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Characterization of *Tuber indicum* (Pezizales, Tuberaceae) mycorrhizae synthesized with four host trees exotic to China

Lan-Lan Huang[1,2] · Alexis Guerin-Laguette[3,4] · Ran Wang[1,5] · Fu-Qiang Yu[1]

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Abstract

The Chinese black truffle *Tuber indicum* is the main commercial truffle species in China and has strong potential for cultivation. Most studies have focused so far on the production and planting of mycorrhizal seedlings of tree species native to China. Here, we selected four exotic tree species, *Quercus pubescens*, *Q. ilex*, *Q. palustris* and *Pinus pinea* and inoculated axenically germinated seedlings, five replicates per tree species, with *T. indicum* spore suspension. As shown by morphological, anatomical and molecular analyses, mycorrhizae were successfully synthesized under greenhouse conditions from 6 months after inoculation and the mycorrhization was stable for at least 24 months in the glasshouse environment. Despite slight morphological variations, *T. indicum* mycorrhizae were similar on all tree species, i.e. swollen, red-brownish with long hyaline emanating hyphae showing right-angle ramifications. Our observations confirmed the similarity of *T. indicum* mycorrhizae with those of *T. melanosporum* regardless of the geographic origin of host trees. Four out of five *T. indicum*-inoculated pine seedlings were cross-contaminated by *T. borchii* that was inoculated to another group of *P. pinea* seedlings raised in the same glasshouse. This is the first study to document the mycorrhization of exotic tree species by *T. indicum* in China and the first report for *Q. palustris*. With two successful out of five inoculated seedlings, *Q. palustris* was less receptive to *T. indicum* mycorrhization than the other two oak species. Further work is needed to assess whether the *T. indicum* symbioses obtained here are maintained after planting in the field and if ascomata can be produced by exotic tree species under Chinese environmental conditions.

Keywords Chinese black truffle · Mycorrhizal synthesis · *Quercus palustris* · *Pinus* · Spore dissemination

1 Introduction

Tuber indicum (Ascomycota, Pezizales) is an ectomycorrhizal hypogeous fungus native to Asia and commonly referred to as the Chinese black truffle (Cooke and Massee 1892). In the wild, *T. indicum* was first discovered growing symbiotically with *Quercus acutissima* Carr., *Q. pannosa* Handl.-Mazz., *Q. monimotricha* Handl.-Mazz., and *Pinus yunnanensis* Franch. in the southwest of China (Zang et al. 1992). It is the main commercial truffle species in China, particularly in the southwest, and has a high economic value (García-Montero et al. 2008; Liu et al. 2011) with a maximum price currently reaching US$ 150/kg (Wang et al. 2020).

The morphological features of *T. indicum*, including the characteristics of its ectomycorrhizae on host trees, are similar to those of the European black truffle (*T. melanosporum* Vittad) (Comandini and Pacioni 1997; Zambonelli et al. 1997; Riousset et al. 2001) to which it is closely related phylogenetically (Chen et al. 2011; Bonito et al. 2013). *Tuber indicum* is distributed over a geographic area distinct from, and much larger than, that of the north Mediterranean species

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✉ Alexis Guerin-Laguette
guerin.myco@gmail.com

✉ Fu-Qiang Yu
fqyu@mail.kib.ac.cn

[1] The Germplasm Bank of Wild Species, Yunnan Key Laboratory for Fungal Diversity and Green Development, Kunming Institute of Botany, Chinese Academy of Science, 132 Lanhei Road, Kunming 650201, Yunnan, China

[2] College of Resource and Environment, Yunnan Agricultural University, Kunming 650100, China

[3] The New Zealand Institute for Plant and Food Research Limited, Gerald Street, Lincoln 7608, New Zealand

[4] Kunming Institute of Botany, Chinese Academy of Sciences, Beijing, China

[5] Department of Crop and Forest Science, University of Lleida, Av. Alcalde Rovira Roure, 191, 25198 Lleida, Spain

T. melanosporum (Chen et al. 2011; Olivier et al. 2018). It is difficult to distinguish these two species from morphological characteristics alone (Bonito et al. 2011). Since the 1990s, *T. indicum* has received more attention from truffle-importing countries, e.g. Europe, Japan, United States, and Australia. Its market value is three to five times less than that of *T. melanosporum* (€500–1000/kg, http://www.truffe-noire.info/accueil-pour-tout-savoir-sur-la-truffe/cours-de-la-truffe/), but when harvested at maturity and correctly graded, its culinary value makes it a good substitute for *T. melanosporum* (Hall et al. 2007; García-Montero et al. 2008; Geng et al. 2009). Consequently, attempts to cultivate *T. indicum* are currently being developed.

Both truffle species have a wide variety of host trees. *T. melanosporum* is predominantly associated with *Quercus* spp. (Fagaceae), *Corylus* and *Carpinus* spp. (Betulaceae) and *Tilia* spp. (Malvaceae) (Olivier et al. 2018; Wang et al. 2019), while the host range of *T. indicum* appears to be wider, including *Quercus*, *Castanea*, and *Castanopsis* spp. (Fagaceae), *Populus* spp. (Salicaceae), *Platycarya* spp. (Juglandaceae) as well as *Pinus* and *Keteleeria* spp. (Pinaceae) (Wang 2012; Wang et al. 2020). Conifer and chestnut species are common hosts of *T. indicum* (Chen et al. 2011; Wang et al. 2020) but are rarely associated with *T. melanosporum* (Olivier et al. 2018). The large array of tree species hosting *T. indicum* is likely to reflect the adaptation of *T. indicum* to a wide range of soil (García-Montero et al. 2008), geographic and climatic conditions (Wang et al. 2006; Deng et al. 2014).

Controlled mycorrhizal synthesis allows the characterization and identification of the mycorrhizae that *T. indicum* forms with different tree species and may be a tool to develop cultivation programs anywhere in the world where climatic conditions are compatible with *T. indicum* ecology. Choosing suitable host trees is a crucial determining factor of whether efficient cultivation from out-planted mycorrhizal seedlings will be successful (Palazón and Barriuso 2007; Benucci et al. 2012). In China, extensive work has been done to cultivate *T. indicum*, starting from mycorrhizal synthesis with potential indigenous hosts as a first step. To date, over 20 species of indigenous trees have been shown to form mycorrhizae with *T. indicum* in nursery containers (Chen 2003; Hu et al. 2004; Lin et al. 2008; Geng et al. 2009; Zhang et al. 2011, 2015; Wang 2012; Deng et al. 2014; Li et al. 2018). The first plantations were established principally in the Guizhou province (Hu et al. 2010). In recent years, many *T. indicum* plantations have been established in various areas of China using indigenous tree hosts (Wang 2012; Reyna and Garcia-Barreda 2014). Several plantations successfully produced ascomata and many more are expected to come into production in the near future (Wang et al. 2020).

Controlled mycorrhization of exotic tree species by *T. indicum* was first attempted by European scientists. Since the middle of the 1990s, massive export of *T. indicum* to Europe and North America threatened the Périgord black truffle industry (Murat et al. 2008) and mycologists started to work on the mycorrhizal synthesis of this species to compare its mycorrhizae with those of *T. melanosporum* on *Q. pubescens* (Comandini and Pacioni 1997), *Q. cerris* and *P. pinea* (Zambonelli et al. 1997). More recently, *T. indicum* was reported to form mycorrhizae with other European and American trees, including *Corylus avellana* (Mabru et al. 2001; Murat et al. 2008), *Q. ilex* (García-Montero et al. 2008), *Carpinus betulus* (Murat et al. 2008), *Pi. taeda* and *Carya illinoinensis* (Bonito et al. 2011). To the best of our knowledge, no *T. indicum* ascoma has yet been produced from truffle orchards outside of China, but Bonito et al. (2011) found one ascoma fruiting naturally from a *Pseudotsuga menziesii* forest in Oregon, USA, with an understory of *Cor. cornuta* var. *californica*. To date, the mycorrhization of exotic tree species by *T. indicum* has been poorly studied in China. However, mycorrhizal synthesis was successful with tree species such as *Car. illinoinensis* (Mei et al. 2019) and *Cor. avellana* (Li Shuhong et al. unpublished), imported to China more than 30 years ago for nut production (Liang 1986; Zhang et al. 2003). Small numbers of exotic trees mycorrhized by *T. indicum* have been planted in China since 2014 and 2017 for *Car. illinoinensis* and *Cor. avellana*, respectively, but truffles haven't been produced so far (Wang Yun and Yang Mei, pers. comm., June 2020).

In this study, we selected four host tree species exotic to China (three native to Europe and one to America) to determine their compatibility with *T. indicum* and to provide further description of the ectomycorrhizae obtained using a combination of microscopy and molecular tools. *Quercus ilex* and *Q. pubescens* are the main host species for *T. melanosporum* cultivation in France (Olivier et al. 2018), *Q. palustris* is a tree species native to North America that has never been tested for mycorrhization by *T. indicum*. *Pinus pinea* is a prime landscaping pine species native to the Mediterranean region that also provides edible nuts. We also aimed to compare the characteristics of the ectomycorrhizae formed by *T. indicum* on these trees with previous reports of mycorrhizae synthesized with host tree species exotic or native to China. Finally, we report the accidental cross-contamination of our *T. indicum*-inoculated *P. pinea* seedlings by *T. borchii* that was itself inoculated onto *P. pinea* seedlings belonging to another trial located in the same glasshouse.

2 Materials and methods

2.1 Seedling production and inoculation with truffle spores

Seeds of *Q. pubescens* and *Q. ilex* were collected from a private truffle plantation near Mérignac (Charente department, France)

in October 2016. Seeds of *Q. palustris* were collected from Beijing Botanical Garden in November 2016. Seeds of *P. pinea* were acquired from Pinoli Limited (Blenheim, New Zealand) in the spring of 2017. The oak seeds were washed in tap water and soaked in water at an initial temperature of 55 °C for 1 day (Mao et al. 2013), then surface sterilized in sodium hypochlorite (2% available chlorine) for 2 h. Seeds of *P. pinea* were washed in tap water and surface sterilized in 30% H_2O_2 for 30 min. After being thoroughly rinsed in distilled water, seeds were sown in a tray on December 2016 (*Quercus* spp.) or June 2017 (*P. pinea*) in an autoclaved (1 h, 121 °C) mixture of perlite and vermiculite (1:1 by volume). Trays were placed in a greenhouse at the Kunming Institute of Botany (KIB) under natural light (e.g., 169 $\mu mol^{-2}\ s^{-1}$ 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. Oaks were inoculated on 27 June 2017 and pines on 21 November 2017, i.e. 6 or 5 months after germination, respectively.

Ascomata of *T. indicum* were obtained from Kunming Mushuihua market, Yunnan, China, in the winter of 2016, confirmed to be of *T. indicum* by morphology and stored in the freezer at −40 °C until use. For oak inoculation, substrate was made of peat (Jiffy, The Netherlands), pumice (Fuyuan, Kunming), pine bark (Mu-mu Biology, Zhejiang) and lime (Kunming) (9:9:2:2 by volume, initial pH of 7.26). For *P. pinea* inoculation, substrate was made of vermiculite (Niu-niu Gardening, Hebei), perlite (Jing-rui, Kunming), peat (Jiffy, The Netherlands) and pine bark (Mu-mu, Biology) (4:2:1:1, initial pH of 6.87). Substrate was sterilized by autoclaving in 10-L bags for 1 h at 121 °C, three times at 48 h intervals. The final pH of the homogenized substrates was adjusted to 7.5 before autoclaving by adding calcium carbonate (0.19 or 0.45 g/L) and magnesium carbonate (0.1 or 0.2 g/L) for the oak or pine substrates, respectively. The spore suspension was prepared by grinding ascomata using a small blender (Philips HR2095, Hong Kong), until the spores were released. For each tree species, each of the five replicate seedlings was inoculated with 10 mL of spore suspension (containing 1×10^6 spores/mL, as determined using a hemocytometer): two doses of 5 mL (equivalent to 1 g fresh truffle per seedling) were distributed around the third upper zone of the root system when planting seedlings in 688-mL square pots (13.2 × 6.4 × 9.1 cm, Xinguanghe Horticulture, Zhejiang). Pots were placed on grid tables in the greenhouse and arranged in groups. Each group consisted of a given tree species inoculated with *T. indicum*: rows of five seedlings. *Tuber indicum*-inoculated *P. pinea* seedlings were placed ~1 m away from *T. borchii*-inoculated *P. pinea* seedlings belonging to an inoculation trial separate from the present study. Pines were on a different table than oaks (~1.5 m apart). Conditions within the area where seedlings grew were fairly homogeneous.

2.2 Morphological observations of ectomycorrhizae

At 6, 12 and 24 months after inoculation, all seedlings were examined in a non-destructive manner (except for mycorrhizal tips mounted on microscope slides) for mycorrhiza formation by light microscopy following the methodology of Wang et al. (2019). The macro-morphological and anatomical characters of *T. indicum* mycorrhizae were examined and photographed under dissecting (Leica S8AP0) and compound (Leica DM2500) microscopes following the method by Agerer (2006). Cross sections were made by hand or using a freezing microtome (Leica CM3050S). In the case of *Q. ilex*, cross sections were made again 28 months after inoculation to obtain better images. All tissues were mounted in distilled water on slides. Morphology and anatomy of *T. indicum* mycorrhizae formed with four host trees were described following the work of Agerer (1987–2008). The identification of *T. indicum* ectomycorrhizae based solely on their morphological characters was insufficient under Chinese conditions since other *Tuber* species show similar mycorrhizal morphology (e.g. *T. pseudoexcavatum*, García-Montero et al. 2008), and a molecular identification was necessary to confirm the fungal identity.

2.3 Molecular analysis of ectomycorrhizae

Genomic DNA from synthesized *T. indicum*-like ectomycorrhizae, and any morphologically different ectomycorrhizae, was extracted from one mycorrhizal tip per mycorrhizal morphotype per tree species 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 et al. 1990; Gardes and Bruns 1993). Each 25 μL PCR consisted of 2.5 μL 10× PCR buffer (Mg^{2+}), 1.5 μL dNTPs (1 mM), 1 μL BSA (0.1%), 1 μL each primer (5 μM), 0.5 μL $MgCl_2$ (25 mM), 1 μL DNA extracts and 1.5 U Taq DNA polymerase (Takara, Takara Biotechnology, Dalian Co. Ltd., China). PCR thermoprofiles consisted of an initial denaturation step of 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 a final extension at 72 °C for 10 min. Three microliters of PCR products were run on 1% (*w*/*v*) agarose gels and visualized by staining with ethidium bromide in a Molecular Imager Gel Doc EX system (Syngene, China). Images were photographed by Gel-Pro 4.5 Analysis software. PCR products were purified and sequenced in both directions using ITS1F and ITS4 primers at TsingKe Biological Technology, Kunming, China. The ITS sequences obtained were compared for similarity with those present in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Mycorrhiza sequences generated in this study were deposited in GenBank.

3 Results

3.1 Morphological and anatomical characterization of synthesized mycorrhizae

Tree seedlings, morphological and anatomical characters of ectomycorrhizae formed by *T. indicum* with *Q. palustris*, *P. pinea*, *Q. pubescens* and *Q. ilex* are shown in Figs. 1 and 2 and Suppl. Figs. 1–2, respectively. Descriptions of their macro-morphological and anatomical characteristics are presented in Table 1. All mycorrhizae obtained showed a *T. indicum*-like morphology, i.e. overall aspect and color (Figs. 1b–c and 2b–c, Suppl. Figs. 1b–c, 2b–c), long, hyaline emanating hyphae (Figs. 1d–e and 2c–e, Suppl. Figs. 1d–e, 2d–e) branching at approximately right angle (Figs. 1e and 2e, Suppl. Fig. 2e), and a jigsaw puzzle pattern of outer mantle cells (Figs. 1f and 2f, Suppl. Figs. 1f, 2f). However, unexpectedly, a different mycorrhizal morphotype was observed on *P. pinea* seedlings: a *Tuber*-like mycorrhiza as per its shape and color (Suppl. Fig. 3b–c), with short, unramified, spiky emanating hyphae (Suppl. Fig. 3b–e) and a *Tuber*-characteristic jigsaw puzzle (Suppl. Fig. 3f).

Mantle cells of *Q. ilex*' *T. indicum* mycorrhizae are slightly bigger than those of *Q. pubescens* and *Q. palustris* (Table 1). The outer mantle cells of *P. pinea* mycorrhizae (Fig. 2f) are longer than for the oaks (Fig. 1f, Suppl. Figs. 1f and 2f). Outer mantle cells of *Q. palustris* (Fig. 1f) are more roundish and less interlocked than those of the other oak species (Suppl. Figs. 1f & 2f). The Hartig net colonizes most cortical cells in *P. pinea* (Figs. 2g–h, Suppl. Fig. 3g–h) while it appears

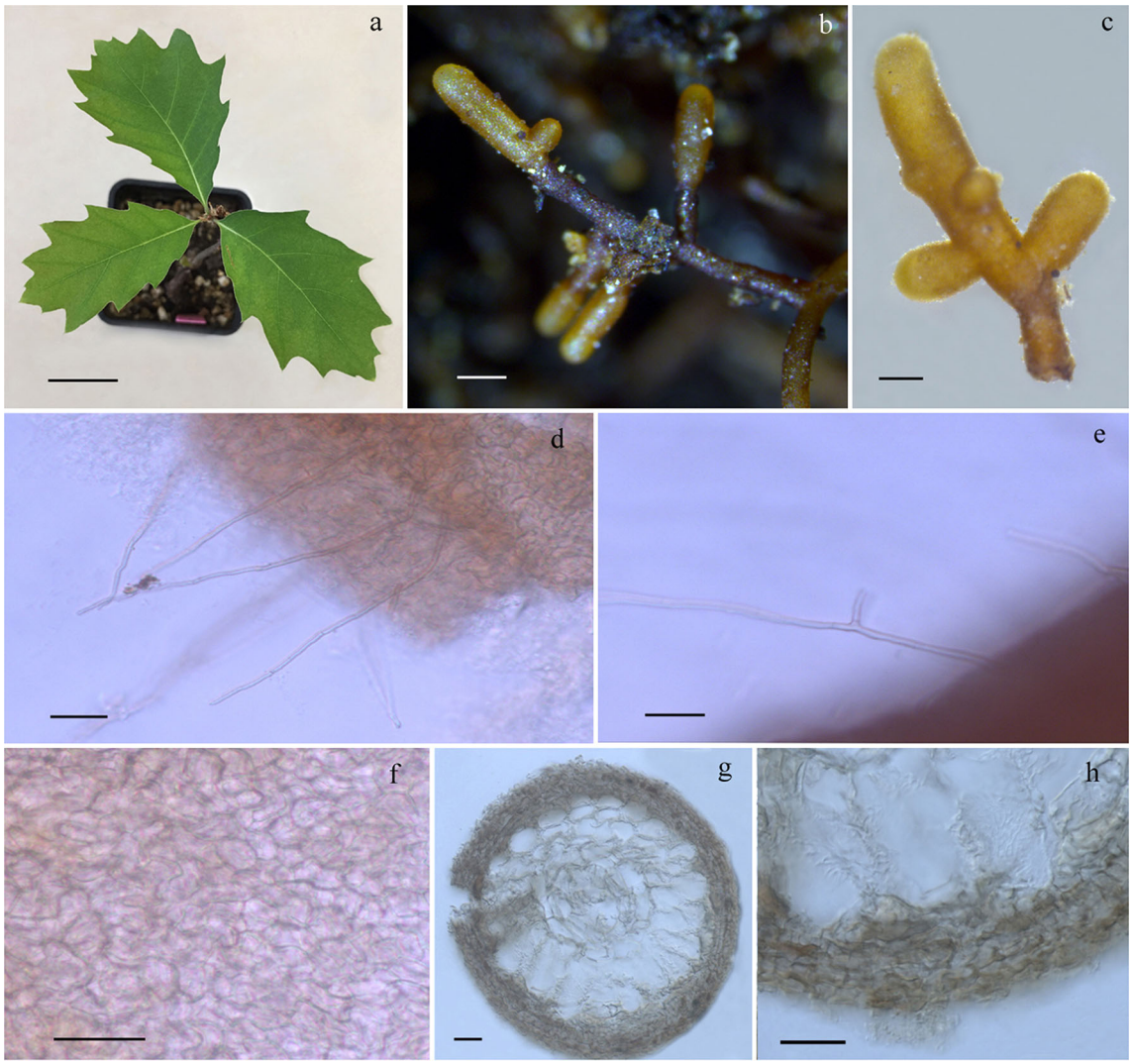

Fig. 1 **a** Seedlings of *Quercus palustris* 6 months after inoculation (*bar* = 5 cm). **b**–**h** Macro-morphological and anatomical characters of artificially synthesized ectomycorrhizae of *Tuber indicum* on *Q. palustris* 6 months after inoculation. **b**–**c** Ectomycorrhizae of *T. indicum* formed with *Q. palustris* (*bars* = 200 μm). **d**–**e** Septate hyphae emanating from the outer mantle layer (**d**), branched (**e**), whole tip mounted in water (*bars* = 20 μm). **f** Outer mantle surface structure (*bar* = 20 μm). **g**–**h** Cross-mycorrhiza sections (*bars* = 20 μm)

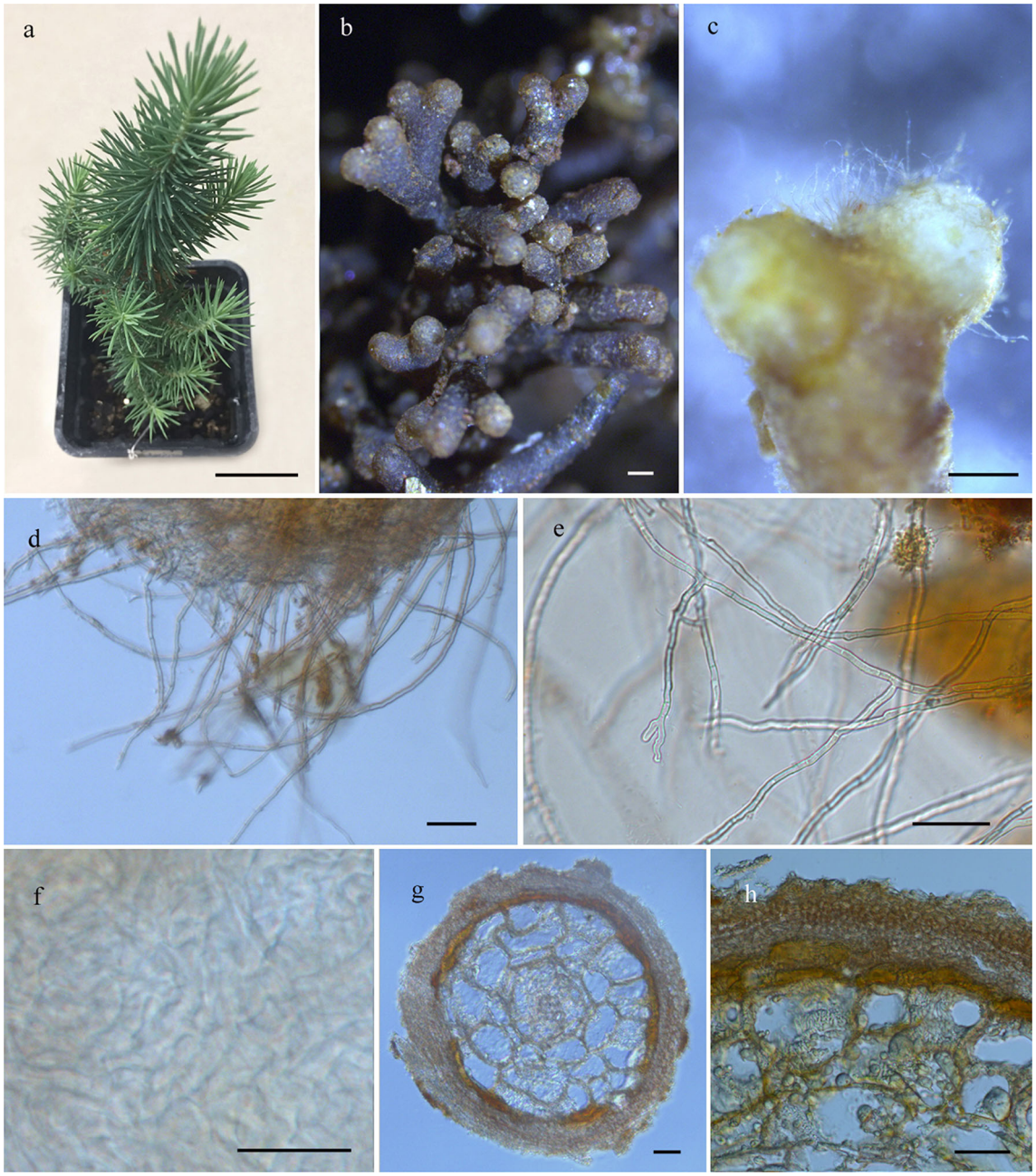

Fig. 2 **a** Seedlings of *Pinus pinea* 6 months after inoculation (*bar* = 5 cm). **b**–**h** Macro-morphological and anatomical characters of artificially synthesized ectomycorrhizae of *Tuber indicum* on *P. pinea* 24 months after inoculation. **b**–**c** Ectomycorrhizae of *T. indicum* formed with *P. pinea* (*bars* = 200 μm). **d**–**e** Emanating hyphae arising from the outer mantle layer (**d**), septate (**e**), whole tip mounted in water (*bars* = 20 μm). **f** Outer mantle surface structure (*bar* = 20 μm). **g**–**h** Cross-mycorrhiza sections (*bars* = 20 μm)

restricted to the outer cortical cell layers in oaks (Fig. 1g–h, Suppl. Figs. 1g–h, 2g–h).

3.2 Molecular identification of truffle species from mycorrhizae

All the ITS sequences obtained from *T. indicum*-like mycorrhizae formed with each *Quercus* species and with *P. pinea* matched *T. indicum* ITS sequences deposited in GenBank. The three ITS sequences from *Quercus* mycorrhizae (all identical) and that from *Pinus* mycorrhizae showed 99.83% and 100% identity with the *T. indicum* sequences GU979080.1 and DQ375516.1, respectively. Furthermore, in the case of *P. pinea* seedlings, the sequence obtained from the *Tuber* mycorrhizal morphotype having unramified spiky emanating hyphae showed 100% similarity with the GenBank sequence of *T. borchii* (KT165350.1). The ITS sequences of *T. indicum* ectomycorrhizae obtained in this study with *Q. pubescens*

Table 1 Macro-morphological and anatomical characteristics of artificially synthesized mycorrhizae of *Tuber indicum* on *Quercus pubescens*, *Q. ilex*, *Q. palustris* and *Pinus pinea*

Type of characteristics	*Q. pubescens*	*Q. ilex*	*Q. palustris*	*P. pinea*
Branching and shape	Simple or ramified in a monopodial- pinnate pattern, up to 2.9 mm long, 2.0 mm wide	Unramified or dichotomous, sometimes almost coralloid type, up to 7.0 mm long, 5.5 mm wide	Simple or ramified or monopodial with lateral, up to 2.7 mm long, 1.7 mm wide	Dichotomous, almost becoming coralloid, up to 3.2 mm long, 2.7 mm wide
Color	Pale yellow - brown - dark brown	Pale brown - ochre - dark brown	Yellowish brown to dark brown	Pale yellow - yellowish brown - dark brown
Unramified ends	Straight, cylindric or club- shaped with rounded ends, up to 2.6 mm long, 0.19–0.34 mm in diam. Some tips had a whitish growing up apex	Straight or slightly bent, cylindric or club-shaped with rounded ends, up to 4.0 mm long, 0.18–0.28 mm in diam. Some tips had a whitish growing up apex	Straight, cylindric or club shaped with rounded ends, up to 2.3 mm long, 0.25–0.39 mm in diam.	Straight, club-shaped or cylindric with rounded ends, up to 1.8 mm long, 0.28–0.43 mm in diam. Some tips had a whitish growing up apex
Mantle surface	Smooth or woolly hyphae	Smooth or woolly hyphae	Smooth or woolly hyphae	Smooth or woolly hyphae
Mantle	19–28 µm thick, three to five layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 4.4–11.3 µm × 3.4–6.3 µm, number of cells in 20 × 20 µm square 3–5	9–19 µm thick, two to four layer composed of epidermoid cells structured in an uneven regular puzzle-like pattern. 3.2–8.2 µm × 2.2–5.5 µm, number of cells in 20 × 20 µm square 3–4	16–30 µm thick, four to six layer composed of ellipse-like cells structured in an uneven regular puzzle-like pattern. 3.6–10.7 µm × 2.6–5.2 µm, number of cells in 20 × 20 µm square 3–5	25–40 µm thick, five to seven layer composed of epidermoid cells structured in an uneven puzzle-like pattern. 6.6–15.3 µm × 3.2–6.1 µm, number of cells in 20 × 20 µm square 2–3
Rhizomorphs	Absent	Absent	Absent	Absent
Emanating hyphae	Simple or branched, smooth, septate, slightly tortuous, pale brown, some with right angle ramification, up to 241 µm long, 1.7–3.4 µm in diam.	Simple or branched, smooth, septate, slightly tortuous, pale brown, some with right angle ramification, up to 389 µm long, 1.7–2.8 µm in diam.	Simple or branched, smooth, septate, pale brown, some with right angle ramification, up to 189 µm long, 3–3.4 µm in diam.	Wooly-like, smooth, septate, slightly tortuous, pale brown, scattered or numerous, up to 168 µm long, 1.4–2.8 µm in diam.
Hartig net	Palmetti type (Mangin 1910)	Palmetti type	Palmetti type	Palmetti type

(mhll-1, accession number MK880411), *Q. ilex* (mhll-2, MK880412), *Q. palustris* (mhll-3, MK880413) and *P. pinea* (mhll-4, MK880414), and that of *T. borchii* obtained with *P. pinea* (mhll-5, MN733243) were deposited in GenBank.

3.3 Mycorrhization success

Mycorrhizae were observed 6 months after inoculation for all combinations of *T. indicum* with *Q. pubescens*, *Q. ilex*, *Q. palustris*, and *P. pinea* (Table 2) but the rate of success (the number of mycorrhized seedlings out of the number of inoculated seedlings) varied with the tree species. All five replicate seedlings of *Q. pubescens* and *Q. ilex* formed well-developed mycorrhizae (Table 2, Suppl. Figs. 1–2) that were branching frequently (Suppl. Figs. 1b, 2b) and distributed all over the root systems, especially at their upper 1/3 (data not shown). For *Q. palustris* seedlings, only two out of five replicates formed mycorrhizae (Table 2, Fig. 1). Although mycorrhizae appeared less abundant than on the other oak species, i.e. required a longer time to be spotted under the microscope, well-developed branched mycorrhizae were also observed on these two seedlings (Fig. 1b–c). No other ectomycorrhizal fungi were detected in any of the oak seedlings. One out of five replicate seedlings of *P. pinea* formed mycorrhizae with *T. indicum* (Table 2, Fig. 2), not distributed everywhere on the root system but including ramified clusters (Fig. 2b). The other four *P. pinea* seedlings had formed mycorrhizae with the contaminant *T. borchii* (Suppl. Fig. 3), distributed all over the root systems (data not shown) and branching frequently (Suppl. Fig. 3b–c).

4 Discussion

This is the first Chinese study to provide detailed morphological characteristics under both dissecting and compound microscopes of mycorrhizae obtained between *T. indicum* and four tree species exotic to China. This is also the first report of successful mycorrhiza synthesis between *T. indicum* and

Table 2 Number of surviving (Surv.) and mycorrhizal (Myc.) seedlings at three assessments (months) following inoculation

Trees	6 months		12 months		24 months	
	Surv.	Myc.	Surv.	Myc.	Surv.	Myc.
QPu	5/5	5/5	5/5	5/5	5/5	5/5
QI	5/5	5/5	4/5	4/4	4/4	4/4
QPa	5/5	2/5	4/5	2/4	4/4	2/4
PP	5/5	1/5	5/5	1/5	5/5	1/5

QPu, *Quercus pubescens*; QI, *Q. ilex*; QPa, *Q. palustris*; PP, *Pinus pinea*

Q. palustris and the first to provide dissecting microscope views of *T. indicum* mycorrhizae synthesized with *Q. pubescens* and *P. pinea*. The ITS sequences of all mycorrhizae synthesized here are identical to reference sequences for *T. indicum* deposited in GenBank, confirming the formation of ectomycorrhizae of *T. indicum*.

The mycorrhizae of *T. indicum* synthesized in our study with four non-indigenous species, three oaks and one pine, all displayed similar colors, shapes and anatomical characteristics. Characteristics include a mantle surface that is smooth or with relatively rare, wooly, transparent, finely walled, emanating hyphae branching at approximately right-angle, and a typical, *Tuber*-like jigsaw puzzle pattern of the mantle cells. Despite the overall similarity of *T. indicum* mycorrhizae synthesized here, slight morphological differences were present between mycorrhizae of different trees species. For example, mycorrhizae of *T. indicum* on *P. pinea* were darker colored and more stout than those on the oak species tested. Geng et al. (2009) observed the same phenomenon on *T. indicum* mycorrhizae obtained on *Pi. armandii* in comparison with those obtained on another Fagaceae species, *Castanea mollissima*. In addition, in our study, *T. indicum* mycorrhizae synthesized on *P. pinea* showed emanating hyphae more frequently that those formed on *Quercus* spp. However, emanating hyphae are not always present or depend on the age of ectomycorrhizae (Guerin-Laguette, unpublished) and more work is required to confirm this difference. When comparing the anatomical characteristics of mycorrhizae obtained from the three *Quercus* species, the mantle cells of mycorrhizae of *T. indicum* with *Q. ilex* were slightly bigger than the same cells of the other two *Quercus* species. Further, the shape of the outer mantle cells of mycorrhizae synthesized with *Q. palustris* were more round than those of the other oak species. Beyond these slight variations between the different tree species found in our study, similar colors, shapes and anatomical characteristics were described for previous syntheses of *T. indicum* mycorrhizae with the same tree species, e.g. *Q. ilex* subsp. *ballota* (García-Montero et al. 2008), *Q. pubescens* (Comandini and Pacioni 1997), and *P. pinea* (Zambonelli et al. 1997), or with other exotic tree species, e.g. *Q. cerris* (Zambonelli et al. 1997), *Pi. taeda* and *Car. illinoinensis* (Bonito et al. 2011). Our work demonstrated that these characteristics remain unchanged with *Q. palustris*.

Mycorrhizae of *T. indicum* were previously synthesized with at least 22 tree species native to China (see review by Wang 2012, Table 3). Of these studies, only four reported on the detailed morphological characteristics of the synthesized mycorrhizae under the compound microscope, i.e. Lin et al. (2008), Geng et al. (2009), Deng et al. (2014) and Zhang et al. (2015). These four studies described mycorrhizal characteristics similar to those observed in the present work: (i) swollen root tips light brown at first, dark brown when ageing, (ii) transparent, finely walled hyphae emanating from the mantle surface and branching at right angle, and (iii) a typical *Tuber*-like Jigsaw puzzle pattern of the mantle cells.

Table 3 Previously published successful mycorrhizal syntheses between *Tuber indicum* and 22 tree species native to China

Tree family	Tree species	References
Pinaceae	*Pinus armandii*	Hu et al. (2004, 2006), Geng et al. (2009)
	Pinus kesiya var. *langbianensis*	Zhang et al. (2015)
	Pinus massoniana	Hu et al. (2004, 2006)
	Pinus yunnanensis	Chen (2003), Hu et al. (2004), Lin et al. (2008)
	Keteleeria evelyniana	Lin et al. (2008)
Fagaceae	*Quercus acutissima*	Hu et al. (2004), Zhang et al. (2019)
	Quercus aliena	Chen (2003), Hu et al. (2004), Li et al. (2018)
	Quercus glauca	Chen (2003), Hu et al. (2004)
	Quercus fabrei	Chen (2003)
	Quercus franchetii	Geng LY (pers. comm.)
	Quercus gilva	Chen (2003)
	Quercus myrsinifolia	Chen (2003)
	Quercus pannosa	Deng et al. (2014)
	Quercus sessilifolia	Chen (2003)
	Castanea molissima	Geng et al. (2009)
	Castanopsis delavayi	Lin et al. (2008)
Salicaceae	*Populus bonatii*	Zhang et al. (2011)
	Populus yunnanensis	Deng et al. (2014)
Betulaceae	*Carpinus pubescens*	Chen (2003)
	Corylus sp.	Hu et al. 2004)
	Corylus yunnanensis	Lin et al. (2008)
Juglandaceae	*Platycarya strobilacea*	Su et al. (2012)

Our present study confirmed and extended the list of tree species that can form mycorrhizae with *T. indicum* under nursery conditions. However, successful mycorrhiza synthesis does not necessarily mean that the association will persist or thrive in nature or in truffle orchards. Of the 22 Chinese indigenous tree species described above, only nine have been shown to host *T. indicum* under natural conditions: *Pi. armandii*, *Pi. yunnanensis*, *Q. pannosa*, *Cas. mollissima*, *Po. yunnanensis* (Deng et al. 2014), *Q. acutissima* (Zang et al. 1992), *Q. franchetii* (Su 2005), *Castanopsis delavayi* and *K. evelyniana* (Lin et al. 2008). More work is required to detect natural associations of *T. indicum* with trees in the wild in China. Studying the persistence and development of *Tuber indicum* mycorrhizae introduced in situ after planting mycorrhizal seedlings of exotic or indigenous origin is also important to further assess the full host spectrum and possible highly adaptive potential of *T. indicum*.

Several authors (Comandini and Pacioni 1997; Zambonelli et al. 1997; García-Montero et al. 2008) noted that the characteristics of *T. indicum* mycorrhizae formed either on Chinese or exotic tree species are very similar to those of *T. melanosporum* formed on either type of host plants. The present study confirms these observations and extends them to *Q. palustris*' mycorrhizae. The well-defined characteristics of *T. melanosporum* mycorrhizae on its most common European host species (Palenzona 1969; Giraud 1988; Guerin-Laguette et al. 2013) have recently been observed on oak species native to China (Wang et al. 2019). *Tuber melanosporum* and *T. indicum* are phylogenetically closely related to each other (Mabru et al. 2001; Douet et al. 2004; Wang et al. 2006; Bonito et al. 2011). Our work further indicated that morphology alone cannot reliably distinguish between mycorrhizae formed by phylogenetically similar *Tuber* species, including *T. indicum*, *T. himalayense* and *T. longispinosum* (Kinoshita et al. 2018), regardless of the host origin. Molecular methods are required to identify such mycorrhizal morphotypes to species level and to monitor the distribution of *T. melanosporum*-related species in the field. The suspected broad host range and very broad pH range of *T. indicum* (5.5–8.5, Wang 2012) could give this species a competitive advantage over *T. melanosporum*.

To the best of our knowledge, this is the first time that *Q. palustris* has been assessed for controlled mycorrhization by a commercial truffle species. It revealed less affinity for *T. indicum* than *Q. ilex* and *Q. pubescens* since only two out of five inoculated *Q. palustris* seedlings formed well-developed ectomycorrhizae within the same period of time. However, these results are based on a small number of replicate plants and more work is required to confirm these initial observations. We speculate that *Pinus pinea* could be more receptive to *T. borchii* than to *T. indicum* since out of five inoculated seedlings only one seedling formed ectomycorrhizae of *T. indicum*, while four heavily formed ectomycorrhizae with the contaminant *T. borchii*. Although obtaining *T. borchii* mycorrhizae on *T. indicum*-inoculated plants was unexpected, previous greenhouse studies have shown that *T. borchii*-inoculated seedlings can cross contaminate seedlings inoculated with other *Tuber* species (Guerin 2015). Our study further confirmed this phenomenon since the *P. pinea* seedlings were only inoculated with *T. indicum* but formed *T. borchii* mycorrhizae. The most likely source of contamination were the *P. pinea* seedlings inoculated with *T. borchii* that were part of another trial in the same glasshouse. Two hypotheses are formulated to explain the development of *T. borchii* mycorrhizae on seedlings that were not inoculated with this species, i) either the accidental dispersal, by insects traveling between plants, of remaining *T. borchii* spores (i.e. from the original *T. borchii* inoculum), and/or ii) the dispersal (by air, insects or splash dispersal while watering) of newly formed, mitotic, asexual *T. borchii* spores formed on *T. borchii*-inoculated seedlings since this species has been shown to produce such vegetative spores in nature (Urban et al. 2004).

In conclusion, we conducted successful mycorrhization trials of *T. indicum* with four exotic tree species, the first time with *Q. palustris*, and provided detailed morphological descriptions and illustrations of these mycorrhizae. This study contributes towards better understanding of the mycorrhization potential of *T. indicum* on exotic tree species and the selection of such host species for *T. indicum* cultivation trials in China. *Quercus ilex* and *Q. pubescens* were promising but more work is required to explore the potential of *Q. palustris* and *P. pinea*. Our study further stressed the need to use a combination of microscopy and molecular tools to distinguish between the mycorrhizae formed by *T. indicum* and *T. melanosporum*-related black *Tuber* spp.. Further study should focus on assessing the persistence and development in the field of *T. indicum* mycorrhizae synthesized with exotic tree species and to monitor ascoma production in trial plantations in China.

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Compliance with ethical standards

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