Mycorrhiza (2009) 19:461–467
DOI 10.1007/s00572-009-0247-0

ORIGINAL PAPER

Mycorrhizal synthesis of *Tuber indicum* with two indigenous hosts, *Castanea mollissima* and *Pinus armandii*

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Received: 23 January 2009 / Accepted: 6 April 2009 / Published online: 29 April 2009

Abstract *Tuber indicum* is one of the most renowned commercialized fungi in China. Mycorrhizal investigations, however, have been carried out mainly with exotic trees. Up to now there is no detailed description of morphology of the mycorrhizae formed with the indigenous hosts of *T. indicum*. Containerized seedlings of two indigenous hosts of the fungus in southwestern China, *Pinus armandii* and *Castanea mollissima*, were inoculated with aqueous spore suspension of *T. indicum* in two kinds of substrates. Mycorrhizae began to form 4 months after inoculation and were harvested at 9 months. The contributing fungus of the mycorrhizae was confirmed to be *T. indicum* by morphological and ITS-rDNA sequence analyses. The morphology of emanating hyphae and epidermoid-like mantle appearance was similar to the mycorrhizae obtained with some European trees. The high morphological variation and the similarity to that of *Tuber melanosporum* makes it difficult to distinguish the mycorrhizae of the two species by morphology alone. The synthesis of mycorrhizae of *T. indicum* with its indigenous hosts will be of great significance for planned cultivation of the Asian black truffles.

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Keywords Asian black truffle · Ectomycorrhizal fungi · ITS-rDNA · Morphology

Introduction

Though the Asian black truffle *Tuber indicum* Cooke & Massee was described early in 1892 (Cooke and Massee 1892), it drew little attention until it was exported to Europe and North America in the 1990s. Presently, with the inclusion of previously separate but very similar species, including *Tuber sinense* K. Tao & B. Liu, *Tuber himalayense* B.C. Zhang & Minter, *Tuber pseudohimalayense* G. Moreno et al., *Tuber formosanum* H.T. Hu, the black truffle originating from Asia has become a well-known edible fungi internationally. The high similarity of *T. indicum* to the Périgord black truffle, *Tuber melanosporum* Vittad. and the potential confusion between the two species led to a series of intensive molecular taxonomic/phylogenetic research (Janex-Favre et al. 1996; Paolocci et al. 1997, 1999; Roux et al. 1999; Mabru et al. 2001; Douet et al. 2004; Zhang et al. 2005; Wang et al. 2006; Murat et al. 2008). With recent studies synonymizing *T. himalayense*, *T. pseudohimalayense*, *T. formosanum*, and *T. sinense* with *T. indicum* (Zhang et al. 2005; Wang et al. 2006; Chen 2007), the accumulation of the above work has provided an accurate identification of *T. indicum* and its allies. The popularly used synonyms and numerous reports from the continent indicate the high morphological variability and broad distribution range of *T. indicum* in Asia (Fukiharu et al. 2006; Wang et al. 2006; Chen 2007).

With the Périgord black truffle threatened by importation of the Asian black truffle to Europe, some European mycologists have been trying to seek feasible and reliable methods to distinguish the two truffles. This prompted

various works on the mycorrhizal synthesis of *T. indicum* with several European trees, including *Corylus avellana* L., *Quercus cerris* L., *Quercus pubescens* Willd., *Quercus ilex* L., and *Pinus pinea* L. (Di Massimo et al. 1996; Comandini and Pacioni 1997; Gandeboeuf et al. 1997; Zambonelli et al. 1997; Mabru et al. 2001). Since the aim of these syntheses was to identify of *T. indicum* mycorrhizae, no detailed information on the factors affecting the formation of mycorrhizae has been published. The most recent data on the mycorrhizae and the substrates of *T. indicum* given by García-Montero et al. (2008), also is based on mycorrhizae synthesized on European trees. In China, attempts to cultivate Asian black truffle by breeding mycorrhizal seedlings have also been made. Mycorrhizal synthesis of *T. indicum* with its possible indigenous hosts in southwestern China, *Castanea mollissima* BL., *Pinus armandii* Franch., *Pinus massonia* Lamb., and *Pinus yunnanensis* Franch. using spores as inocula were recently reported by Chen (2003) and Hu et al. (2004, 2006). Hu et al. (2005) declared that ascocarps of *T. formosanum* [= *T. indicum*, according to Chen (2007)] were successfully harvested 8 years after planting the truffle-infected seedlings of *Cyclobalanopsis glauca* (Thunb.) Oerst. to the field (10 years after inoculation). Yamanaka et al. (2000, 2001) show that *T. indicum* var. *yunnanense* Yamanaka formed dichotomous ectomycorrhizae with *P. armandii* and gave a general description and provided photographs of these mycorrhizae. However, up to now, there has been no detailed morphological data of the mycorrhizae on the indigenous trees.

As a result of excessive and undue exploration and utilization, the natural resource of *T. indicum* in China is seriously threatened (Wang and Hall 2001; Wang et al. 2007). It is urgent to conserve the Asian black truffle, either by protecting the habitats, limiting hunting activities, or by producing ascocarps with methods of planting truffle-infected seedlings to meet the market demands. The limited knowledge of the morphology of mycorrhizae and the influence of biological and non-biological factors that ensure success, such as substrates, host plants, inocula, and inoculating methods necessitates the research on the establishment of mycorrhizae of *T. indicum* on its indigenous hosts.

Asian black truffles were reported in association with a variety of species, including *Alnus nepalensis* D. Don., *Cyclobalanopsis glauca*, *P. armandii*, *P. yunnanensis*, *Pinus taiwanensis* Hayata., *Quercus* spp. *Keteleeria evelyniana* Mast. (Zhang and Minter 1988; Zhang and Wang 1990; Hu 1992; Zang et al. 1992; Chen et al. 1998; Chen 2007; Wang et al. 2007). The symbiotic relationships of *T. indicum* with some of these trees, however, are still to be confirmed by morphological, molecular, or experimental proofs. Among the indigenous hosts confirmed (Hu 1992; Hu et al. 2006; Chen 2007), *P. armandii* and *C. mollissima* are two main trees that are economically important and popularly planted in the south and north of China. Combining the cultivation of mycorrhizal edible fungi with afforestation of economic plants will have a practical significance in the mountainous area of developing countries. The successful cultivation of valuable truffles and quality indigenous trees could have great economic potentialities for the local people in marginal regions. Since morphologic identification of mycorrhizae will be an important criteria and feasible target for judging the success of synthesis, the objectives of this work are to provide detailed descriptions of the *T. indicum* mycorrhizae formed with *C. mollissima* and *P. armandii*.

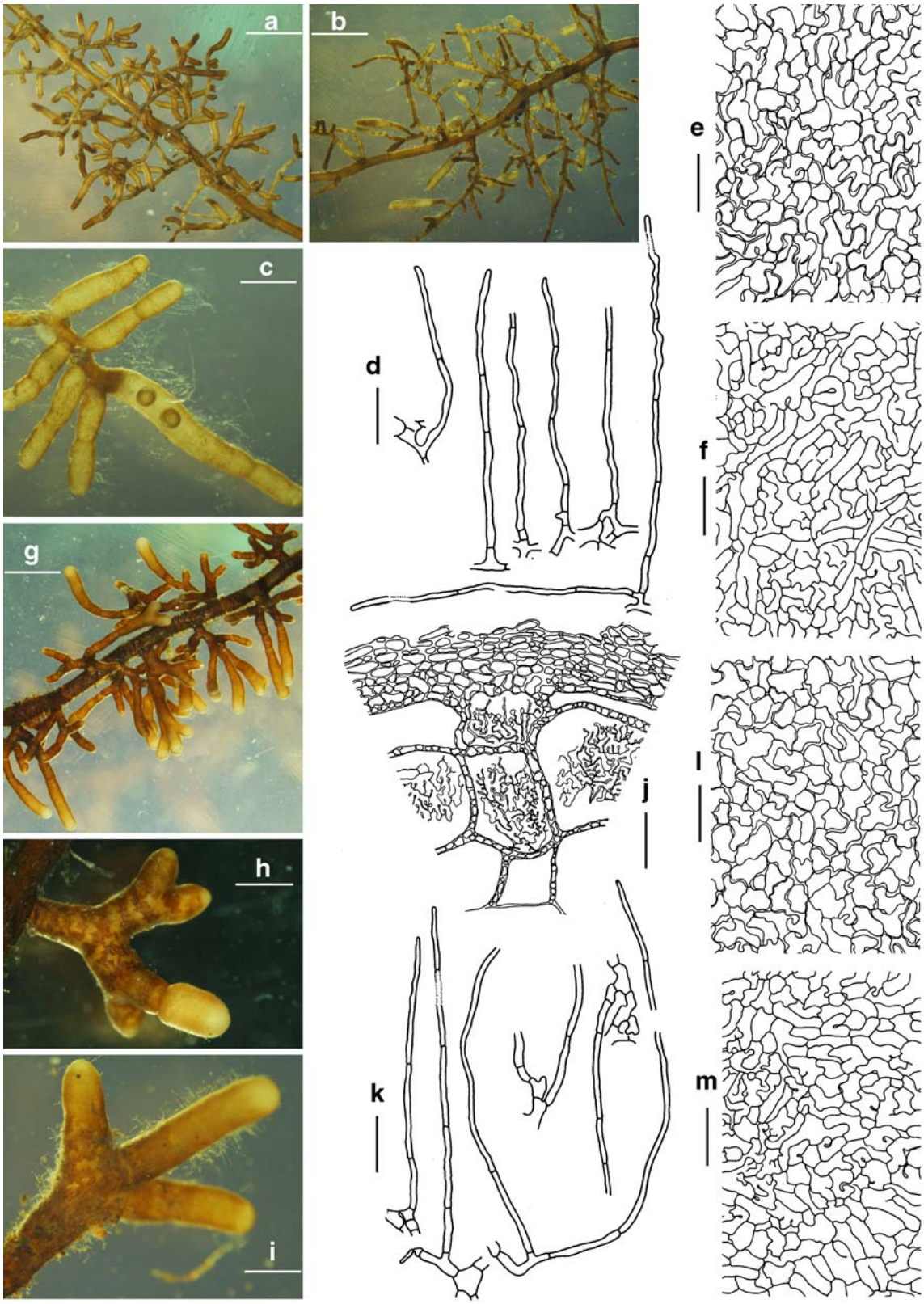
Fig. 1 Morphological-anatomical characteristics of mycorrhizae of *Tuber indicum* synthesized with *Castanea mollissima* (**a–f**) and *Pinus armandii* (**g–m**). **a** monopodial-pyramidal ECM system; **b** simple ECM system; **c** ECM tips with whitish flocculent emanating hyphae; **d** emanating hyphae; **e** puzzle-like outer mantle layers with epidermoid cells; **f** inner mantle lays with some hyphae in a Hartig net pattern; **g** dichotomous and irregularly pinnate ECM systems; **h** ECM tips without emanating hyphae; **i** ECM tips with emanating hyphae; **j** transverse section of ECM; **k** emanating hyphae; **l** puzzle-like outer mantle layers with epidermoid cells; **m** inner mantle lays with some hyphae in a Hartig net pattern. **a**, **b**, **g** *bars*=2 mm; **c**, **h–i** *bars*=600 μm; **d–f**, **j–m** *bars*=25 μm

Materials and methods

Mycorrhizal synthesis

Ascocarps of *T. indicum* were commercially acquired from Kunming market, Yunnan, China and said to be collected in Yunnan at the end of February, 2006. After cleaned with tap and distilled water, and sterilized with 75% alcohol, the ascocarps were stored at −20°C until use. Aqueous spore suspension was prepared by blending the chopped ascocarps with a blender, until the spores were released. Spore concentration was measured with a hemacytometer and the bulk spore suspension was then serially diluted with sterilized water into 10^6 spores/ml.

Seeds of *C. mollissima* and *P. armandii* (commercially acquired from Kunming market, China in the autumn of 2005) were surface sterilized with 30% H_2O_2 for 1 and 4 h, respectively. After washing three times with distilled water, seeds were sown in a square plastic container, which was filled with sterilized perlite/vermiculite (1/1, *v*/*v*). When the seedlings were 3-month-old they were transplanted in container (1.5 l) with 1,000 ml transplant substrate, consisting of humus/vermiculite/peat moss (1/1/1, *v*/*v*) and humus/soil/limestone (4/4/1, *v*/*v*), previously steam sterilized for 3 h. The final pH of both substrates were adjusted to seven by adding slaked lime. Each seedling per treatment received 50 ml spore suspension, at a rate of 5×10^7 spores/seedling.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One hundred forty seedlings for humus/vermiculate/peat and thirty five seedlings for humus/soil/limestone were inoculated both on *C. mollissima* and *P. armandii.* Ten seedlings acted as a control for each treatment. All pots were maintained in the greenhouse condition. Plants were watered when necessary. No fertilizers were applied to the plants.

After 9 months, seventeen seedlings randomly sampled in the treatment humus/vermiculate/peat and five in humus/soil/limestone were examined for the observation of ectomycorrhizae for both *C. mollissima* and *P. armandii*. Molecular analysis was also performed on these *T. indicum*-like mycorrhizae.

Morphological observation

Mycorrhizae were photographed under a Nikon SMZ1500 stereoscope and all microscopical drawings were made under a drawing tube installed in a Nikon E400 microscope with transmitted light. All anatomical sections were prepared from fresh root material by hand. Section of outer mantle layers was made by peeling the mycorrhiza along longitudinal axes and then putting the mantle part on the slide with its outer surface upwards. Inner mantle layers were obtained in the same way but with the inner surface upwards (according to Agerer (1991)). When transverse section was prepared, a mycorrhiza was sandwiched in filter paper for 3–4 min for dehydrating and then cut it into thin slides. All sections were made with newly opened sharp razor blade. For all slides 5% KOH then Congo Rid aqueous solution were used for observing and illustrating, except that presence or absence of a matrix was checked in water medium. The typing of mantle in Table 2 followed Agerer (1987–2002).

Molecular analysis

DNA was extracted from fresh mycorrhizae using the protocol of Doyle and Doyle (1987). DNA sequence data were obtained from ITS region for rDNA. The primers and PCR protocols were described previously (White et al. 1990). DNA sequences were initially aligned using Clustal X (Thompson et al. 1997), followed by manual alignment in the data editor of BioEdit ver. 7.0.1 (Hall 1999). Maximum parsimony analyses were conducted with PAUP*4.0b10 (Swofford 2002) with the heuristic search option (TBR and MULTREES on) and 1,000 replicates of random addition sequence.

Twenty complete ITS sequences of three black *Tuber* species (*T. indicum*, *T. melanosporum*, *Tuber brumale*) belonging to the *Melanosporum* group were used for analysis, among which six were obtained from fresh mycorrhizae and fourteen were downloaded from NCBI website (http://www.ncbi.nlm.nih.gov/). The *T. brumale* was selected as outgroup. The phylogenetic tree revealed three major, well-supported clades. The Clade I and II correspond to the two *T. indicum* clades was already suggested by Zhang et al. (2005) and Wang et al. (2006), and Clade III was basal to Clade I and Clade II. Our six *T. indicum* sequences obtained from mycorrhizae of *P. armandii* and *C. mollissima* truffle inoculated seedlings were identical and clustered in the *T. indicum* (Clade II). This clade comprises most samples from Huili (Sichuan Province), Chuxiong (Yunnan Province), and Kunming (Yunnan Province) (Zhang et al. 2005; Wang et al. 2006). As a result of the high phylogeographic structure of *T. indicum*, it is suggested that the ascocarps used for seedlings inoculation were probably harvested from these regions of China.

Results and discussion

Mycorrhizae formed 5 months after inoculation for *T. indicum* with *C. mollissima* and 4 months with *P. armandii*, respectively. All seedlings were formed well mycorrhizal systems with *T. indicum* without contaminating ectomycorrhizae of other fungi (Fig. 1a–b, g). Tables 1 and 2 show the morphological and anatomical characteristics of these ectomycorrhizae.

The mycorrhizae produced by *P. armandii* and *C. mollissima* with *T. indicum* are identical in the puzzle-like on the outer mantle layers the type H inner mantle layers and the thin-walled filamentous emanating hyphae with right-angle branching. The mycorrhizae of *P. armandii* and *C. mollissima* with *T. indicum* are typical truffle mycorrhizae (Zambonelli et al. 1997; Comandini and Pacioni 1997; Smith and Read 2008). By comparison, mycorrhizae on *P. armandii* are more dark-colored and stouter than those on *C. mollissima.* One distinctive character of the mycorrhizae on *P. armandii* is the stripe-like color pattern on the surface, which are more visible at the basal part or well-developed individuals (Fig. 1g–i). There were rich variations in dimensions of epidermal cells, degrees of interlocking between adjacent cells, and colors found between different individuals of mycorrhizae or even different parts of the same mycorrhiza.

Before this, a simple description of the mycorrhizae formed between *T. formosanum* (= *T. indicum*) and its indigenous host plant of *C. glauca* in Taiwan, China was provided (Hu 1992), which is similar to the ones produced by *C. mollissima* except that the color of emanating hyphae is golden yellowish in *T. formosanum* (under stereoscope). The mantle in the former description is thinner than that described here, which might partly be attributed to the preparation of slides for SEM observation.

Morphologically, the mycorrhizae of *C. mollissima* with *T. indicum* are not different from the ones produced by *T.*

Table 1 Morphological characters of ectomycorrhizae of *Tuber indicum* with *Castanea mollissima* & *Pinus armandii*

Species	*T. indicum* with *C. mollissima*	*T. indicum* with *P. armandii*
Mycorrhizae systems	Unramified to monopodial-pyramidal, with 0–1 order of ramification, up to 6.5 mm long (Fig. 1a–c)	Mostly dichotomous or irregularly pinnate (dichotomous-like), sometimes almost coralloid or between the above two types, with 1–4 orders of ramification, sometimes ramifications with 3–4 orders densely clustered along root rarely unramified, 2–4.6 mm long (Fig. 1g–i)
Main axes	0.2–0.4 mm in diameter	0.25–0.35 mm in diameter
Unramified ends	Straight, rarely slightly bent, usually cylindrical or slightly tapering, up to 1.9 mm long, 0.2–0.3 mm in diameter	Straight, cylindrical, 0.8–3.9 mm long, (0.22) 0.35–0.5 mm in diameter
Surface of unramified ends	Smooth or woolly with whitish emanating hyphae, very tips yellowish-white to pale ochraceous, older part concolorous or darker, the very base concolorous or brown; mantle distinct, not transparent, no transversal stripe-like color patterns, epidermal cells visible through mantle; cortical cells not visible	Almost smooth or loosely woolly with a few or numerous emanating hyphae, very tips yellowish to whitish, rarely yellowish brown to reddish brown, becoming darker towards base, often forming transversal stripe-like color patterns, whole mycorrhiza discoloring darker with age; mantle distinct, not transparent; cortical cells not visible
Emanating hyphae	Whitish, long, distinct, numerous, distributed from middle part to apex	Whitish, long, distinct, scattered or numerous, not specifically distributed

indicum with European species, such as *Q. pubescens* (Comandini and Pacioni 1997), *Q. cerris* (Zambonelli et al. 1997) and *Q. ilex* (García-Montero et al. 2008) except that the cells in outer mantle layers of our mycorrhizae seem bigger. The mycorrhizae of *T. indicum* reported by García-Montero et al. (2008) are much stouter (diameter of unramified ends 500–680 μm) than other reports (Comandini and Pacioni 1997; Zambonelli et al. 1997).

The anatomical characters of mycorrhizae of *T. indicum* are very similar to those of *T. melanosporum* (Comandini and Pacioni 1997; Zambonelli et al. 1997; García-Montero et al. 2008). Even compared with the description and illustrations of *T. melanosporum* on *Nothofagus* spp. (Pérez et al. 2007), the mycorrhizae obtained here do not show significant difference. It is not surprising that the mycorrhizae produced by *T. melanosporum* and *T. indicum* are

Table 2 Anatomical characters of ectomycorrhizae of *Tuber indicum* with *Castanea mollissima* & *Pinus armandii*

Species	*T. indicum* with *C. mollissima*	*T. indicum* with *P. armandii*
Mantle	(20) 25–40 (50) μm thick in transverse section, composed of (5) 6–9 layers of hyphal cells, cells round to elliptical from transverse section, 2–12 (14)×2–6 μm in diameter	20–30 (35) μm thick in transverse section, composed of (6) 7–10 layers of hyphal cells; cells round to elliptical from transverse section, 2–20 (25)×2–7 (8) μm in diameter (Fig. 1j)
Outer mantle layers	Pseudoparenchymatous with polygonal and interlocking cells arranged in a puzzle-like pattern in plan views (mantle type M), cells 10–35 (42)×5–20 μm, 6–10 cells in a square of 20×20 μm, slightly thick-walled, with yellowish brown walls, 0.5–1 μm thick, not gelatinous (Fig. 1e)	Pseudoparenchymatous with polygonal and interlocking cells arranged in a puzzle-like pattern in plan views (mantle type M), cells 10–25×(5) 8–11 μm, 7–10 cells in a square of 20×20 μm, thin-walled or slightly thick-walled, with yellowish brown walls, often locally transversal lightly dark-colored, surface smooth and no adhering, not gelatinous (Fig. 1l)
Inner mantle	Pseudoparenchymatous or a transitional type between plectenchymatous to pseudoparenchymatous in plan views (close to type H), partially Hartig net-like; cells 5–30 (35)×4–10 (15) μm, 9–14 cells in a square of 20×20 μm, often thin-walled (Fig. 1f)	Pseudoparenchymatous or a transitional type between plectenchymatous to pseudoparenchymatous in plan views (close to type H), partially Hartig net-like; cells 6–30 (35)×4–14 μm, 9–13 cells in a square of 20×20 μm, often thin-walled (Fig. 1m)
Emanating hyphae	Emerging from the outer mantle, scattered or abundant, 100–300 (400) μm long, 2–3 μm in diameter at the middle, 3.5–5 μm in diameter at the base, septate, sections between two septa 20–55 μm long, terminal section (17) 25–50 μm long, cylindrical, not inflated, often branching in approximately 90° angle near base, thin-walled, yellowish brown, long, surface smooth, tips cylindrical or tapering (Fig. 1d)	Emerging from the outer mantle, scattered, 100–300 (400) μm long, 2–2.5 μm in diameter at the middle, 3–5 μm in diameter at the base, septate, sections between two septa (14) 20–45 μm long, terminal sections (12) 15–40 (60) μm long, cylindrical, not inflated, sometimes branching in approximately 90° angle near base, slightly thick-walled at the base, elsewhere thin-walled with yellowish brown walls, short, surface smooth, tips cylindrical or tapering (Fig. 1k)
Very tip	With mantle structure as in other parts of the mycorrhiza but with small-sized cells	Idem

very similar because these two truffle species are closely related to each other phylogenetically (Janex-Favre et al. 1996; Paolocci et al. 1997, 1999; Roux et al. 1999; Mabru et al. 2001; Douet et al. 2004; Zhang et al. 2005; Wang et al. 2006).

Zambonelli et al. (1997) and García-Montero et al. (2008) found the mycorrhizae of *T. indicum* had smaller but less lobed polygonal pseudocells than those of *T. melanosporum*. Our result, however, shows that the cells in the outer layers are always bigger than those in *T. melanosporum.* Since the interlocking degree, namely more lobate or not is very variable, depending on different individuals of mycorrhizae and different parts of the same mycorrhiza, it is, therefore, not reliable enough to distinguish the mycorrhizae of the two black truffles by this difference alone. Molecular methods should be employed when tracing exotic species.

Mabru et al. (2001) reported that the formation of mycorrhizae of *T. indicum* was earlier and more abundant compared with *T. melanosporum.* According to García-Montero et al. (2008), *T. indicum* and *T. melanosporum* were able to form mycorrhizae 2.5 months after inoculation. In our work, however, the earliest mycorrhizae of *P. armandii* occurred 4 months after inoculation and those of *C. mollissima* even later. Moreover, inoculation of *C. glauca* with *T. formosanum* (= *T. indicum*) needed 5–6 months to form ectomycorrhizae (Hu 1992). By comparison, mycorrhization of *T. melanosporum* with fagaceous trees (*Nothofagus*, *Quercus*) over 40% of the seedlings and over 8% of root tips were colonized 6 months after inoculation (Pérez et al. 2007). Seedlings, 50–88%, were infected 5 months after inoculation with *Tuber maculatum* Vittad. (Parladé et al. 1996). These results indicate that infecting time of *T. indicum* seems not to be as short as other known truffle mycorrhization using spores as inocula. The differences of time needed for mycorrhizal initiation might be caused by using different methods for inoculation, different species of truffles and host plants, substrate, temperature, or other incubation conditions.

The spores rate of *T. indicum* used in our experiment was much higher than compared with 1.2×10^5 for *T. formosanum* (= *T. indicum*) in Hu et al. (2005), 10^2–10^4 for *T. maculatum* in Parladé et al. (1996), 5×10^6 for *Tuber aestivum* Vittad. (syn. *Tuber uncinatum* Chatin) recommended by Wedén (2004). In our experiment, on average one seedling received around 3 g fresh ascocarps. This is not an economic consumption. Future study is needed to find out what is the best rate of spores of *T. indicum* for inoculation.

To our knowledge, this is the first time that detailed morphological descriptions of the mycorrhizae of *T. indicum* on its indigenous hosts were provided. This will help to identify mycorrhizae of Asian black truffle with its indigenous hosts. Based on this, further experiments and statistical analyses should be stressed to promote our understanding on the optimized conditions in mycorrhization of the species *T. indicum*.

Acknowledgements The authors are very grateful to Dr. Christine Fischer, Dr. Carlos Colinas, and Dr. Alexis Guerin for their kind help on gathering literatures; Dr. Pierre Sourzat and Dr. Yun Wang are highly appreciated for helping to check the mycorrhizae formed; and Dr. Yun Wang, Dr. Christine Fischer, Dr. Alessandra Zambonelli, and Dr. Randy Molina critically reviewed the manuscript and kindly offered invaluable suggestions. The work was supported by "West Light" Program of Chinese Academy of Sciences (2004), National Natural Science Foundation of China (No. 30470011, 30770007), the Knowledge Innovation Program of the Chinese Academy of Sciences (No. KSCX2-YW-G-025), the president fund of the Chinese Academy of Sciences (1022, 1035), the supportive poverty program of Chinese Academy of Sciences, Natural Science Foundation of Yunnan Province (No. 2004C0050M) and the Joint Founds of the National Science Foundation of China and Yunnan Province Government (No. U0836604).

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