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Mycorrhizal synthesis of *Tuber pseudohimalayense* with seven broad-leaved trees and *Pinus armandii*

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ABSTRACT

Truffle cultivation is successfully applied throughout the world for several truffles of European origin. However, just two Chinese black truffles (*Tuber indicum* and *T. himalayense*) have been cultivated with a favorable outcome so far. *Tuber pseudohimalayense* is a black truffle of significant economic relevance in China, but little is known about its mycorrhizal ecology and it is not cultivated in orchards yet. Here, we selected seven broad-leaved tree species (*Quercus fabrei*, *Q. aliena*, *Castanea mollissima*, *Carya illinoinensis*, *Q. glauca*, *Castanopsis orthacantha*, *Betula costata*), and one coniferous tree (*Pinus armandii*), and inoculated them with *T. pseudohimalayense* spore suspension using axenically germinated seedlings under greenhouse conditions. The obtained mycorrhizae, well-developed, were analyzed from the morpho-anatomical and molecular points of view, and their main characteristics described. Synthesized *T. pseudohimalayense* mycorrhizae showed similar characters on all tree species, with a typical interlocking pseudoparenchymatous mantle and Hartig net, swollen appearance, yellow-brownish color, and long hyaline emanating hyphae with right-angle ramifications. These features are similar to those reported for mycorrhizae formed by related black truffle species. The successful mycorrhizal synthesis of *T. pseudohimalayense* on multiple trees species indicates that it has potential for cultivation in China.

Keywords: *Ascomycota*, Chinese black truffles, hypogeous fungi, mycorrhizal characteristics

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1. Introduction

Tuber pseudohimalayense G. Moreno, Manjón, J. Díez & García-Mont. [MycoBank #437558] is a Chinese black truffle, the second for economic relevance of its market in China after *T. indicum* Cooke & Massee. It was first described by Moreno and colleagues in 1997 on the basis of Chinese specimens that had been exported to Spain (Moreno, Manjón, Díez, García-Montero, & Di Massimo, 1997). *Tuber pseudohimalayense* is widely distributed in Yunnan and Sichuan provinces of southwestern China (Fan, Cao, & Li, 2013). It grows naturally in *Pinus armandii* Franch. or *P. yunnanensis* Franch. forests (Chen & Liu, 2011).

On one hand, mycorrhizal synthesis is a valuable tool for confirming the compatibility between host tree species and their mycobionts following the identification of mycobionts in naturally-occurring ectomycorrhizae. On the other hand, it also allows to develop cultivation programs and explore the potential of different combinations of host plant and truffle species in tree orchards. *Tuber pseudohimalayense* is frequently sold in local market in southwestern China (Wang & Liu, 2011) and is also exported to Spain, France and Italy, as mentioned above (Wang et al., 1998; García-Montero, Di Massimo, Manjón, & García-Abril, 2008). However, there are currently no plantations of *T. pseudohimalayense* in China, and the entire stock of truffles sold in markets comes from collections in natural stands, which poses a significant threat to the conservation of selected ecosystems. Therefore, for both economic and environmental reasons, it is important and urgent to explore the possibility to cultivate *T. pseudohimalayense* in its homeland.

Tuber species are known to form mycorrhizae with a wide range of host plants (both trees and shrubs), including *Fagaceae* (e.g., *Quercus*, *Castanea*, *Fagus*), *Betulaceae* (e.g., *Corylus*, *Carpinus*), *Salicaceae* (e.g., *Populus*), *Pinaceae* (e.g., *Pinus*, *Keteleeria*), *Juglandaceae* (e.g., *Carya*, *Platycarya*), *Cistaceae* (*Cistus*, *Helianthemum*) and so on (Geng, 2009; Wang, 2012; Zambonelli, Iotti, & Murat 2016; Olivier, Savignac, & Sourzat, 2018; Wang, Guerin-Laguette, Butler, Huang, & Yu, 2019; Wang et al., 2020; Huang, Guerin-Laguette, Wang, & Yu, 2020). Despite its economic and ecological relevance, the mycorrhizal biology of *T. pseudohimalayense* is

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poorly understood. To the best of our knowledge, naturally-occurring mycorrhizae have not been reported so far. Only one host has been described to form mycorrhizae with *T. pseudohimalayense* at the seedling stage under controlled environmental conditions, namely *Quercus ilex* subsp. *ballota*, a plant that is not present in the natural range of this truffle (Manjón, García-Montero, & Di Massimo, 1998; García-Montero et al., 2008; Manjón, García-Montero, Alvardo, Moreno, & Di Massimo, 2009).

It is thus necessary to increase the current knowledge of *T. pseudohimalayense*' potential host range and ectomycorrhizae. In this study, we selected eight host tree species to determine their compatibility with *T. pseudohimalayense* in pot culture conditions and provided a description and comparison of the ectomycorrhizae obtained using a combination of microscopy and molecular tools. The results of this study can prove critical in establishing the sustainable cultivation of *T. pseudohimalayense* in a foreseeable future.

2. Materials and methods

2.1. Mycorrhizal synthesis

Seeds' origin of the eight species of host plants used for mycorrhization experiments are shown in Table 1. Before germination, broad-leaved tree seeds were soaked in tap water for one day, with an initial water temperature of 55 °C (Mao et al., 2013), and seeds were subsequently surface-sterilized in sodium hypochlorite (2% available chlorine) for 2 h. Seeds of *Pinus armandii* were washed in tap water and surface-sterilized in 30% H_2O_2 for 30 min. After washing three times with tap water, all seeds were sown in large plastic crates lined with a cotton mesh which held a sterilized mixture of perlite and vermiculite (50:50, v:v) in Dec 2017. Plastic crates were placed in a greenhouse at the Kunming Institute of Botany under natural light. One ascoma of *T. pseudohimalayense* was commercially obtained from Kunming market, Yunnan, China, in Nov 2017, and stored in the freezer at -40 °C until use. Spore suspension was prepared by grinding the ascoma using a small blender, until the spores were released. Spore concentration was measured with a hemocytometer and the final spore suspension was diluted to 10^6 spores/mL. Substrate for inoculation was made of peat, vermiculite, and perlite (2:3:1, by volume). The pH of the substrate was adjusted to about 7 before autoclaving by adding calcium carbonate (0.19 g/L). Before use, substrate was sterilized by autoclaving in 10-L bags for 1 h at 121 °C, twice at 48 h intervals. For each tree species, each of five replicate seedlings was inoculated with 10 mL of spore suspension. Two doses of 5 mL (equivalent to 1 g fresh truffle per seedling) were distributed around the third upper zone of the root system, planting seedlings in 688-mL square pots on 5 May 2017. For each tree species, five seedlings acted as control non-inoculated seedlings. All pots were placed on grid tables in the same greenhouse at the Kunming Institute of Botany under natural light (e.g., 169 μmol m^{-2} s^{-1} inside in June) in a 28.5 m^2 modern design glasshouse, fitted with roof panels that could be opened and with an extractor fan cooling system with a maximal midday temperature of 30 °C in summer months. Pots were arranged in groups consisting of a given tree species inoculated, or not, with *T. pseudohimalayense*: two rows of five seedlings for each tree species. Plants were watered once a week or when necessary. No fertilizer was added during the whole inoculation test.

2.2. Morphological observations of ectomycorrhizae

Eight mo after inoculation (early Jan 2018), all seedlings were examined for observation of ectomycorrhizae. Five to eight mycorrhizal tips were examined per seedling, i. e . more than 30 tips for each host plant. The macro-morphological and anatomical characters of *T. pseudohimalayense* mycorrhizae were observed and photographed under dissecting (Leica S8APO, Leica Microsytems, Wetzlar, Germany) and compound (Leica DM2500, Leica Microsytems, Wetzlar, Germany) microscopes following the method by Agerer (2006). Anatomical cross sections were prepared from fresh root material using a freezing microtome (Leica CM3050S, Leica Microsytems, Wetzlar, Germany). All tissues were mounted on slides in distilled water. Morphology and anatomy of *T. pseudohimalayense* mycorrhizae formed with all eight host trees were described following the indications of Agerer (1987–2008).

2.3. Molecular analysis of the ascoma and ectomycorrhizae

To confirm successful ectomycorrhizal synthesis in each combination, genomic DNAs from the *T. pseudohimalayense* ascoma and from synthesized ectomycorrhizae were extracted using the protocol of Doyle and Doyle (1987). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified with the ITS1F/ITS4 primer pair (White, Bruns, Lee, & Taylor, 1990; Gardes & Bruns, 1993). PCR analyses were carried out on a LifeECO thermocycler (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China) in a final volume of 25 μL containing 1 μL DNA template, 1 μL (5 μM) of each primer, 12.5 μL 2x Taq Mastermix, 9.5 μL ddH_2O. The amplifications were performed with the following cycle parameters: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and with a final extension at 72 °C for 10 min. Three microliters of each PCR product were run on 1% (w/v) agarose gels and visualized by staining with ethidium bromide in Gel Doc™ EZ gel imaging system (Bio-Rad, Hercules, USA). PCR products were purified and sequenced in one direction by TsingKe Biological Technology, Kunming, China, using ITS1F. All the sequences obtained, either from the ascoma or from synthesized mycorrhizae, were compared for similarity with sequences published and deposited in GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTn algorithm for identification. A DNA sequences similarity ≥ 98% was required to conclude to the

Table 1. The origin of the seeds of eight species of host trees used in this study for mycorrhizal synthesis with *Tuber pseudohimalayense*.

Species	Collection date	Origin
Castanea mollissima Blume	02-11-2017	Changsha, Hunan, China
Quercus fabrei Hance	02-11-2017	Changsha, Hunan, China
Quercus aliena Blume	31-10-2017	Guiyang, Guizhou, China
Carya illinoinensis (Wangenh.) K. Koch	28-10-2017	Kunming, Yunnan, China
Quercus glauca Thunb.	30-10-2017	Kunming, Yunnan, China
Castanopsis orthacantha Franch.	30-10-2017	Kunming, Yunnan, China
Betula costata Trautv.	17-10-2017	Wulumuqi, Xinjiang, China
Pinus armandii Franch.	26-08-2017	Kungming, Yunnan, China

same species. Ascoma and mycorrhizae sequences generated in this study have been deposited in GenBank (see Results).

3. Results

3.1. Mycorrhization outcome

Eight mo after inoculation, all combinations of *T. pseudohimalayense* with *Quercus fabrei*, *Q. aliena*, *Castanea mollissima*, *Carya illinoinensis*, *Quercus glauca*, *Castanopsis orthacantha*, *Betula costata* and *Pinus armandii* formed mycorrhizae. Only a single mycorrhizal morphotype was found in all the inoculated seedlings. All replicate seedlings formed abundant and well-developed mycorrhizae except *B. costata* + *T. pseudohimalayense* combination. Indeed, only five to ten mycorrhizal tips could be found on the *B. costata* roots system for each seedling. In all cases, no ectomycorrhiza was found on the roots of control seedlings. No contaminant due to other ectomycorrhizal fungi was detected in any of the seedlings.

3.2. Morphological and anatomical characterization

Tree seedlings and morphological characteristics of ectomycorrhizae formed by *T. pseudohimalayense* are shown in Figs. 1–4. Description of their macromorphological and anatomical characters are provided in Table 2. All mycorrhizae obtained showed a '*Tuber*-like' morphology, as all have similar ectomycorrhizal systems and color, long and obvious emanating hyphae branching at

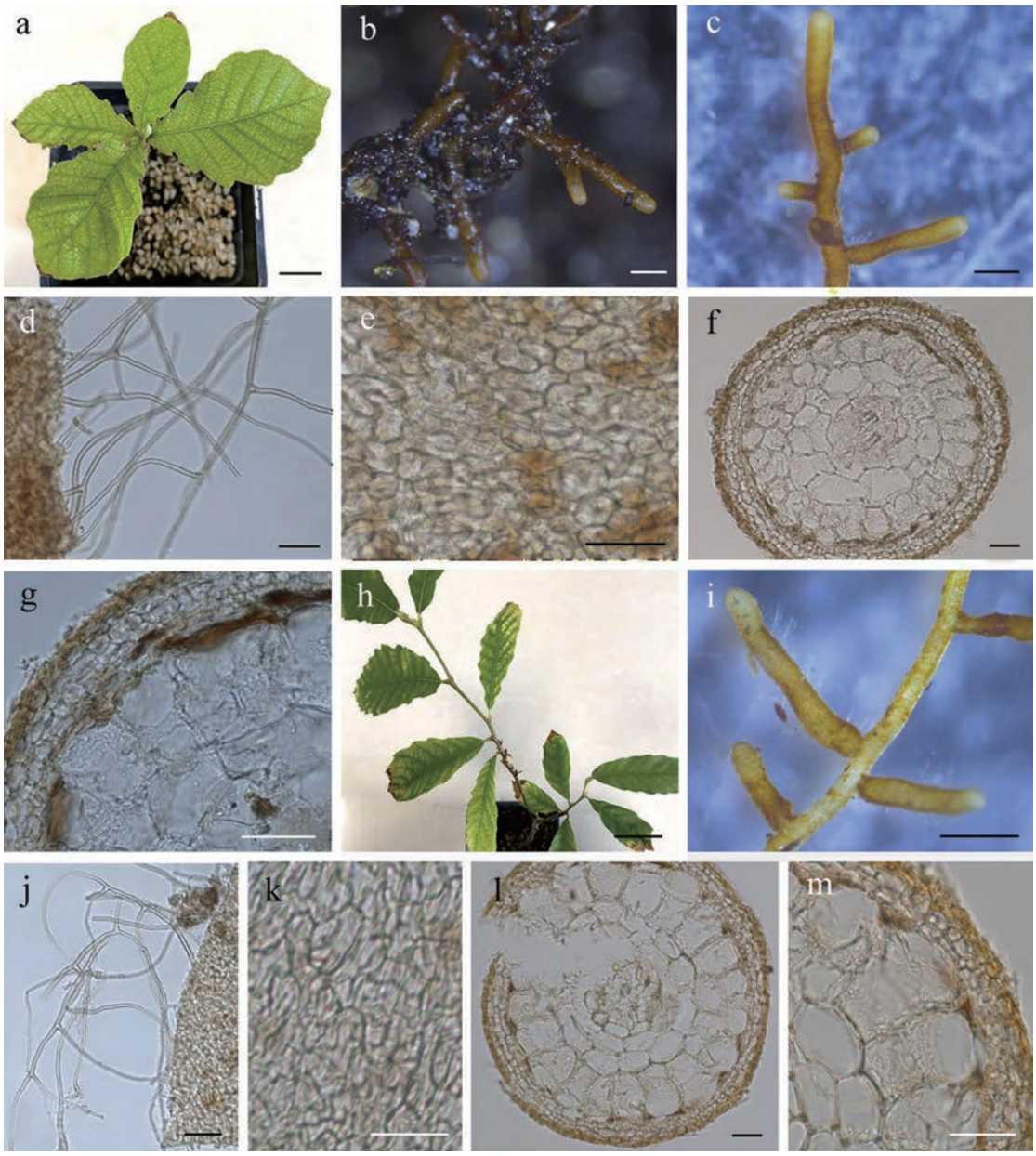

Figure 1. A–G: Ectomycorrhizal synthesis between *Quercus fabrei* and *Tuber pseudohimalayense* 8 mo after inoculation. A: Seedling. B, C: Dissecting microscope views of ectomycorrhizae. D: Septate hyphae emanating from the outer mantle layer. E: Outer mantle surface structure. F, G: ectomycorrhizae cross-sections. H–M: Ectomycorrhizal synthesis between *Castanea mollissima* and *T. pseudohimalayense* 8 mo after inoculation. H: Seedling. I: Dissecting microscope view of ectomycorrhizae. J: Septate hyphae emanating from the outer mantle layer. K: Outer mantle surface structure. L, M: Ectomycorrhizae cross-sections. *Bars*: A 2 cm; B, C, I 500 μm; D–G, J–M 25 μm; H 4.5 cm.

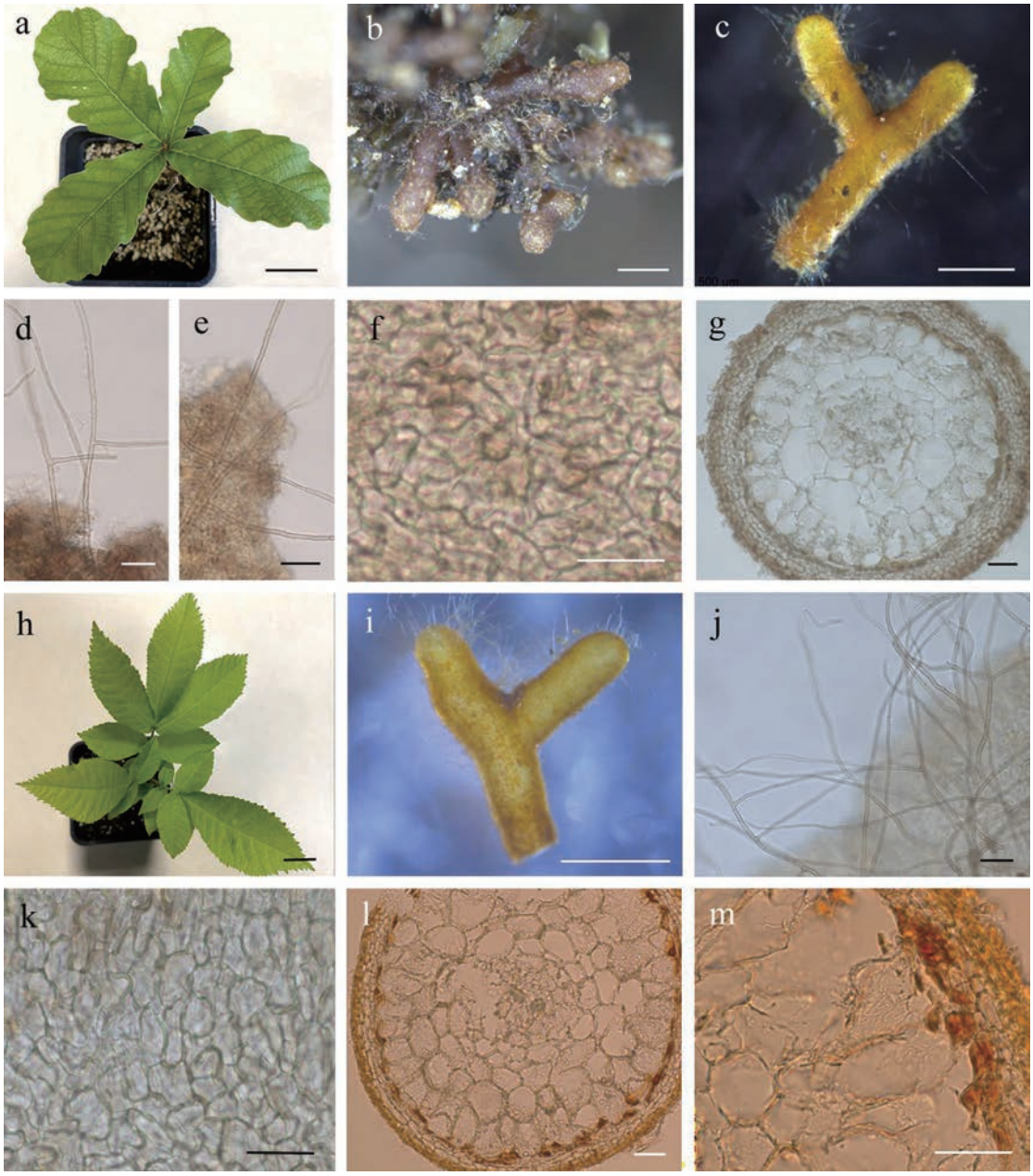

Figure 2. A–G: Ectomycorrhizal synthesis between *Quercus aliena* and *Tuber pseudohimalayense* 8 mo after inoculation. A: Seedling. B, C: Dissecting microscope views of ectomycorrhizae. D, E: Septate hyphae emanating from the outer mantle layer. F: Outer mantle surface structure. G: Ectomycorrhizae cross-sections. H–M: Ectomycorrhizal synthesis between *Carya illinoinensis* and *T. pseudohimalayense* 8 mo after inoculation. H: Seedling. I: Dissecting microscope view of ectomycorrhizae. J: Septate hyphae emanating from the outer mantle layer. K: Outer mantle surface structure. L, M: Ectomycorrhizae cross-sections. *Bars*: A, H 3 cm; B, C, I 500 μm; D–G, J–M 25 μm.

approximately right angle, and a jigsaw puzzle pattern of outer mantle cells.

3.3. Molecular identification

All the ITS sequences obtained from the ascoma and from *T. pseudohimalayense*-like mycorrhizae formed with each host species were deposited in GenBank. The ITS sequences from mycorrhizae of *Quercus fabrei*, *Q. aliena*, *Castanea mollissima*, *Quercus glauca* and *Betula costata* (identical between all of them) showed 99.37% identity with a *T. pseudohimalayense* reference sequence (GU979045.1), and ITS sequences from mycorrhizae on the other three hosts (*Carya illinoinensis, Castanopsis orthacantha* and *Pinus armandii*) showed 99.34% identity with *T. pseudoexcavatum* = *T. pseudohimalayense* sequence GU979039.1. The ITS sequences of *T. pseudohimalayense* ectomycorrhizae/ascoma obtained in this study and deposited in GenBank are: *Q. fabrei* (mhll-7, accession number MT446220), *Q. aliena* (mhll-8, MT446221), *Castanea mollissima* (mhll-10, MT446223), *Carya illinoinensis* (mhll-9, MT446222), *Quercus glauca* (mhll-6, MT446219), *Castanopsis orthacantha* (mhll-11, MT446224), *Pinus armandii* (mhll-12, MT705328) *Betula costata* (mhll-5, MT446218), and *T. pseudohimalayense* ascoma (ahll-1, MT446225).

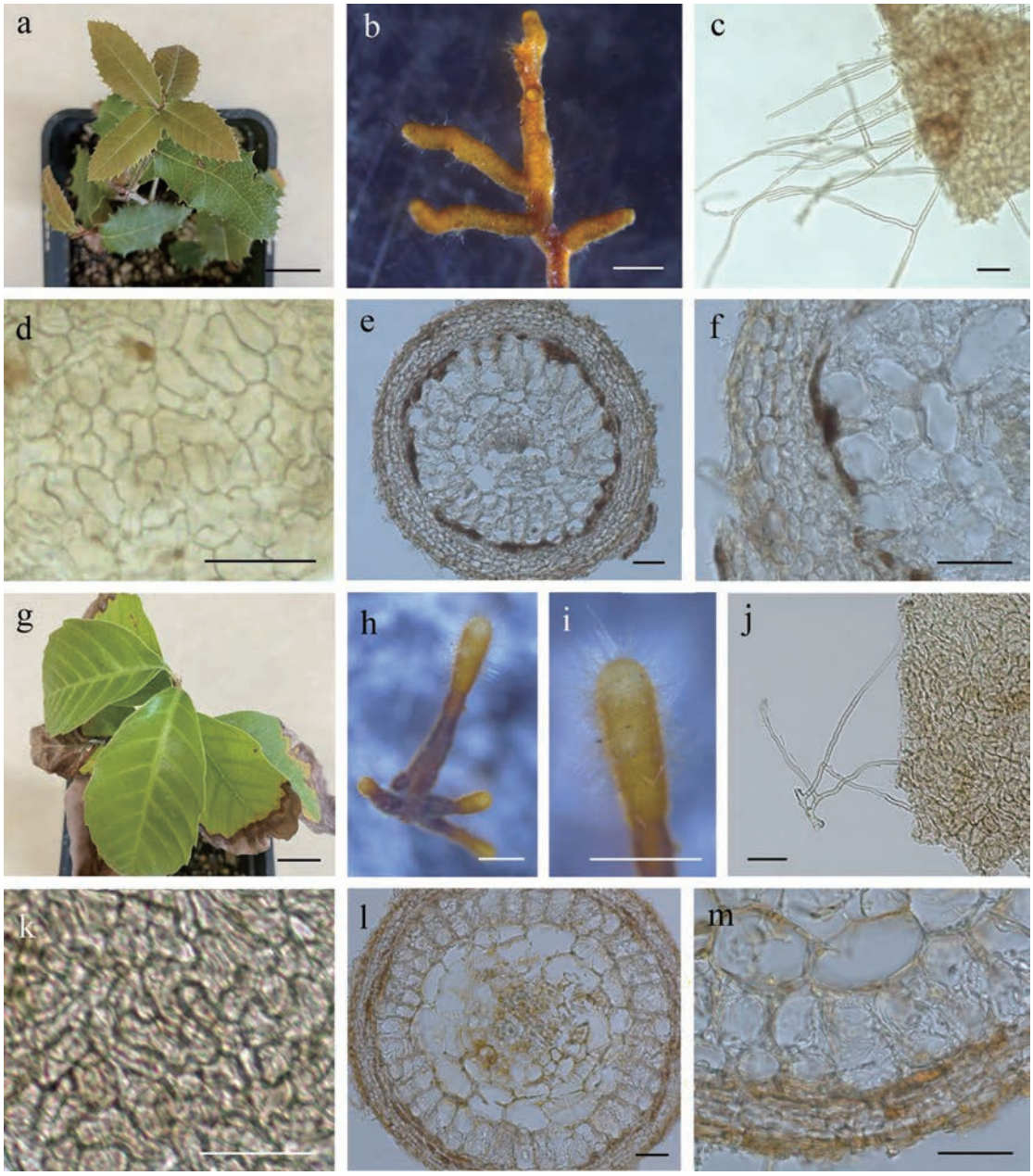

Figure 3. A–F: Ectomycorrhizal synthesis between *Quercus glauca* and *Tuber pseudohimalayense* 8 mo after inoculation. A: Seedling. B: Dissecting microscope view of ectomycorrhizae. C: Septate hyphae emanating from the outer mantle layer. D: Outer mantle surface structure. E, F: Ectomycorrhizae cross-sections. G–M: Ectomycorrhizal synthesis between *Castanopsis orthacantha* and *T. pseudohimalayense* 8 mo after inoculation. G: Seedling. H, I: Dissecting microscope views of ectomycorrhizae. J: Septate hyphae emanating from the outer mantle layer. K: Outer mantle surface structure. L, M: Ectomycorrhizae cross-sections. *Bars*: A 2 cm; B, H, I 500 μm; C–F, J–M 25 μm; G 3 cm.

4. Discussion

This work reports the successful mycorrhizal synthesis between *Tuber pseudohimalayense* and eight host plants, namely seven broad-leaved trees (*Betula costata*, *Carya illinoinensis, Castanea mollissima*, *Castanopsis orthacantha*, *Quercus glauca*, *Quercus aliena, Quercus fabrei*) and one coniferous tree (*Pinus armandii*). The detailed analysis of the morphological characteristics under both the dissecting and compound microscopes revealed that all the mycorrhizae obtained in this study display similar colors, shapes and anatomical characteristics, which include a mantle surface that is smooth or with wooly hyphae, long emanating hyphae branching at approximately right-angle, and a typical, *Tuber*-like, jigsaw puzzle pattern of the mantle cells. The similarity of these features among the synthesized mycorrhizae is not surprising, since they depend on the mycobiont identity. The ITS sequences of all the synthesized mycorrhizae matched with that of the ascoma used for inoculation, thus confirming the identity of the mycobiont in all cases, i.e., *T. pseudohimalayense*.

Mycorrhizae of *T. pseudohimalayense* were previously synthesized only with *Quercus ilex* subsp. *ballota* (Manjón et al., 1998, 2009; García-Montero et al., 2008). Therefore, the present study considerably increases our knowledge of mycorrhizal synthesis with *T. pseudohimalayense*. and demonstrate that this truffle species can associate, under nursery pot conditions, with a diverse range of host plants, mostly native to China but including one exot-

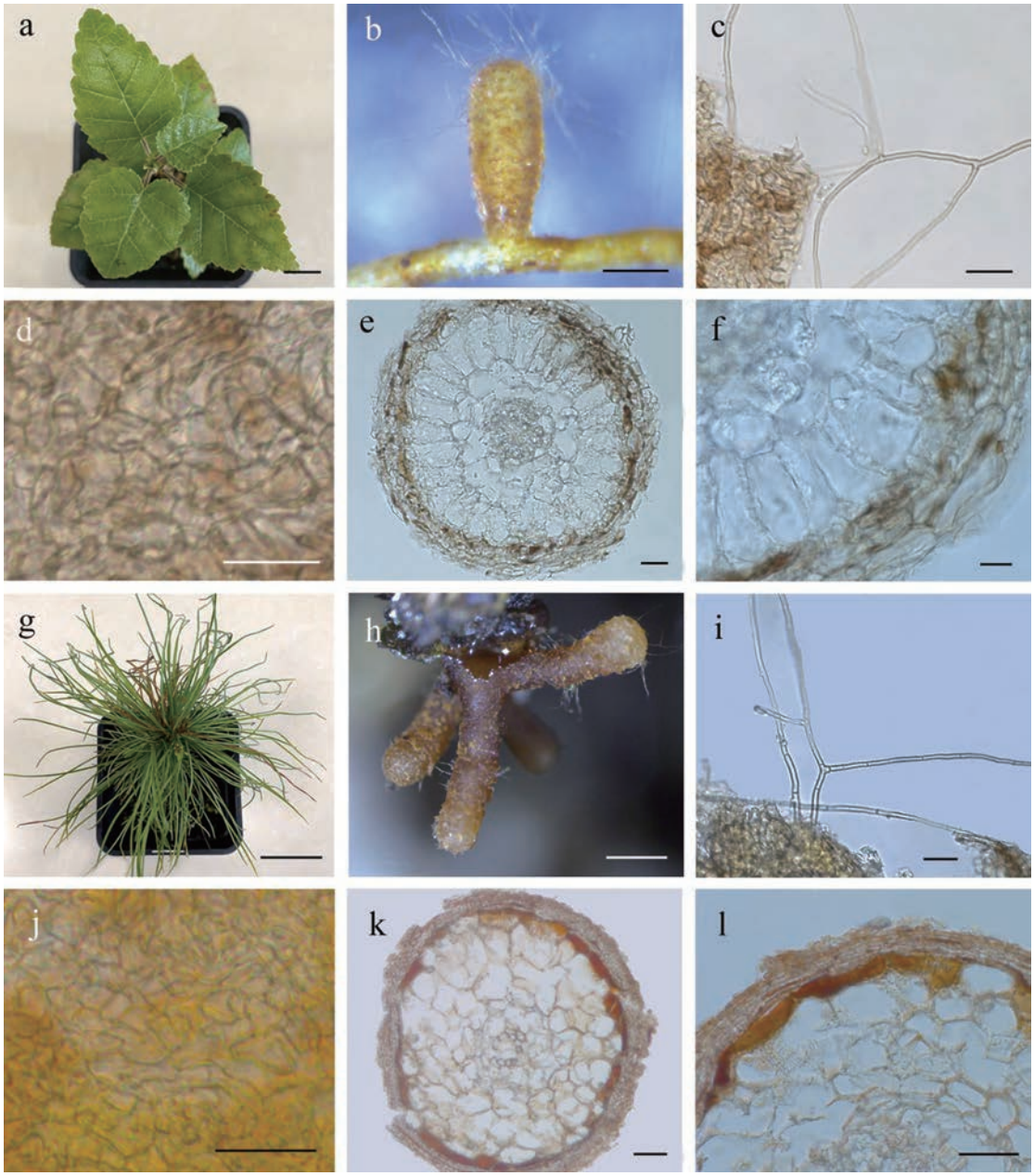

Figure 4. A–F: Ectomycorrhizal synthesis between *Betula costata* and *Tuber pseudohimalayense* 8 mo after inoculation. A: Seedling. B: Dissecting microscope view of an ectomycorrhiza. C: Septate hyphae emanating from the outer mantle layer. D: Outer mantle surface structure. E, F: Ectomycorrhizae cross-sections. G–L: Ectomycorrhizal synthesis between *Pinus armandii* and *T. pseudohimalayense* 24 mo after inoculation. G: Seedling. H: Dissecting microscope view of ectomycorrhizae. I: Septate hyphae emanating from the outer mantle layer. J: Outer mantle surface structure. K, L: Ectomycorrhizae cross-sections. *Bars*: A, G 2 cm; B 200 μm; C–F, I, J 25 μm; H 500 μm; K, L 50 μm.

ic species, i.e., *Carya illinoinensis*. Most characteristics of the mycorrhizae we obtained coincide with the description in previous studies, such as smooth surface with long emanating hyphae often branching at approximately right-angle, absence of rhizomorphs, pseudoparenchymatous cells and outer mantle surface with irregular polygonal to sinuous cells. However, the color of the mycorrhizal tips was slightly different, being dark brown in previous studies but rather yellowish brown in our cases. The color of mycorrhizae is a variable and age-dependent characteristic and might also be influenced by the specific conditions applied to seedling cultivation (Wang et al., 2019). The mycorrhizae of *T. pseudohimalayense* with different host species obtained in our study also showed slight differences among themselves. For example, cortical cells in the external layer of *T. pseudohimalayense* mycorrhiza on *Betula costata* seems elongated compared with those of other species. Further, the shape of the outer mantle cells of mycorrhizae synthesized with *C. illinoinensis* were rounder than those of the other host species.

The main features of *T. pseudohimalayense* mycorrhizae we described here are similar to those of *T. indicum* and *T. melanosporum* Vittad. (often used as a reference species for black truffles, because of its prevalence in international truffle markets) synthesized with several other broad-leafed and coniferous hosts (Comandini & Pacioni 1997; García-Montero et al., 2008; Lin et al., 2008; Geng et al., 2009; Zhang et al., 2011; Deng, Yu, & Liu, 2014; Wang et al., 2019; Huang et al., 2020), not surprisingly indeed, given the close phylogenetic relationship of these groups of Asian black truffles

Table 2 Macro-morphological and anatomical characteristics of artificially synthesized mycorrhizae of *Tuber pseudohimalayense* on *Quercus fabrei*, *Q. aliena*, *Castanea mollissima*, *Carya illinoinensis*, *Quercus glauca*, *Castanopsis orthacantha* and *Betula costata*.

Type of characteristics	*Q. fabrei*	*Q. aliena*	*Castanea mollissima*	*Carya illinoinensis*
Ectomycorrhizal systems	Simple or ramified in a monopodial-pinnate pattern, up to 4.2 mm long, 2.3 mm wide	Dichotomous or ramified in a monopodial-pinnate pattern, up to 3.4 mm long, 2.5 mm wide	Simple or monopodial with lateral or dichotomous, up to 2.4 mm long, 1.3 mm wide	Simple or monopodial with lateral or ramified in a monopodial-pinnate pattern, up to 3.8 mm long, 1.9 mm wide
Color	Pale yellow - brown - dark brown	Yellowish brown - ochre - dark brown	Yellowish brown - ochre	Yellowish brown - ochre - dark brown
Unramified ends	Straight or slightly bent, cylindric or club-shaped with rounded ends, up to 1.6 mm long, 0.19–0.24 mm in diam. Some tips had a whitish growing up apex	Straight, cylindric or club-shaped with rounded ends, up to 2.1 mm long, 0.27–0.32 mm in diam.	Straight or slightly bent, cylindric or club-shaped with rounded ends, up to 2.3 mm long, 0.23–0.27 mm in diam. Some tips had a whitish growing up apex	Straight or slightly bent, cylindric or club-shaped with rounded ends, up to 2.1 mm long, 0.24–0.31 mm in diam.
Mantle surface	Smooth or wooly hyphae	wooly hyphae	wooly hyphae	Smooth or wooly hyphae
Mantle	16–25 μm thick, three to five layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.3–14.6 μm × 2.5–4.6 μm, number of cells in 20 ×20 μm square 3–5	25–32 μm thick, four to six layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.3–15.8 μm × 3.6–7.1 μm, number of cells in 20 × 20 μm square 3–6	15–20 μm thick, two to three layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 3.7–11.9 μm × 3.3–5.9 μm, number of cells in 20 × 20 μm square 3–6	16–22 μm thick, three to five layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.1–12.7 μm × 3.4–6.3 μm, number of cells in 20 ×20 μm square 3–5
Rhizomorphs	Absent	Absent	Absent	Absent
Emanating hyphae	Simple or branched, concentrated proximally, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 389 μm long, 2.0–4.4 μm diam	Simple or branched, not specifically distributed, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 392 μm long, 2.4–3.9 μm diam	Simple or branched, not specifically distributed, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 380 μm long, 1.7–2.9 μm diam	Simple or branched, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 426 μm long, 1.8–3.2 μm diam
Hartig net	Palmetti	Palmetti	Palmetti	Palmetti

Type of characteristics	*Quercus glauca*	*Castanopsis orthacantha*	*Betula costata*	*Pinus armandii*
Ectomycorrhizal systems	Simple or dichotomous, sometimes almost coralloid type, up to 4.7 mm long, 2.3 mm wide	Simple or monopodial with lateral or ramified in a monopodial - pyramidal, up to 4.4 mm long, 3.1 mm wide	Simple or monopodial with lateral, up to 1.3 mm long, 0.8 mm wide	Dichotomous or irregularly pinnate, sometimes almost coralloid type, up to 2.5 mm long, 1.6 mm wide
Color	Pale brown - ochre - dark brown	Pale brown - ochre - dark brown	Pale brown - ochre	Yellowish brown - ochre - dark brown
Unramified ends	Slightly bent, cylindric or club-shaped with rounded ends, up to 1.4 mm long, 0.15–0.22 mm in diam. Some tips had a whitish growing up apex	Straight, cylindric or club-shaped with rounded ends, up to 1.7 mm long, 0.17–0.28 mm in diam	Straight, cylindric or club-shaped with rounded ends, up to 1.1 mm long, 0.14–0.21 mm in diam	Straight, cylindricl, up to 1.4 mm long, 0.29–0.45 mm in diam. Some tips had a whitish growing up apex
Mantle surface	wooly hyphae	Smooth or wooly hyphae	Smooth or wooly hyphae	Smooth or wooly hyphae
Mantle	26–35 μm thick, three to five layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.9–16.3 μm × 2.6–10.8 μm, number of cells in 20 × 20 μm square 3–5	17–30 μm thick, three to four layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.9–13.0 μm × 3.2–5.7 μm, number of cells in 20 × 20 μm square 4–5	19–29 μm thick, two to four layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.7–13.7 μm × 3.5–7.9 μm, number of cells in 20 × 20 μm square 3–6	23–38 μm thick, four to six layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 6.2–12.1 μm × 3.8–11.2 μm, number of cells in 20 × 20 μm square 5–6
Rhizomorphs	Absent	Absent	Absent	Absent
Emanating hyphae	Simple or branched, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 392 μm long, 2.3–3.4 μm diam	Simple or branched, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 368 μm long, 1.7–2.8 μm diam	Simple or branched, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 375 μm long, 1.9–2.8 μm diam	Simple or branched, smooth, septate, straight to slightly irregularly inflated, pale brown, most with right angle ramification, up to 438 μm long, 2.6–4.3 μm diam
Hartig net	Palmetti	Palmetti	Palmetti	Palmetti

(Wang et al., 2006a, 2006b).

Spore inoculation is widely used for truffle mycorrhiza synthesis. Ferrara & Palenzona (2001) and García-Montero et al. (2008) found that the spores of *T. indicum* and *T. pseudohimalayense* had high germination capacity and formed young mycorrhizae 2.5-3 mo after inoculation. However, most studies in the literature indicate that *Tuber* mycorrhizae would form about 4–6 mo after inoculation (Hu, 1992; Geng et al., 2009; Wan et al., 2016; Kinoshita, Obase, & Yamanaka, 2018; Wang et al., 2019). Longer inoculation periods might be necessary for *Tuber* mycorrhization to occur and

develop. Benucci, Bonito, Falini, & Bencivenga (2012) observed well-developed *T. borchii* Vittad. and *T. aestivum* Vittad. ectomycorrhizae on Pecan seedlings 10 mo after inoculation. In our study, after 8 mo of inoculation we found a well-developed mycorrhizal system in all tested host plants, with the notable exception of *Betula costata*, observing mycorrhiza formation being concentrated in the proximal and median part of the root system. These findings indicate that mycorrhizae of *T. pseudohimalayense* start to develop in the span of time reported in most previous *Tuber* mycorrhization studies.

Regarding host plants, we used eight tree species of three families from *Fagaceae*, *Juglandaceae* and *Pinaceae*, six of which are common host trees of *Tuber* spp. in southern China, to test the colonizing ability of *T. pseudohimalayense*. As a typical ectomycorrhizal tree, *Betula costata* is a potential candidate for truffle cultivation in northern China since it is well-adapted to the local pedo-climatic conditions. We planned to use a wide array of hosts in order to understand the range of plants that could potentially be used in *T. pseudohimalayense* truffle orchards. From the results, we succeeded obtaining mycorrhizae with all tested trees species, although the level of mycorrhization in *B. costata* was limited. This indicates that all these broad-leafed and coniferous trees species have potential for cultivation of *T. pesudohimalayense*. As for coniferous hosts, Wang (2012) reported finding *T. pseudohimalayense* ascomata under *Pinus yunnanensis*, but did not give any information about mycorrhizae. Here, we provide details on the formation of *T. pseudohimalayense* ectomycorrhizae on *P. armandii*, which confirm that coniferous trees might be suitable hosts. However, more work is needed in order to ascertain which plant host would perform better in cultivation orchards. Also, little is known about the characterization of *T. pseudohimalayense* mycorrhizae under natural conditions, a type of information that obviously would drive cultivation efforts and would reduce unproductive attempts.

In conclusion, we demonstrated successful mycorrhization trials of *T. pseudohimalayense* with seven broad-leafed and one coniferous tree species and provided detailed morphological descriptions and illustrations of these mycorrhizae. This study contributes significantly towards a better understanding of the mycorrhization potential of *T. pseudohimalayense* on a wide range of tree species, an essential preliminary information that is much needed to select suitable host species for making it possible to cultivate *T. pseudohimalayense* in truffle orchards in China. We are now working towards establishing *T. pseudohimalayense* trial orchards in both Guizhou and Yunnan provinces, southwest of China. Future experimental work will assess the persistence and development in the field of *T. pseudohimalayense* mycorrhizae synthesized with these tree species, and monitor *T. pseudohimalayense* ascomata production in trial plantations.

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Reference

Agerer, R. (2006). Fungal relationships and structural identity of their ectomycorrhizae. *Mycological Progress*, *5*, 67–107. https://doi.org/10.1007/s11557-006-0505-x

Agerer, R. (1987–2008). *Colour atlas of ectomycorrhizae.* EinhornVerlag, Schwäbisch Gmünd, 1st–13th.

Benucci, G. M. N., Bonito, G., Falini, L. B., & Bencivenga, M. (2012). Mycorrhization of Pecan trees (*Carya illinoinensis*) with commercial truffle species: *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. *Mycorrhiza*, *22*, 383–392. https://doi.org/10.1007/s00572-011-0413-z

Chen, J., & Liu, P. G. (2011). Delimitation of *Tuber pseudohimalayense* and *T. pseudoexcavatum* based on morphological and molecular data. Cryptogamie Mycologie, 32, 83–93. https://doi.org/10.7872/crym.v32.iss1.2012.083

Comandini, O., & Pacioni, G. (1997). Mycorrhizae of Asian black truffles, *Tuber himalayense* and *T. indicum*. *Mycotaxon*, *63*, 77–86.

Deng, X. J., Yu, F. Q., & Liu, P. G. (2014). Contribution to confirmed & synthesized on mycorrhizae of *Tuber indicum* s.l. with two dominated & subalpine broadleaf trees in southwestern China. *American Journal of Plant Sciences*, *5*, 3269–3279. http://doi.org/10.4236/ajps.2014.521341

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissues. *Phytochemistry Bull*, *19*, 11–15.

Ferrara, A. M., & Palenzona, M. (2001). Impiego di marcatori biochimici perl' identificacione di sporofi, miceli, e micorrize di *Tuber indicum* Cooke and Massee. In: M. Courvoisier, J. M. Olivier, & G. Chevalier (Eds.), *Actes du V Congres International: Science et Culture de la Truffe. Federation Française des trufficulteurs* (pp. 76-77). Aix-en-Provence.

Fan, L., Cao, J. Z., & Li, Y. (2013). A reassessment of excavated *Tuber* species from China based on morphology and ITS rDNA sequence data. *Mycotaxon*, *124*, 155–163. http://doi.org/10.5248/124.155

García-Montero, L. G., Di Massimo, G., Manjón, J. L., & García-Abril, A. (2008). New data on ectomycorrhizae and soils of the Chinese truffles *Tuber pseudoexcavatum* and *T. indicum*, and their impact on truffle cultivation. *Mycorrhiza*, *19*, 7–14. https://doi.org/10.1007/s00572-008-0198-x

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. *Molecular Ecology Resources*, *2*, 113–118. http://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Geng, L. Y. (2009). Mycorrhizal synthesis of *Tuber indicum* with *Pinus armandii*, *Castanea mollissima* and *Quercus franchetii*. Master dissertation. Lanzhou University.

Geng, L. Y., Wang, X. H., Yu, F. Q., Deng, X. J., Tian, X. F., Shi, X. F., Xie, X. D., Liu, P. G., & Shen, Y. Y. (2009). Mycorrhizal synthesis of *Tuber indicum* with two indigenous hosts, *Castanea mollissima* and *Pinus armandii*. *Mycorrhiza*, *19*, 461–467. https://doi.org/10.1007/s00572-009-0247-0

Hu, H. T. (1992). *Tuber formosanum* sp. nov. and its mycorrhizal associations. *Quarterly Journal of the Experimental Forest of National Taiwan University*, *6*, 79–86.

Huang, L. L., Guerin-Laguette, A., Wang, R., & Yu, F. Q. (2020). Characterization of *Tuber indicum* (Pezizales, Tuberaceae) mycorrhizae synthesized with four host trees exotic to China. *Symbiosis*, *89*, 215–224. https://doi.org/10.1007/s13199-020-00715-7

Kinoshita, A., Obase, K., & Yamanaka, T. (2018). Ectomycorrhizae formed by three Japanese truffle species (*Tuber japonicum*, *T. longispinosum*, and *T. himalayense*) on indigenous oak and pine species. *Mycorrhiza*, *28*, 679–690. https://doi.org/10.1007/s00572-018-0860-x

Lin, Q., Li, R. W., Han, D., Liu, C. Y., Sha, W. Y., & Zhang, F. C. (2008). Researches on the range of symbiotic host plants of commercial truffle species and *Tuber indicum* in Panzhihua, Sichuan. *Journal of Sichuan Forestry Science and Technology*, *29*, 17–20. (in Chinese, with English abstract)

Mao, L. G., Yan, D. D., Wu, Z. F., Ma, T. T., Wang, Q. X., Li, Y., Guo, M. X., Ouyang, C. B., Cao, A. C., & Zheng, J. Q. (2013). Research progress in seed treatment. *China Vegetables*, *10*, 9–15. (in Chinese, with English abstract)

Manjón, J. L., García-Montero, L. G., & Di Massimo, G. (1998). Mycorrhizal synthesis of *Tuber pseudohimalayayense* and *T. pseudoexcavatum*, two truffles from Asia. Abstracts of the Second International Conference on Mycorrhizae (ICOM II), Uppsala, Sweden, p. 113.

Manjón, J. L., García-Montero, L. G., Alvarado, P., Moreno, G., & Di Massimo, G. (2009). *Tuber pseudoexcavatum* versus *T. pseudohimalayense* - new data on the molecular taxonomy and mycorrhizae of Chinese truffles. *Mycotaxon*, *110*, 399–412. http://doi.org/10.5248/110.399

Moreno, G., Manjón, J. L., Díez, J., García-Montero, L. G., & Di Massimo, G. (1997). *Tuber pseudohimalayense* sp. nov. an Asiatic species commercialised in Spain, similar to Perigord truffle. *Mycotaxon*, *63*, 217–224.

Olivier, J. M., Savignac, J. C., & Sourzat, P. (2018). Truffe et trufficulture. [Truffle and Truffle cultivation] (in French). Fanlac, France, 4th edition, 343 pp.

Wan, S. P., Yu, F. Q., Tang, L., Wang, R., Wang, Y., Liu, P. G., Wang, X. H., & Zheng, Y. (2016). Ectomycorrhizae of *Tuber huidongense* and *T. liyuanum* with *Castanea mollissima* and *Pinus armandii*. *Mycorrhiza*, *26*, 249–256. http://doi.

org/10.1007/s00572-015-0663-2
Wang, R., Guerin-Laguette, A., Butler, R., Huang, L. L., & Yu, F. Q. (2019). The European delicacy *Tuber melanosporum* forms mycorrhizae with some indigenous Chinese *Quercus* species and promotes growth of the oak seedlings. *Mycorrhiza*, *29*, 649–661. http://doi.org/10.1007/s00572-019-00925-y
Wang, X. H. (2012). Truffle cultivation in China. In: A. Zambonelli, G. M. Bonito (Eds.), *Edible Ectomycorrhizal Mushrooms* (pp. 227–240). Springer Verlag. https://doi.org/10.1007/978-3-642-33823-6_13
Wang, Y., Moreno, G., Riousset, L. J., Manjón, J. L., Riousset, G., Fourré, G., Di Massimo, G., García-Montero, L. G., & Díez, J. (1998). *Tuber pseudoexcavatum* sp. nov. a Chinese species commercialised in Spain, France and Italy with comments on Chinese truffles. *Cryptogamie Mycologie*, *19*, 113–120. http://doi.org/10.1371/journal.pone.0132496
Wang, Y., Yu, F. Q., Zhang, C., Liu, C., Yang, M., & Li, S. (2020). Edible ectomycorrhizal fungi and their cultivation in China. In: J. Pérez-Moreno, R. Flores-Arzú, A. Guerin-Laguette, F. Q. Yu (Eds.), *Mushrooms, humans, and nature in a changing world: Perspectives from ecological, agricultural and social sciences* (pp. 31–60). Springer Nature Switzerland. https://doi.org/10.1007/978-3-030-37378-8_2
Wang, Y., Tan, Z. M., Zhang, D. C., Murat, C., Jeandroz, S., & Le Tacon, F. (2006a). Phylogenetic relationships between *Tuber pseudoexcavatum*, a Chinese truffle, and other *Tuber* species based on parsimony and distance analysis of four different gene sequences. *FEMS Microbiology Letters*, *259*, 269–281. http://doi.org/10.1111/j.1574-6968.2006.00283.x
Wang, Y., Tan, Z. M., Zhang, D. C., Murat, C., Jeandroz, S., & Le Tacon, F. (2006b). Phylogenetic and populational study of *Tuber indicum* complex. *Mycological Research*, *110*, 1034–1045. http://doi.org/10.1016/j.mycres.2006.06.013
Wang, Y., & Liu, P. G. (2011). Verification of Chinese Names of Truffles and Their Conservation in Natural Habitats. Plant Diversity and Resources, 33(6): 625-542. (in Chinese, with English abstract)
White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.) *PCR Protocols: a guide to methods and applications* (pp. 315–322). Academic Press.
Zambonelli, A., Iotti, M., & Murat, C. (2016). *True Truffle (Tuber spp.) in the World.* Soil Biology, 47. Springer, Cham.
Zhang, X. L., Li, S. H., Li, S. B., Yang, L. F., Ma, M. F., & Su, K. M. (2011). Techniques for the synthesis of mycorrhizal seedings of *Populus bonatii* with *Tuber indicum*. *Edible Fungi of China*, *30*, 18–20. (in Chinese, with English abstract)