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Lu-Min Vaario · Shu-Tang Xing · Zong-Qiang Xie · Zhi-Ming Lun · Xue Sun · Yu Hua Li

In situ and in vitro colonization of *Cathaya argyrophylla* (Pinaceae) by ectomycorrhizal fungi

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Abstract *Cathaya argyrophylla*, a critically endangered conifer, is found to grow at four isolated areas located in subtropical mountains of China. To examine the involvement and usefulness of mycorrhizas for sustaining the population of this tree, we compared the root system, morphology, and structure of mycorrhizal roots of *C. argyrophylla*, which were collected from a natural stand and an artificial stand, each grown at a different location. More mycorrhizal roots were found for trees from an artificial stand. The presence of extramatrical mycelium, mantle, and Hartig net revealed that *C. argyrophylla* formed an ectomycorrhizal association in both sampling sites. Starch granules were found in mycorrhizal roots collected only from a natural stand. The aseptic synthesis of *C. argyrophylla* and *Cenococcum geophilum* was established for the first time in vitro. Typical ectomycorrhizas formed on seedlings on RM medium containing 0.1 g/l glucose, 5 weeks after inoculation. By light microscopy, the synthesized mycorrhizas showed a thin mantle from which emanated extramatrical hyphae and highly branched Hartig net. A simple, rapid, and convenient mycorrhiza synthesis system was developed, which facilitates further studies on ectomycorrhizal development of *C. argyrophylla*.

Keywords *Cathaya argyrophylla* · Ectomycorrhizal fungi · In vitro · In situ · Hartig net

L.-M. Vaario · S.-T. Xing · Z.-M. Lun · X. Sun · Y. H. Li (✉)
Research Institute of Flower Biotechnology,
Northeast Forestry University,
Harbin 150040, China
e-mail: yuhuali05@163.com
Tel.: +86-451-82191738
Fax: +86-451-82191738

Z.-Q. Xie
The Laboratory of Quantitative Vegetation Ecology,
The Institute of Botany, CAS,
Beijing 100093, China

Introduction

Cathaya argyrophylla, which is a relic species of a monotypic genus endemic to China (Silba 1986; Vidakovic 1991), is distributed in provinces of Guangxi, Hunan, Sichuan, and Guizhou (Fig. 1; Wang 1990). It grows at narrow mountain ridges with sharp slopes, at the top of solitary rocky mountains, or in the crevice of sheer precipices and overhanging rocks at altitudes of 940–1,870 m. Pollen fossils of this plant have been found in the Asia–Europe continental deposits of the Tertiary period; thus, *Cathaya* is honored to be called as "Panda" of the plant kingdom. The development of the embryo in *Cathaya* is similar to that of *Pinus* (Hu et al. 1976; Hu and Wang 1984; Wang 1990). *C. argyrophylla*, which is native to the subtropical mountain regions of the northern hemisphere, has great ecological importance within natural forests (Xie 1995). However, this tree is now facing problems in natural propagation, which include seed loss due to predispersal predation and the decreased seed germination rates, and competition with shade-forming broad-leaved trees. Thus, it is threatened by habitat loss (Ying et al. 1983). The population is decreasing year by year. It is reported that there are less than 5,000 trees in China (Xie et al. 1999). This species is listed as endangered by the State Environmental Protection Administration of China (1992).

As mycorrhizas can improve fitness of plants, knowledge of mycorrhizas is particularly important for ecological restoration, preservation, and maintenance of endangered species (Smith and Read 1997). In particular, ectomycorrhizal fungi provide a major link between carbon fixed by primary producers and other trophic levels in plant ecosystems. Information on the mycorrhizal status of *C. argyrophylla* can be crucial for the implementation of successful conservation strategies; however, it is little available so far.

Our present study had two objectives. The first one was to verify whether *C. argyrophylla* is a mycorrhizal species and to compare the mycorrhizal status of these trees from both a natural stand and an artificial stand. The second was to characterize in vitro the interaction between *C. argyrophylla*

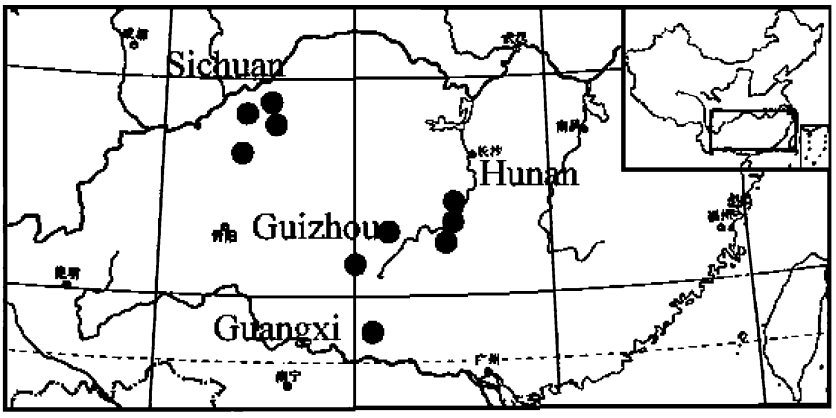

Fig. 1 Distribution map of *Cathaya argyrophylla* (from http://www.plant.csdb.cn/sdb/teyou/ty.htm)

and several ectomycorrhizal fungi with broad host ranges and to establish a mycorrhizal seedling culture system in *C. argyrophylla*.

Materials and methods

Study sites and sampling of soil

C. argyrophylla trees were investigated at two locations. First site is a small plantation of 30 transplanted *C. argyrophylla* trees at 23–28 years old, located at Guangxi Institute of Botany, Guangxi Zhuangzu Autonomous Region, and The Chinese Academy of Sciences, Yanshan, Guilin, China (elevation of 195 m, 25°04′10″N, 110°17′59″). Second site is a natural forest, where there were 219 *C. argyrophylla* trees, located at Dayaoshan, Jinxiu, Guangxi, China (elevation of 1,100 m, 24°9′–24°24′N, 109°27′E). The oldest tree of them is more than 500 years old. Five soil samples were randomly collected from each site and stored at 4°C until analysis. Soil pH (Thomas 1996), the contents of organic carbon, total nitrogen (The Standards of Forest Systems, P. R. China, 2000), and nitrate-nitrogen and ammonium-nitrogen (a Skalar SAN Plus segmented flow analyzer, Skalar, Inc., Norcross, GA, USA) were measured.

Collection and classification of root samples

Soil samples with five replicates for each site were collected by digging out a soil core of 10×10×10 cm near a *C. argyrophylla* tree. They were put in paper bags, taken to a laboratory, and kept at 4°C. The soil core with roots of *C. argyrophylla* was soaked in tap water of a beaker. Root tips were observed under a stereomicroscope and categorized by color as black, brown, white, or other color according to Agerer (1987–2002). Percentage of colonization by ectomycorrhizal fungi was measured using the gridline intersection method (Brundrett et al. 1996). Only fine roots with less than 2-mm diameter were counted in quantification because wider roots were not colonized.

Fungal culture

Cenococcum geophilum and *Pisolithus tinctorius*, both of which were deposited in the culture collection of Laboratory of Forest Botany, University of Tokyo, Japan, as strains FBCg1 and Pt2, were kindly provided by Prof. Suzuki. *Boletus edulis* was kindly provided by Prof. X-F Yan, Northeast Forestry University, China. These three isolates were maintained at 23±2°C in darkness on modified Melin–Norkran medium (MMN; Marx 1969), which contained 10 g/l glucose instead of sucrose and was solidified with 1.5% agar.

Preparation of plant material

Seeds of *C. argyrophylla* were collected in a natural forest in Guangxi Province, China, in 2002. They were air-dried and stored in a polyethylene bag in darkness at 4°C. The seeds were surface-sterilized in 70% ethanol for 1 min and then in 30% H_2O_2 solution for 30 min. Following air drying, they were placed on agar medium containing 5 g/l glucose for 7 days, and then, the seed capsule was removed with a knife and forceps. After germination, the seedlings were transplanted on modified MS media (Murashige and Skoog 1962), which contained 0.2 mg/l 6-benzylaminopurine (6-BAP) and 0.02 mg/l α-naphthaleneacetic acid (NAA) with one fourth strength of macroelements, a strength of microelements, and organic elements prescribed for MS medium, 20 g/l sucrose, and 8 g/l agar.

Aseptic synthesis of ectomycorrhizas

Clear plastic culture plates (200×90×10 mm) were filled with 80 ml RM medium (Vaario et al. 2002). For each culture plate, one sterile seedling was laid directly on the agar surface and covered with a sheet of autoclaved filter paper (Hangzhou Xinhua Paper Ltd.) to maintain root surface moisture. The plates were then incubated for 1 week, after which the seedlings were well attached to agar medium. The cover paper was then aseptically removed, and

subsequently, three plugs of fungal mycelium of 6-mm diameter were placed on the medium adjacent to lateral roots of a seedling. The plates were sealed with Parafilm (American Can Company, Detroit, MI, USA). The lower part of the plate, where both the host root system and ectomycorrhizal (ECM) fungus were developing, was covered with aluminum foil. Fifty-eight seedlings were selected for ectomycorrhiza synthesis including 20 for control, 20 inoculated with *C. geophilum*, 10 inoculated with *P. tinctorius*, and 8 with *B. edulis*.

All incubations were carried out at 3,000 lx by diffuse fluorescent light at 23±2°C at a 16-h photoperiod.

Preparation for studying ECM structure

Mycorrhizal and control root samples collected at both field and laboratory conditions were immersed in 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer (pH 7.2) overnight at 4°C. They were then rinsed three times in 0.1 M phosphate buffer (pH 7.2), postfixed for 90 min in 2% OsO_4 (SPI-CHEM TM, West Chester, PA, USA) in the same buffer, and dehydrated in an ascending acetone series in 20% increments, each for 20 min, followed by three changes, each for 15 min, of 100% propylene oxide. The mycorrhizal roots were finally embedded in Epon 812 resin (Heidelberg, New York). Thin sections were cut with a glass knife on an ultramicrotome (Leica Ultraunt R) and gently heat-fixed to glass microscope slides. The sections were then stained in 0.1% toluidine blue in 1% acetate buffer for 10 min, kept in tap water for 15 min, air-dried, then mounted in 100% glycerin beneath a cover slip, and examined with an Olympus BX51 light microscope.

Results

Site and soil investigation

Investigation of pedological soil showed a difference between soils collected in a natural stand at Dayaoshan and an artificial stand at Yanshan (Table 1). Dayaoshan soil had a higher percentage of organic C and N as well as a higher concentration of NO_3^--N than Yanshan soil. There was no significant difference in NH_4^+-N concentration of soil between both sites. The pH of both site soils was lower than 3.8, especially Dayaoshan soil, being strongly acidic at pH 2.7.

Ectomycorrhizal roots were found in both sites. There were four main types of mycorrhizal roots, which had such characteristics in color of fungal mantle, type of ramification, and features of mantle surface as shown in Table 2. Two types were found in both sites. Many mycorrhizal roots of white, glossy type were found at the Yanshan site but not at Dayaoshan. Also, a much higher percentage of mycorrhization was observed at the Yanshan site (Table 1).

Table 1 Comparison of two sampling sites of *Cathaya argyrophylla*

Site	Dayaoshan	Yanshan
Type of stand	Natural stand	Artificial stand
Altitude (m)	1,100	190–200
No. of *C. argyrophylla* trees	219	30
Soil type	Red soil	Yellow soil
Humus type	Fulvic acid type	Fulvic acid type
Soil pH	2.7±0.3	3.8±0.2
C (%)	6.4±2.9	1.2±0.2
N (%)	0.5±0.0	0.2±0.0
NO_3^--N (ppm)	33.8±5.9	18.4±7.0
NH_4^+-N (ppm)	24.1±16.8	9.2±1.3
Mycorrhizal colonization (%)	34.9±11.5	54.1±8.9[a]

[a]Using the gridline intersection method (Brundrett et al. 1996)

Microscopic features of excavated roots

Microscopic observation revealed detailed characteristics of mycorrhizal roots from both sites. A yellowish and unramified type was selected from both sites for microscopic observation (Fig. 2a). In root samples from Dayaoshan, thick mantles and big tannin cells were found (Fig. 2b,c). Intercellular space between cortical cells are colonized by fungal hyphae (Fig. 2b,c). The highly branched, distinctively multilobed, and fan-shaped Hartig net was confirmed in plan view (Fig. 2d). In the root samples from Yanshan, distinct mantles and Hartig net were also found (Fig. 2e). In contrast, nonmycorrhizal roots from both sites had root hairs but no Hartig net structure (data not shown).

Development of mycorrhizal roots in vitro

C. argyrophylla + *C. geophilum* Three weeks after inoculation, a taproot produced short lateral roots, some of which came into contact with hyphae arising from the fungal inocula. Lateral root formation occurred in all

Table 2 The macroscopic characteristics of ectomycorrhizas collected from both a natural stand and an artificial stand

Type of mycorrhizas	Dayaoshan	Yanshan
Black, unramified, smooth mantle	++	−
Light brown, dichotomous, smooth mantle	+	+
Yellow, unramified or dichotomous, reticulate	+	+
White with gloss, monopodial pyramidal, smooth	−	+++

*-, absent;
+, less than 25% tested samples
++, 25–50% tested samples
+++, more than 75% tested samples

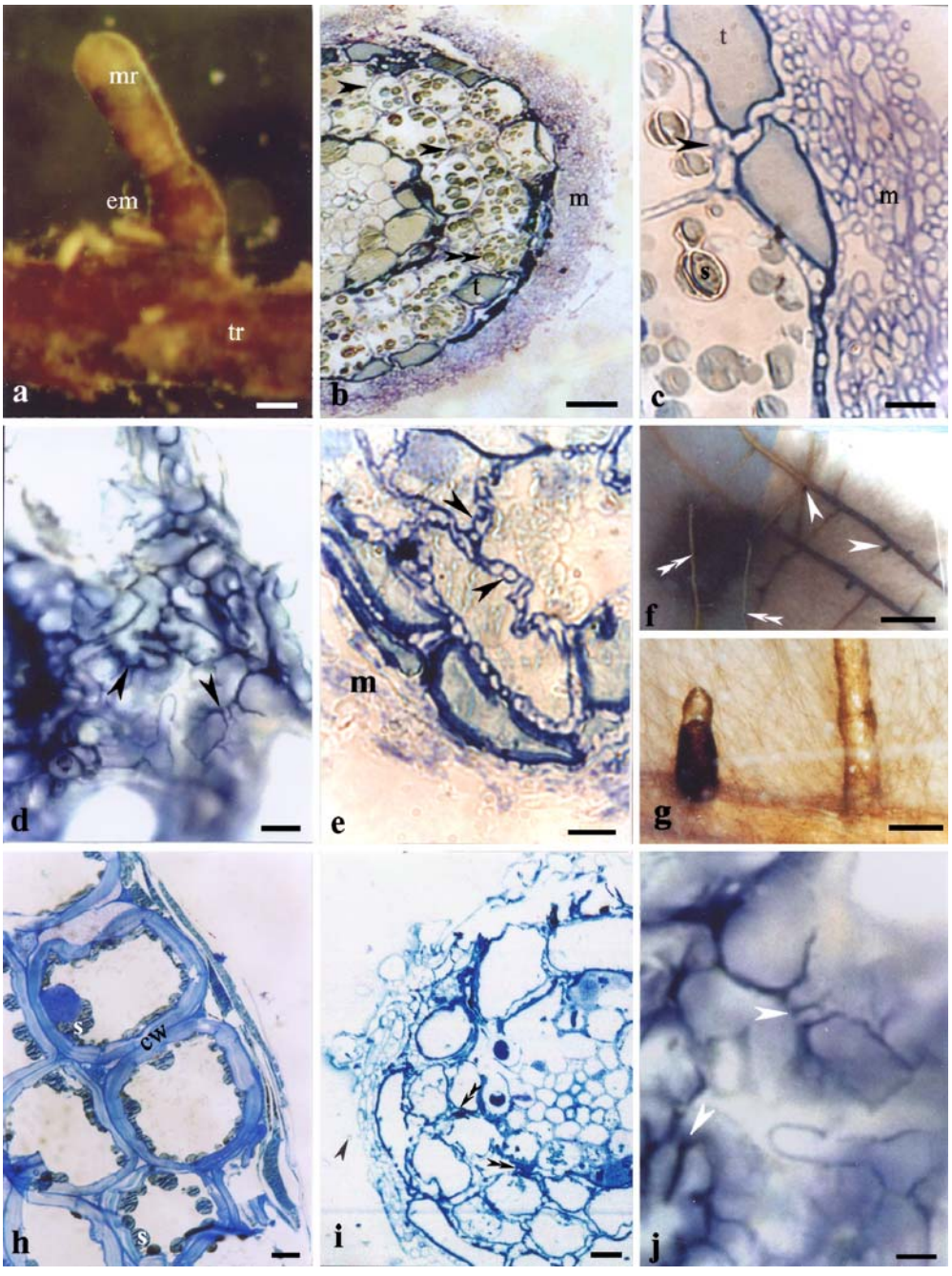

Fig. 2 External morphology and light micrographs of *C. argyrophylla* mycorrhizal roots and nonmycorrhizal roots. **a** External morphology of *C. argyrophylla* mycorrhizal roots showing taproot (*tr*), mycorrhizal root (*mr*), and external mycelium (*em*). *Bar*, 500 μm. **b** Light micrograph of a mycorrhiza collected at Dayaoshan. The thick mantle (*m*) overlies the epidermis, which is filled with tannins (*t*). A number of starch granules (*double arrowheads*) are present in the cortical cells. The intercellular Hartig net (*arrowheads*) colonized the epidermis and cortex. *Bar*, 100 μm. **c** Magnification of **b** showing thick mantle (*m*), tannin cells (*t*), and Hartig net structure (*arrowheads*). *Bar*, 20 μm. **d** Longitudinal section of mycorrhizal roots collected at Dayaoshan showing the distinctively multibranched pseudoparenchymatous structure of the Hartig net in plan view (*arrowheads*). *Bar*, 10 μm. **e** Light micrograph of a mycorrhiza collected at Yanshan. Mantle (*m*) overlies the epidermis. The intercellular Hartig net (*arrowheads*) colonized the epidermis and cortex. No starch granule is present. *Bar*, 20 μm. **f** The view of an in vitro mycorrhization between *C. argyrophylla* and *Cenococcum geophilum* showing unramified short mycorrhizal root (*arrowheads*) and elongated roots with a hyphal mantle at their base (*double arrowheads*). *Bar*, 1 cm. **g** Magnification of **f** showing typical *C. geophilum* mycorrhiza with black mantle. *Bar*, 2 mm. **h** Light micrograph of in vitro uninfected root showing distinctive thick cell wall (*cw*) of cortical cells and a number of starch granules (*s*) in cortical cells. *Bar*, 30 μm. **i** Light micrograph of in vitro mycorrhizal roots. A loose and thin mantle (*arrowheads*) lies on roots. Hartig net (*double arrowheads*) colonizes epidermis and cortex with no starch granules present in the cell. *Bar*, 50 μm. **j** Longitudinal section through the distal cortex of a mycorrhiza showing the distinctively multibranched pseudoparenchymatous structure of the Hartig net (*arrowheads*). *Bar*, 10 μm

Fig. 3 A mature tree of *C. argyrophylla* at approximately 26 years old, growing at the artificial stand at Yanshan, Guangxi, China

seedlings inoculated with ectomycorrhizal fungi and also occurred in 14 out of 20 control seedlings. After 5 weeks, a network of black extraradical hyphae was formed around the bases of short lateral roots, and some roots stopped elongating. Some still elongated but failed to form root hairs in *C. argyrophylla* + *C.geophilum* plates (Fig. 2f). After 6–7 weeks, *C. geophilum* had completely enveloped the entire lateral root and formed black mycorrhizas with a rough mantle surface (Fig. 2g). However, some lateral roots did not stop elongating and were only enveloped by a mantle at the basal portion (Fig. 2f). *C. argyrophylla* + *C. geophilum* mycorrhizas were straight and unramified (Fig. 2f,g) and consisted of a thin, loose fungal mantle. Mantle hyphae gave rise to profuse hyphae, which invaded and colonized the epidermal and cortical cell walls, and developed a partially thick Hartig net (Fig. 2i). Hartig net hyphae invaded host cortex and enveloped host cells with a uniseriate or multiseriate layer of fungal cells but did not penetrate them (Fig. 2i,j). In cross section of an uninoculated control *C. argyrophylla* root grown in the same culture plate, thicker cell walls of cortex were observed. Furthermore, there were starch granules in cortical cells (Fig. 2h). However, these starch granules were not found in mycorrhizal roots (Fig. 2i).

C. argyrophylla + *P. tinctorius* Fungal hyphae contacted lateral roots 3 weeks after inoculation but did not envelop roots. Later, roots still elongated and formed root hairs. No mycorrhization was confirmed by microscopy.

C. argyrophylla + *B. edulis* Fungal hyphae grew quickly and covered the whole roots 3 weeks after inoculation. Finally, hyphae overgrew the whole of a seedling, the needles turned yellow, and then the seedling died. No mycorrhization was found between these two partners.

Discussion

It was proved that *C. argyrophylla* was a typical ectomycorrhizal tree according to both studies in fields and laboratories. Although mycorrhizas of *C. argyrophylla* were reported previously in a Chinese language publication (Wang 1990), this was the first time that ectomycorrhizas were described in situ and in vitro. It was shown that *C. argyrophylla* formed typical ectomycorrhizas similar to other pine trees.

According to investigation carried out at two sites, *C. argyrophylla* growing in an artificial stand had exuberant foliage with high branching, fresh green needles, and a sturdy trunk (Fig. 3). In contrast, *C. argyrophylla* in a natural stand had sparse foliage, especially in young trees, although they gained vigor once they grew over forest canopy. With regard to growth behavior, some studies suggested that *Cathaya* trees grew suitably in nutrient-poor soils and that they were not shade-tolerant (Xie et al. 1999). *C. argyrophylla* trees grew well in gaps formed in the natural forests; however, adult trees were stressed by inadequate light (Xie 1995). In an artificial stand, sun light was provided enough for growth, and the contents of C and N in soil were lower with a higher frequency of mycorrhiza formation (Table 1). These results suggested that environmental conditions in both stands of *C. argyrophylla* influenced tree growth and also mycorrhizal formation.

In the present study, there was a noticeable difference in amount of starch in cortical cells between mycorrhizal roots collected from two sites. Starch granules were frequently observed in cortical cells of nonmycorrhizal roots formed by trees in fields (data not shown) and in vitro seedlings (Fig. 2h). There were starch granules in mycorrhizal roots collected in a natural stand at Danyaoshan (Fig. 2b,c). However, no starch granules were found in mycorrhizal roots in an artificial stand at Yanshan (Fig. 2e) and in those of *C. argyrophylla* + *C.geophilum* in vitro (Fig. 2h). Root starch is a kind of energy storage for trees so that the presence of starch may imply relatively low metabolism in roots. Once a mycorrhizal root forms, fungal hyphae will utilize a carbon source provided by a host tree for their growth and functions in roots and soil. One possibility for the presence of many starch granules at the Dayaoshan site might be that mycorrhizas were not functional anymore when sampled, or mycorrhizas at the Yanshan site might have been more active than those at the Dayaoshan site. Jordy et al. (1998) reported that the density of starch grains increased in plant cells of *Paxillus involutus–Betulapendula* ectomycorrhizas, especially at the early infection stage.

The long-term goal of our study is to understand the physiological and biochemical events that control ectomycorrhization of *Cathaya* trees. Therefore, it is necessary to establish a simple, rapid, and convenient mycorrhiza synthesis system. In this study, *C. geophilum* was selected as a candidate for in vitro ectomycorrhizal synthesis in *Cathaya* since *C. geophilum* has frequently been described in

ectomycorrhizal association with economically important tree families such as Myrtaceae, Salicaeae, and Pinaceae (Trappe 1962; Heslin and Douglas 1986; Danielson and Pruden 1989). Furthermore, *C. geophilum* is considered an excellent model for the isolation and regeneration of protoplasts from ascomycetous ectomycorrhizal fungi (Stülten et al. 1995). Mycorrhizas synthesized by our seedling culture system showed typical characteristics of ectomycorrhizas. The presence of a mantle and a Hartig net indicated that the mycorrhizal roots examined were typical ectomycorrhizal associations between *C. argyrophylla* and *C. geophilum* under the applied culture conditions.

Although both *P. tinctorius* and *B. edulis* are broad-host-range fungi (Carroll 1992; Hall et al. 1998), they failed to produce mycorrhizas in *C. argyrophylla*. Recently, Martin et al. (2002) focused on host-fungus specificity of *Pisolithus* species to show that each species of *Pisolithus* was confined to hosts originated from a single biogeographical area. This suggests a need to test more isolates of mycorrhizal fungi or isolates collected much closer to *Cathaya* trees.

An established in vitro system ensures that inoculum is produced in laboratory conditions enough to apply practically to the forests. Furthermore, this system will enable us to follow the morphological, physiological, molecular, and biochemical changes during development of *C. argyrophylla* + *C. geophilum* mycorrhizas and other host-fungus ectomycorrhizas. Such a variety of application of this synthesis protocol ensures that it continued to be used further in ectomycorrhizal studies in vitro.

In conclusion, an endangered tree, *C. argyrophylla*, was a typical ectomycorrhizal tree. However, whether mycorrhization substantially helps to reestablish the endangered trees is still a question. At present, it is necessary to make a longer-term comparison between mycorrhizal and non-mycorrhizal seedlings under controlled conditions.

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