ spores/ml water. Spore suspensions were kept at 4°C and used as an inoculum. In the case of in vitro mycorrhizal synthesis, cultured *Astraeus* mycelia on an MNC agar plate were cut into 2–3 mm^3 blocks, and four blocks were transferred into 20 ml MNC liquid media in a 75-ml glass bottle. After 2 months, mycelia colonies were used as inocula for mycorrhizal synthesis.

Sterilized pine seedlings were prepared following the method of Yamada et al. (1999). Pine seeds were washed three times in sterilized 0.01% (w/w) Tween-80 solution using a vortex mixer, surface sterilized in 2% (w/w) calcium hypochlorite solution for 10 min, rinsed three times

Table 1 Reported mycorrhization success of *Astraeus* in vitro by using cultured mycelium inoculum

Astraeus species	Host plant	Reference
A. hygrometricus	*Pinus banksiana*	Danielson (1984)
A. pteridis	*Alnus incana*	Molina (1981)
	A. rubra	Molina (1979)
	A. sinuata	Molina (1981)
	Arbutus menziesii	Molina (1982)
	Arctostaphylos uva-ursi	Molina (1982)
	Eucalyptus spp.	Malajczuk et al. (1982)
	Larix occidentalis	Molina and Trappe (1982)
	Picea sitchensis	Molina and Trappe (1982)
	Pinus contorta	Molina and Trappe (1982)
	P. monticola	Molina and Trappe (1982)
	P. ponderosa	Molina and Trappe (1982)
	P. radiata	Malajczuk et al. (1982)
	Pseudotsuga menziesii	Molina and Trappe (1982), Trappe (1967)
	Tsuga heterophylla	Molina and Trappe (1982)

Table 2 List of *Astraeus* strains established in this study

Astraeus species	Strain number	Origin			Remarks
		Country	Location	Putative host plant	
A. hygrometricus	Aswan16	Japan	Sukagawa, Fukushima	(Commercially harvested)	NBRC 106048
	Aswan17	Japan	Sukagawa, Fukushima	(Commercially harvested)	
	Aswan18	Japan	Kooriyama, Fukushima	(Commercially harvested)	
	Aswan19	Japan	Machida, Tokyo	*Quercus* sp.	
	Aswan20	Japan	Okaya, Nagano	*Pinus densiflora*	
	Aswan28	Japan	Kumakougen, Ehime	*Quercus* sp.	NBRC106049
	Aswan45	Japan	Kayanokougen, Nagano	*P. densiflora*	
	Aswan46	Japan	Kayanokougen, Nagano	*P. densiflora*	
	Aswan53	Japan	Tsukuba, Ibaraki	(not determined)	
	Aswan54	Japan	Ina, Nagano	*P. densiflora*	NBRC106050
	Aswan56	Japan	Matsumoto, Nagano	*Cedrus deodara*	
A. odoratus	Aswan27	Thailand	Bankog, Uttaradit,	Dipterocarpaceae	
	Aswan29	Thailand	Numpad, Uttaradit	Dipterocarpaceae	
	Aswan32	Thailand	Numpad, Uttaradit	Dipterocarpaceae	
	Aswan35	Thailand	Numpad, Uttaradit	Dipterocarpaceae	
	Aswan80	Thailand	Numpad, Uttaradit	Dipterocarpaceae	

NBRC, National Institute of Technology and Evaluation, Biologica Resource Center, Japan

with sterilized distilled water, and placed on MNC agar plates and incubated at 23°C. Seeds germinated in 7–15 days.

In the spore inoculation experiment, 1-week-old *P. densiflora* seedlings were individually transplanted into 200-ml glass bottles containing 100 cm^3 autoclaved forest soil, and 10 ml of the $\geq 10^6$ basidiospore suspension of *A. odoratus* or *A. hygrometricus* was inoculated onto the seedling by dropping the spore suspension near the seedling stem. In the in vitro mycorrhizal synthesis, a sterilized vermiculite–peat moss mixture (80:1, v/v) containing ~60% (v/v) MNC liquid medium with 0.2% glucose (Yamada et al. 1999) was used. Cultured mycelia of both species were dissected into several segments with fine forceps and dispersed throughout the substrate. At the same time, one aseptically germinated seedling (3–7 days old) was transplanted into a sterilized 200-ml glass bottle containing 100 cm^3 vermiculite–peat moss mixture as described above. All inoculated glass bottles were incubated at 20°C under continuous 140 $\mu mol^{-2}\ m^{-2}\ s^{-1}$ light for

4–6 months. Ectomycorrhizal formation was checked every week by observing the inoculated bottles under a dissecting microscope.

PCR–RFLP analysis of synthesized mycorrhizae

To confirm that ectomycorrhizae formed from the *Astraeus* inoculum, the internal transcribed spacer (ITS) region of the ribosomal DNA was analyzed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (Gardes and Bruns 1993). Total DNA was extracted from a few milligrams of mycelium on agar plates or from 3–5 ectomycorrhizal tips. PCR amplification of the ITS region was done with a 25-μl reaction mixture including 1× PCR buffer, 2.5 U of Taq DNA polymerase (TaKaRa, Tokyo, Japan), 0.2 mM of each dNTP, 0.5 μM of each primer pair, and 1.5 mM $MgCl_2$. The PCR was performed with a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 3 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min 30 s; and a final step of 72°C for 10 min. The primer pair ITS1F/ITS4B was used for the first-round PCR and ITS1/ITS4 for the second-round PCR. PCR products were electrophoresed on 1.5% agarose gel immersed in TBE buffer [90 mM Tris-borate, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and visualized by ethidium bromide staining. Amplified products were digested with *Hae*III, *Rsa*I, and *Cfr*13 (TaKaRa, Tokyo, Japan) at 37°C for 3 h. The digested samples were electrophoresed on 3% agarose gel to determine the restriction fragment length polymorphisms (RFLP). The restriction fragments were compared to reference RFLPs from *A. hygrometricus* and *A. odoratus* that could be estimated from the sequence data in GenBank.

Morphological and anatomical characterization of ectomycorrhizae

Seedlings that were inoculated with *A. odoratus* or *A. hygrometricus* were removed from the glass bottles, and ectomycorrhizal root systems were cut off. The external morphology of mycorrhizae was examined and photographed under a dissection microscope (Zeiss Stemi 2000C). Morphological descriptions of mycorrhizae followed Agerer (1986, 1987–2006) and Brundrett et al. (1996). Further microscopic characterizations were made using a DIC Nomarski microscope (Yamada et al. 1999). To confirm ectomycorrhizal anatomy, i.e., the presence of a Hartig net, we prepared transverse sections of mycorrhizal tips from each fungal inoculation. Photographic images of hyphae, ectomycorrhizal root tips, emanating hyphae, and mantle cell size were analyzed from at least 50 portions by using Adobe Photoshop (Adobe Systems) and NIH imaging software.

Results

Culture characterization of *Astraeus*

A total of 5 strains of *A. odoratus* from Thailand and 11 strains of *A. hygrometricus* from Japan, respectively, were established. All *A. odoratus* strains had yellowish-brown to slightly dark greenish-brown velvety mycelia on MNC agar plates (Fig. 1a). All *A. hygrometricus* strains had dark brown to dark greenish-brown velvety mycelia on MNC agar plates (Fig. 1b), which were darker than those of *A. odoratus*. Both species had abundant light brown aerial mycelia and matted or smooth mycelia submerged in the medium with an almost regular margin. Both species produced a yellowish to reddish, dark brown pigment that diffused through the media, causing a change in the original color. The release of these pigments was greater in *A. hygrometricus*. In both *A. odoratus* and *A. hygrometricus*, the vegetative hyphae varied in shape from straight to somewhat curved and formed abundant clamp connections on septa (Fig. 1c–e). Hyphal diameters of *A. odoratus* and *A. hygrometricus* were 2.63–5.39 and 2.19–3.54 μm, respectively. Pigmented knobs of hyphae were formed laterally or intercalarily (Fig. 1g). Rhizomorphs (Fig. 1f, h) were observed in every strain of both *Astraeus* species, which had similar to highly differentiated rhizomorphs in ectomycorrhizal tips (see following). *Astraeus hygrometricus* had brownish intracellular pigments on the mycelium and young rhizomorph (Fig. 1h).

Ectomycorrhiza formation

Four weeks after inoculation of cultured mycelium, the mycelium was growing well on particles of vermiculite–peat substrate. However, the mycelial growth was obscured in soil that had been inoculated with spores. Ectomycorrhizal tips were first observed 6–8 or 8–10 weeks after inoculations with cultured mycelia or spores, respectively. In *A. hygrometricus*, both types of inocula formed ectomycorrhizae on pine seedlings. However, in *A. odoratus*, no ectomycorrhiza formation was observed. The PCR products of the ITS regions of *A. hygrometricus* ectomycorrhizae from both types of inoculation were the same size, approximately 700 bp (Fig. 2). All PCR products had the same RFLP pattern with the three endonucleases. In addition, the RFLP pattern matched the estimated RFLP pattern from the sequence data of Japanese *A. hygrometricus* (accession number AB507397). Therefore, all

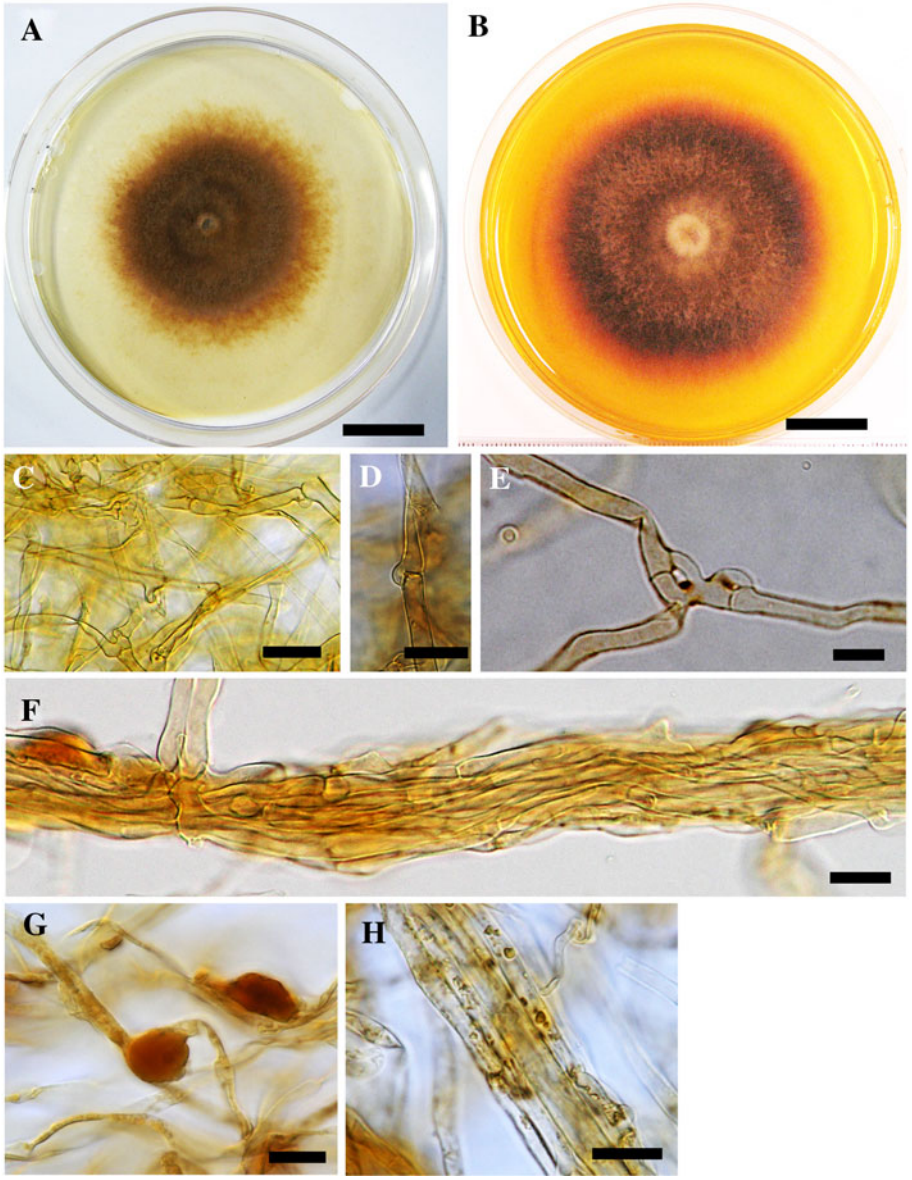

Fig. 1 Pure culture characteristics of *Astraeus odoratus* and *Astraeus hygrometricus*. *A. odoratus* strain Aswan 32 (**a**) and *A. hygrometricus* strain Aswan 21 (**b**) were cultured on modified Norkrans's "C" agar (MNC) medium for 40 days. Vegetative hyphae of *A. odoratus* were varied in shape (**c**) and formed clamp connections on septa (**d**). Vegetative hyphae of *A. hygrometricus* with clamp connections on septa (**e**). Rhizomorphs of *A. odoratus* on MNC medium (**f**). Pigmented knobs of hyphae (**g**) of *A. hygrometricus*. Greenish-brown intracellular pigments (**h**) observed on the rhizomorph hyphae of *A. hygrometricus*. *Bars* **a**–**b** 2 cm, **c**–**h** 5 μm

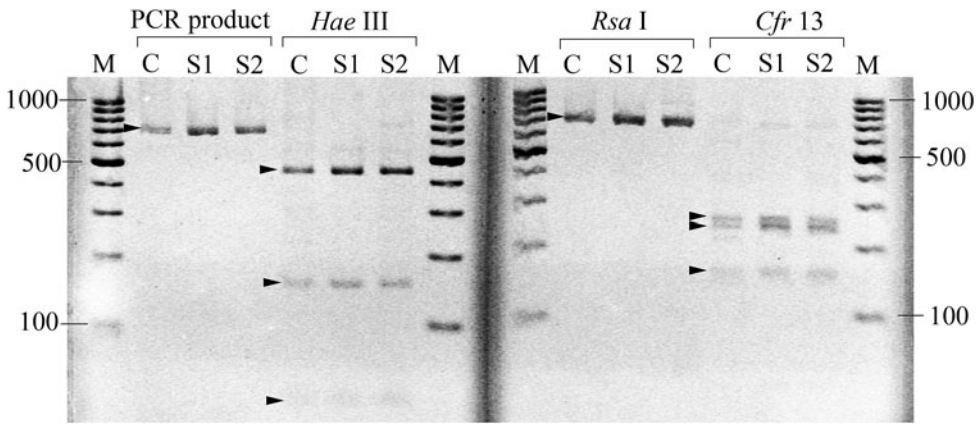

Fig. 2 Restriction fragment length polymorphism (RFLP) patterns of polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS) region from inoculum and synthesized mycorrhizae. *C*, cultured mycelium of strain Aswan 56; *S1*, ectomycorrhizal root tips from the mycelium inoculation; *S2*, ectomycorrhizal root tips from spore inoculation; *M*, molecular markers (100–1000 bp). *Arrows* indicate distinct DNA fragments

synthesized ectomycorrhizae were formed by the colonization of *A. hygrometricus*.

Morphological and anatomical description of synthesized *A. hygrometricus* ectomycorrhizae

Ectomycorrhizal root systems from both types of inoculation showed few differences under a dissecting microscope. Mycorrhizae formed from the spore inoculum had well-developed fungal sheaths, extraradical mycelia, and many rhizomorphs. In the cultured mycelium inoculation, the fungal sheath was thinner and the rhizomorphs were finer. The color of mycorrhizae from spore inoculation tended to be darker. The Hartig net structure was similar in size and pattern between inocula. Abundant crystals were observed within rhizomorphs, on the surface of emanating hyphae, and the fungal mantle of mycorrhizae in both inoculation treatments. The ectomycorrhiza of *A. hygrometricus* on *P. densiflora* is as follows.

Macromorphology

Mycorrhizal root systems forked and were dichotomously ramified. The unramified end was straight to somewhat slightly curved, 1.35–4.80 mm in length (Fig. 3a). Mycorrhizal root tips were white to light yellow when young, then became light to pale yellow, and were dark brown when older. Dark brown rhizomorphs were attached to the fungal mantles at restricted points. Numerous light brown emanating hyphae grew out from the mantle surface and were concentrated in the tips or older parts of the ectomycorrhizae. The mantle surface was shiny, bright yellowish, and densely covered by many smooth concolorous fine rhizomorphs. No color change was observed when the fungal sheath was injured (Fig. 3b).

Micromorphology and anatomy

Emanating hyphae connected to the fungal sheath were straight, 2.76–4.75 mm in diameter, and with clamp connections; rhizomorph types e and f in texture; central thick hyphae with thick cell walls; clamp connections observed on single hyphae in the rhizomorphs, especially on anastomosing hyphae; surface outer mantle layer (Fig. 3e) of fungal sheath of the net transitional type between plectenchymatous and pseudoparenchymatous; constructed hyphae 3.88–11.12 μm in diameter with intercellular crystals (Fig. 3c); crystals fine, granular in shape, also present on surfaces of external hyphae and rhizomorphs; surface mantle layer with oblong cystidia (Fig. 3d), middle mantle layer net plectenchymatous (Fig. 3f); hyphae rather irregularly arranged, no special pattern discernible, but hyphae often growing longitudinal to root orientation; constructed hyphae 2.56–4.89 mm in diameter; inner mantle layer plectenchymatous, with ringlike arrangement of hyphal bundles; constructed hyphae 2.29–5.12 μm in diameter; Hartig net developed continuously at the cortex of the roots (Fig. 3g); the thin-walled, interwoven net intrudes between cells of root cortex.

Discussion

Although *Astraeus* is a common member of ectomycorrhizal fungal communities, little is known about the mycorrhizal morphology, host specificity, and fruiting ecology of this genus. *Astraeus odoratus* and *A. asiaticus* in Thailand were clearly distinguished by Phosri et al. (2007) in terms of their morphology and DNA sequences of fruiting bodies. However, these species lack studies on the mycorrhizae and pure culture. The observed culture characteristics, e.g., mycelial growth pattern, colony, and pigment color, differed between *A. odoratus* and *A. hygrometricus*. Sims et al. (1999) reported that cultural characteristics enabled clear species separation of isolates and, together with growth rates, suggested geographically linked intraspecific variation in populations of *Pisolithus*, which is closely related to *Astraeus* (Binder and Bresinsky 2002).

A few weeks after inoculations, *Astraeus* mycelia were growing well around particles of substrate in the cultured mycelium inoculation but not in the spore inoculation. This observation suggests an advantage of mycelium inoculation because the mycelium can maintain structural integrity and is completely protected from microbial competition. In addition, the vermiculite substrate itself might be advantageous for mycelium growth compared to substrates such as sand, perlite, or cereal grains (Smith and Read 2008). Ectomycorrhizal tips were first observed 6–8 weeks after inoculation with cultured mycelia and 8–10 weeks after inoculation with spores. Inoculation with mycelia gave more rapid mycorrhization than with spores, which may be explained by the colonization dynamic of the propagules used, i.e., hyphae, spores, sclerotia, and mycelial strands. Small propagules, such as basidiospores, are not capable of extensive growth in soil for long distances, so colonization to roots from spores needs sufficient numbers to make contact with the roots. Much larger propagules, i.e., sclerotia and rhizomorphs, are generally capable of growing through soil for considerable distances, and high infection levels will be obtained from fewer propagules (Bowen 1994). In some ectomycorrhizal associations, mycelial strands associated with live roots are an effective inoculum source over large distances (Fleming 1983).

No ectomycorrhizal formation was observed in *A. odoratus* inoculations throughout the 6-month incubation

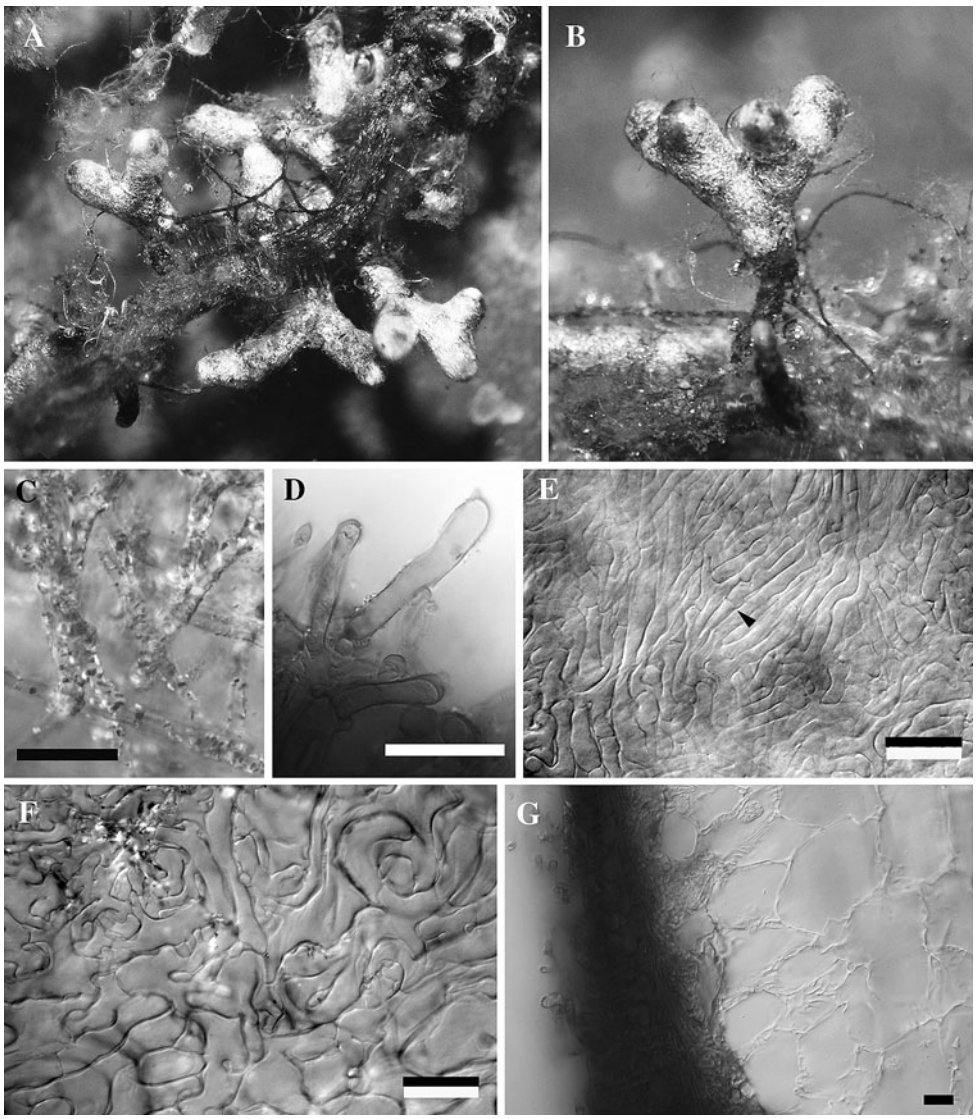

Fig. 3 Morphology and anatomy of *Astraeus hygrometricus* ectomycorrhizae. An ectomycorrhizal system colonized by spore inoculation (**a**, **b**) shows dichotomous branching mycorrhizal tips with a well-developed fungal mantle, extraradical mycelium, and many rhizomorphs. Small particles of crystalline ornamentation (**c**) occur on emanating hyphae. Planar view of the surface mantle layer with oblong cystidia (**d**), subsurface mantle layer (**e**) showing plectenchymatous hyphae having clamp connections (*arrowhead*), and middle mantle layer shows densely plectenchymatous organization (**f**). A transverse section of the mycorrhiza shows the Hartig net structure at the root cortex (**g**). *Bars* **c**–**g** 5 μm

period, suggesting that *A. odoratus* showed host specificity and did not establish a mycorrhizal association with the pine host. This result follows the ecological specificity of *A. odoratus* mycorrhization in nature. Phosri et al. (2004) and Petcharat (2004) reported that *A. odoratus* habitats are sandy or lateritic soil in dry lowland dipterocarp forests in northern and northeastern areas of Thailand. No case of *A. odoratus* fruiting under pine forests has been documented. Our data suggest that *A. odoratus* lacks the ability to form ectomycorrhizae with *P. densiflora*. Test plants closely related to dipterocarp tree species should be examined for mycorrhizal synthesis in future experiments. In the case of *A. hygrometricus*, both spore and mycelium inoculation formed ectomycorrhizae on *P. densiflora* seedlings. *Astraeus hygrometricus* forms ectomycorrhizal associations with several host tree species, including pines, e.g., *Pinus banksiana* and *Pinus virginiana* (De Roman et al. 2005).

This is the first report on mycorrhization between *A. hygrometricus* and *P. densiflora* in vitro. However, in vitro mycorrhizal syntheses of *A. hygrometricus* and *A. pteridis* with other host plants have been reported (Trappe 1967; Malajczuk et al. 1982; Danielson 1984). In the present study, mycorrhizal tips of *A. hygrometricus* had a pale yellow mantle that became dark brown to black with age, with dark brown or blackish rhizomorphs, similar to mycorrhizae formed between *A. hygrometricus* and *P. banksiana* (Danielson 1984), and *A. pteridis* and *Pseudotsuga menziesii* (Trappe 1967). Trappe (1967) also described tough rhizomorphs that darken with age in pure cultures which resemble clustered rhizomorphs at the bases of basidiocarps, similar to our results. In addition, we showed that the surface outer mantle layer was a transition between plectenchymatous and pseudoparenchymatous, with intercellular crystals. The color of the

A. hygrometricus ectomycorrhizae is considered a distinctive trait, as are the well-developed fungal sheath and the abundance of rhizomorphs. In *Pisolithus tinctorius*, mycorrhizal color is yellowish brown to bright yellow, which is different from *Astraeus* sp. (García-Rodríguez et al. 2006). Our study provides the first description of cultured mycelia of *Astraeus* on nutrient medium, as well as morphological and anatomical descriptions of *A. hygrometricus* ectomycorrhizae with *Pinus densiflora.*

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