Mycorrhiza (2008) 19:7–14
DOI 10.1007/s00572-008-0198-x

ORIGINAL PAPER

New data on ectomycorrhizae and soils of the Chinese truffles *Tuber pseudoexcavatum* and *Tuber indicum*, and their impact on truffle cultivation

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Received: 7 April 2008 / Accepted: 16 August 2008 / Published online: 24 September 2008

Abstract Chinese truffles serve as a good complement to the market for *Tuber melanosporum* (Périgord black truffle). However, Chinese truffles could be introduced accidentally or fraudulently into the plantations of Mediterranean truffles, and they could have a negative effect on truffle production and natural ecosystems. The study of *Tuber* species from China which are commercialized in Europe began 14 years ago. *Tuber pseudoexcavatum* was proposed as a new species, and this has been validated by some authors based on molecular and phylogenetic studies. We synthesize their ectomycorrhizae using samples from the type collection, and we compare *T. pseudoexcavatum* and *Tuber indicum* ectomycorrhizae. The ectomycorrhizae of these species have a morphology which is related to the ectomycorrhizae of *T. melanosporum*. We provide useful information for the rapid screening of the above-mentioned Chinese truffles ectomycorrhizae, for the quality control of commercial plants mycorrhized with *Tuber*. Moreover, we analyze the soil tolerance and the host plant affinity of *T. pseudoexcavatum* and *T. indicum*, in order to assess the capacity of both Chinese truffles to penetrate *T. melanosporum* plantations and habitats.

Keywords Chinese truffle · Ectomycorrhizae · Truffle cultivation · Truffle ecology · Truffle yield

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Introduction

Many *Tuber melanosporum* Vittad. (Périgord black truffle) crops have a high carpophore production, but uncontrolled factors still induce a great deal of variability in production from year to year. Chevalier and Frochot (1997) and Lefevre and Hall (2001) indicate that contamination of truffières (truffle production sites) by other competing ectomycorrhizal fungi poses formidable problems for researchers working to optimize production of truffle species. Several authors have expressed their concern over the risk that commercial Chinese truffles (*Tuber* species) might be introduced accidentally or fraudulently into the plantations of Mediterranean truffles. Murat et al. (2008) have confirmed the appearance of *T. indicum* DNA in root tips and soil samples from a *T. melanosporum* plantation in Piedmont (Italy).

The international truffle market has seen regular shipments of Chinese truffles between China and Europe, Japan, United States, and Australia, for the last 14 years (Rey 2001; Wang and Hall 2001; Yamanaka et al. 2001; Yang 2001; García-Montero et al. 2005). Chinese truffles act as complements to the market for *T. melanosporum* because they are cheaper, have a milder aroma and the carpophores are of good quality for culinary purposes (Rey 2001).

New Chinese truffle species have been discovered in European markets, such as *Tuber pseudoexcavatum* Wang, G. Moreno, L. G. Riousset, J. L. Manjón, and G. Riousset (Wang et al. 1998). From the taxonomical point of view, *T. pseudoexcavatum* species were confirmed by several studies on phylogeny and taxonomy based on morphological characteristics, and DNA methods (Riousset et al. 2001; Zhang et al. 2005; Wang et al. 2006b). In the University of Alcalá, we synthesized *T. pseudoexcavatum* ectomycorrhizae using samples of fresh carpophores from the type collections. We observed that the ectomycorrhizae of this species had a similar morphology to the ectomycorrhizae of *T. melanosporum* (Manjón et al. 1998). However, we have not yet published a description of *T. pseudoexcavatum* mycorrhizae, as we are awaiting further information which would enable us to determine the taxonomy of the vast quantity of Chinese truffles that are being discovered and marketed.

Di Massimo et al. (1996), Zambonelli et al. (1996, 1997) and Comandini and Pacioni (1997) synthesized *Tuber indicum* Cooke and Massee ectomycorrhizae. They showed that these ectomycorrhizae also have a considerable affinity with *T. melanosporum* ectomycorrhizae. These authors, as well as García-Montero et al. (1997a, b, 2005) and Ferrara and Palenzona (2001), indicated the need to increase quality controls of mycorrhized plants in truffle culture, and warned that the Chinese truffles could displace truffles with a greater economic value and penetrate the Mediterranean *Tuber* ecosystems. In this regard, the latest trends for distinguishing the various *Tuber* ectomycorrhizae are based simultaneously on morphological taxonomical characters and a range of biochemical and DNA-based methods (Ferrara and Palenzona 2001; Mabru et al. 2001; Douet et al. 2004; Iotti and Zambonelli 2006).

It is, therefore, necessary to increase the current knowledge of the Chinese truffles ectomycorrhizae and to determine their ecological requirements and restrictions. We report the morphological characterization of *T. pseudoexcavatum* ectomycorrhizae, and we provide useful information for the rapid morphological screening of *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae versus *T. melanosporum* ectomycorrhizae. We also determine whether *T. pseudoexcavatum* and *T. indicum* could be easily introduced into Mediterranean habitats of *T. melanosporum*, depending on the characteristics of the soils and host plants common to *T. melanosporum*. We did this by analyzing samples of soils inhabited by *T. melanosporum* in natural Spanish woods, and we used these soil samples to synthesize *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae with the principal host plant of *T. melanosporum* in many Mediterranean areas, *Quercus ilex* L.

Materials and methods

Synthesis and study of Chinese truffles ectomycorrhizae

We synthesized *T. indicum* ectomycorrhizae using samples of fresh carpophores, and *T. pseudoexcavatum* ectomycorrhizae using samples of fresh carpophores from the same commercial batches as the type collections (*isotypus* AH-18383 and AH-18384). The taxonomical identifications are presented in Manjón et al. (1995) and Wang et al. (1998). The mycorrhizal synthesis of these Chinese species was done by applying a method of intensive mycorrhization on six *Quercus ilex* subsp. *ballota* (Desf.) Samp. plants, respectively, as described by Bencivenga (1982), but modified according to Manjón and García-Montero (1996). We also mycorrhized *T. melanosporum* with six plants as a comparative control test.

The *Quercus ilex* acorns and truffle carpophores were carefully washed, sterilized on the surface, and conserved in a fridge at a temperature of +4°C. The acorns were sown in semi-rigid plastic containers with a volume of 1,000 cc. The substrate used for seeding was a mixture of two calcareous soils from a natural *T. melanosporum* truffière, mixed with vermiculite and perlite, which was sterilized in an autoclave at 120°C for 4 h. At the moment of sowing, they were inoculated with a water suspension of ascospores obtained from finely-chopped carpophores. An acorn and a suspension containing 2 g of fruit body were placed in each container. The plants were kept under controlled environmental conditions in the Juan Carlos I Royal Botanical Garden at the University of Alcalá (Madrid, Spain). They were housed in a greenhouse with an average daily temperature of 20 to 25°C; relative humidity of 60 to 70%; watered by micro-sprinkler between one and three times a day for 1–3 min depending on the time of year; and under natural light conditions, with no artificial lighting. These parameters were controlled automatically using a computer. The plants were not fertilized. We obtained a batch of 18 well-lined, disease-free plants, with an average height of 17 cm and a diameter at the base of the plant of 2 cm.

The degree of mycorrhization of each plant was expressed as the number of *Tuber* mycorrhizae of the total number of apices counted, according to Bencivenga et al. (1987). The ectomycorrhizae were identified with a stereoscopic microscope (photo-Leica WildMZ8) and a microscope (photo-Leica LeitzDMRB) following the terms, descriptions, and recommendations of Agerer (1987–2002), Bencivenga et al. (1995) and Granetti (1995). This study was done with the criterion of selecting the macroscopic and microscopic characteristics of these ectomycorrhizae which would be most useful in the control procedures for mycorrhized plants in truffle culture, according to Bencivenga et al. (1987) and Granetti (1995). We, therefore, observed the morphological

Table 1 Morphological characters of *Tuber melanosporum*, *T. indicum* and *T. pseudoecavatum* ectomycorrhizae

Species	*T. melanosporum*	*T. melanosporum*	*T. indicum*	*T. indicum*	*T. pseudoexcavatum*
Synthesis source	Granetti 1995; Granetti et al. 2005; Zambonelli et al. 1993	Present study	Comandini and Pacioni 1997; Zambonelli et al. 1997	Present study	Present study
Geographical location	Mediterranean Europe	Spain	China	China	China
Host plant	*Quercus pubescens*, *Corylus avellana*, *Pinus sylvestris*	*Quercus ilex* subsp. *ballota*	*Quercus pubescens* and *Q. cerris*	*Quercus ilex* subsp. *ballota*	*Quercus ilex* subsp. *ballota*
Mode of ramification	Simple with clavate shape and round apex, or with monopodial-pinnate ramifications	Idem	May be simple, monopodial-pinnate, or dichotomic branched	Idem	From slightly club-shaped to almost cylindrical, without ramifications in very young forms, or with monopodial-pinnate ramifications
Length of unramified ends	From 200 to 4,000 µm	From 250 to 2,800 µm	From 150 to 1,620 µm	From 500 to 2,700 µm	Up to 2,500 µm
Diameter of unramified ends	From 230 to 450 µm	From 200 to 350 µm	From 120 to 540 µm	From 500 to 680 µm	Up to 240 µm
Structure of the surface	Smooth with long cystidia especially on the tip.	Idem	Smooth; long loose cystidia especially on the tip. Older specimens seem to lose cystidia	Idem	Smooth with long cystidia especially on the tip.
Color of unramified ends	Dark amber to ochre	Idem	7.5 YR 6/6 ocherous-amber	Idem	7.5 YR 6/6 dark brown

Comparisons include ectomycorrhizae synthesized in the present and previous studies

characters of the ectomycorrhizae, such as shape, size, type of ramifications, color, and mantle surface. The ectomycorrhizal color was described following Munsell (1976) standard soil color charts. For the analysis of the anatomical characters, we chose the median parts of adult ectomycorrhizae, as indicated by Agerer (1987–2002) and we observed the outer mantle surface and the mycelium. We measured the different characters, and maximum and minimum ranges of values were established by taking the necessary measurements until regularity was attained in the values observed, with a minimum of 30 measurements.

The morphological characteristics of *T. indicum*, and *T. melanosporum* ectomycorrhizae were described within the ranges and descriptions proposed by Granetti (1995), Comandini and Pacioni (1997), Zambonelli et al. (1997) and Granetti et al. (2005).

Soil analysis

In the mycorrhizal synthesis of the Chinese truffles, we used soil samples from natural Spanish woods inhabited by *T. melanosporum*. We took the soil samples from around a *T. melanosporum* truffière associated to *Quercus ilex* subsp. *ballota* and *Q. faginea* Lam., with a maximum carpophore production of up to 5,000 g/year. A more detailed description of the study area, the soils and the truffle habitats can be found in García-Montero et al. (2006).

We analyzed two soil samples. One soil sample was taken from the bare soil in the interior of this truffière, and the other at a distance of 5 m from the truffière. Only the first 30 cm of soil profile were taken and studied, as *T. melanosporum* usually bears fruit in this range. Sampling was done according to the FAO (1990). The following soil determinations were made: pH, total organic carbon (T.O.C.), total calcium carbonate (equivalent calcium carbonate), granulometric analysis, cation exchange capacity (C.E.C.) and the exchangeable cation saturation percentage (% V), following the methods of the ISRIC (1995); the textures are classified according to the International Society of Soil Science (I.S.S.S.); the total nitrogen was analyzed with the variant of Bouat and Crouzet (1965); and the active carbonate (calcium carbonate extractable with ammonium oxalate) was determined according to AFNOR (1982). The exchangeable cations of Ca^{2+} and Mg^{2+} were determined

Table 2 Anatomical characters of *Tuber melanosporum*, *Tuber indicum* and *Tuber pseudoecavatum* ectomycorrhizae

Species	*T. melanosporum*	*T. melanosporum*	*T. indicum*	*T. indicum*	*T. pseudoexcavatum*
Synthesis source	Granetti 1995; Granetti et al. 2005; Zambonelli et al. 1993	Present study	Comandini and Pacioni 1997; Zambonelli et al. 1997	Present study	Present study
Host plant	*Quercus pubescens*, *Corylus avellana*, *Pinus sylvestris*	*Quercus ilex* subsp. *ballota*	*Quercus pubescens* and *Q. cerris*	*Quercus ilex* subsp. *ballota*	*Quercus ilex* subsp. *ballota*
Outer structure of the mantle	Pseudoparenchymatous. Hyphal pattern is formed by roundish cells, with well-defined irregular lobes, which give a puzzle-like appearance	Idem	Pseudoparenchymatous, very heterogeneous, formed by slightly roundish regular pseudocells, with puzzle-like appearance. More polygonal pseudocells which are smaller on average and less lobed than *Tuber melanosporum*	Idem	Pseudoparenchymatous, homogeneous, formed by pseudocells with a sinuous form. The general feature of the surface is its regular puzzle-like appearance.
Outer dimensions of hyphal cells	Mean length 10.6 (±2.4) µm, mean width 4.6 (±1) µm	Length between (10) 15–20 µm, width between 5–10 mm	Length between (8) 10–16 (24) µm, width between (4) 5–6 (10) µm	Length between 10–15 µm, width between 5–8 mm	Length between (8) 10–25 (26) µm, width between (5) 6–16 (17) µm
Cystidia form	Length up to 300 µm; straight; frequent right angle-like and 45° ramifications	Idem	Length up to 300 µm, with frequent right angle-like ramifications	Idem	Very long, sinuous to straight cystidia, sectioned, 15% right angle-like ramifications
Cystidia color	Hyaline	Idem	Pale yellow	Idem	Yellowish
Cystidia diameter	Plinth diameter mean (2.8) 3.5 (4.4) µm,	Plinth diameter mean 2 µm	Plinth diameter from 2 to 4 (8) µm, tip diameter from 2 to 3 (6) µm	Plinth diameter mean 2 µm	Plinth diameter from 2 to 4 µm
Septa distance	–	10–26	25–35 µm	12–29 µm	From 25 to 35 µm

Comparisons include ectomycorrhizae synthesized in the present and previous studies

using AAS (Philips UP9100x), and K^+ and Na^+ with a flame photometer (Sherwood 410).

Results

The 18 seed plants of *Quercus ilex* subsp. *ballota* showed that the roots had between 40 to 60% of their tips mycorrhized with *T. pseudoexcavatum*, *T. indicum*, and *T. melanosporum*, respectively, without contaminating ectomycorrhizae of other fungi. Tables 1 and 2 show the morphological characteristics of these ectomycorrhizae, to assist in their rapid identification in the routine control procedures for mycorrhized plants in truffle culture.

The ectomycorrhizae of *T. pseudoexcavatum* and *T. indicum* have a considerable morphological similarity with the ectomycorrhizae of *T. melanosporum*, due to the presence of cystidia with right angle-like ramifications and outer mantle pseudocells with a sinuous puzzle-like form, which up to now have been considered the most distinctive characteristics of *T. melanosporum* ectomycorrhizae (Palenzona 1969; Zambonelli et al. 1993; Granetti 1995; Granetti et al. 2005). However, some morphological characteristics of *T. pseudoexcavatum* ectomycorrhizae make it possible to differentiate this species from *T. indicum*, and *T. melanosporum*. Specifically, the length of the unramified ends of the ectomycorrhizae in *T. pseudoexcavatum* is much greater than in the other Chinese truffles ectomycorrhizae, and the *T. pseudoexcavatum* ectomycorrhizae are darker in color than *T. indicum* and *T. melanosporum* ectomycorrhizae. Moreover, the outer mantle pseudocells and cystidia diameter of *T. pseudoexcavatum* are larger than the pseudocells and cystidia of *T. indicum* and *T. melanosporum* (Tables 1 and 2; Figs. 1 and 2).

We obtained the *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae using two soil samples with the following properties (Table 3): a relative abundance of sand and clay and moderate level of silt, which generates a texture tending towards sandy clay loam. These soils have a moderately basic pH, low levels of total carbonates but an

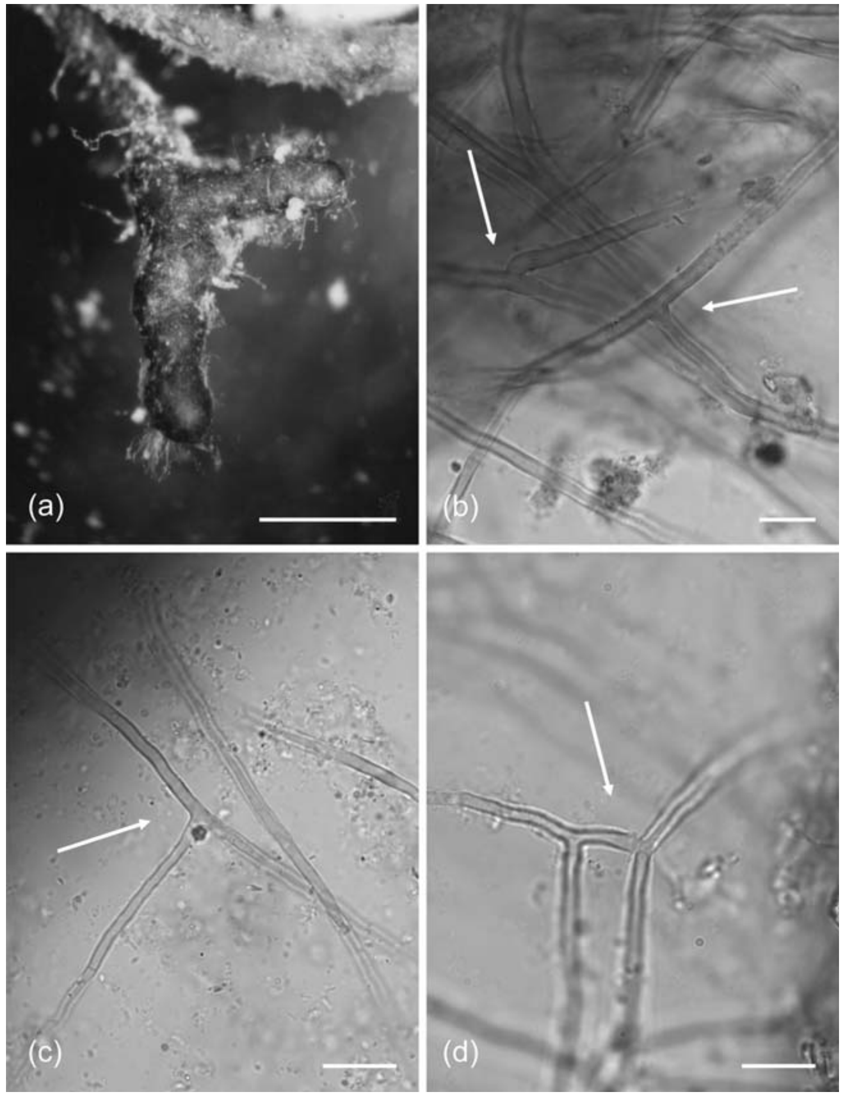

Fig. 1 **a** Macroscopic appearance of *T. pseudoexcavatum* mycorrhizae (50×) (*bar*= 1000 μm); **b** detail of *T. pseudoexcavatum* cystidia with right angle-like ramifications (630×; *bar*=10 μm); **c** detail of *T. indicum* cystidia with right angle-like ramifications (630×; *bar*=10 μm); **d** detail of *T. melanosporum* cystidia with right angle-like ramifications (630×; *bar*=10 μm)

elevated level of active carbonate. Levels of organic carbon and total nitrogen are moderate and the C/N ratio is 13 to 15. They have high values of exchangeable cation complex and the degree of saturation of exchangeable cations is 100%, with a good proportion of exchangeable Ca^{2+} and Mg^{2+}, K^{+} in relatively large concentrations, and scarce Na^{+}. These soils have a good structure with a granular tendency and an abundance of pores.

Discussion

Wang and Hall (2001) confirm that, in Europe, some of the Chinese truffles that were similar to *T. melanosporum* were originally identified as *T. indicum*. However, these authors and Wang and He (2002) indicated that most of the truffles that were being exported from southwest China to Europe were *T. sinense* Tao and Liu and *T. pseudoexcavatum*, in hundreds of tons a year. *T. pseudoexcavatum* has also been detected in Japanese truffle markets (Yamanaka et al. 2001).

Our results show that the morphological characteristics of *T. pseudoexcavatum* ectomycorrhizae enable this species to be differentiated from *T. indicum* ectomycorrhizae. We also show that the ectomycorrhizae of *T. pseudoexcavatum* have a similar morphology to the ectomycorrhizae of *T. melanosporum*, although there are some morphological characteristics which make it possible to differentiate both species. In any case, it is necessary to use molecular tools to positively separate these species. It would, therefore, be very useful to have a specific primer for *T. pseudoexcavatum* to be used in conjunction with the other black truffle specific primers already designed by Paolocci et al. (1999) for use in multiplex PCRs.

From the soil point of view, very few studies have been done so far on Chinese truffles. Most Chinese truffles have been found in calcareous soils. *Tuber furfuraceum* Hu and

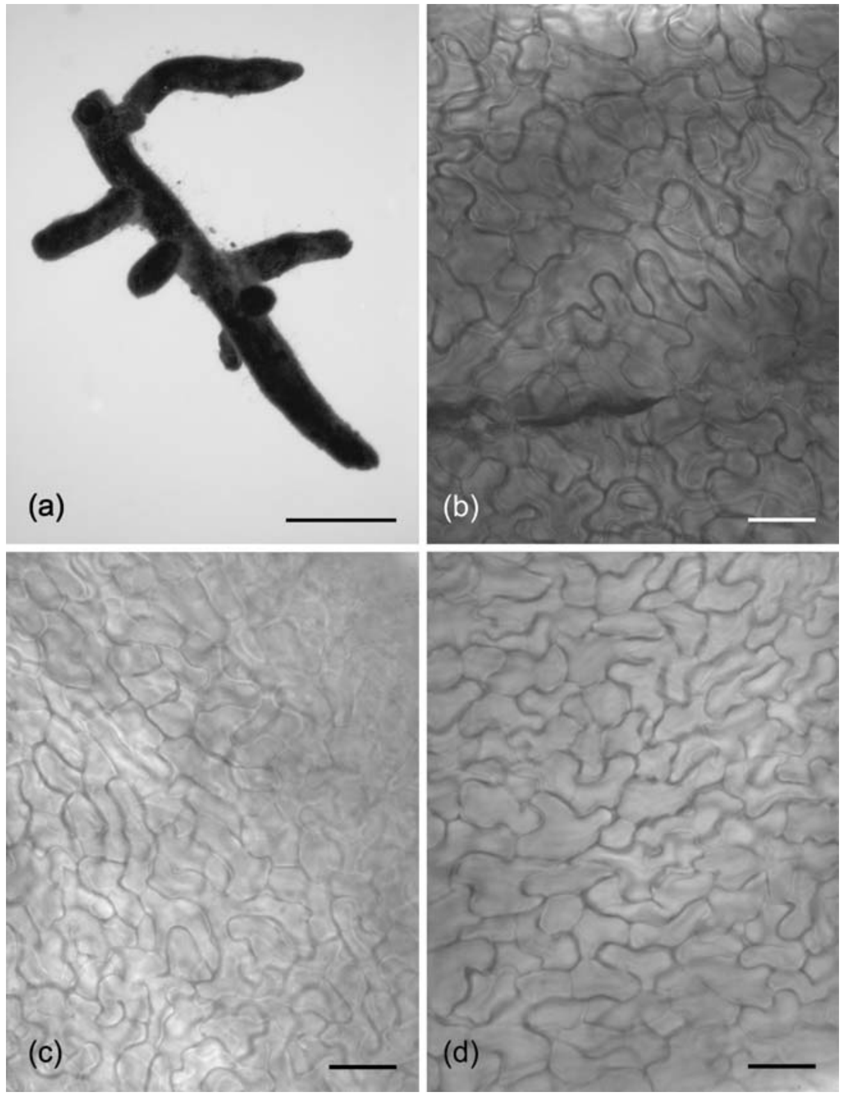

Fig. 2 **a** Macroscopic appearance of *T. indicum* mycorrhizae (50×; *bar*=2,000 μm); **b** hyphal pseudocells of the outer surface of the *T. indicum* mantle with a sinuous form (630×; *bar*= 10 μm); **c** hyphal pseudocells of the outer surface of the *T. melanosporum* mantle with a sinuous form (630×; *bar*= 10 μm); **d** hyphal pseudocells of the outer surface of the *T. pseudoexcavatum* mantle with a sinuous form (630×; *bar*= 10 μm)

Y. Wang and *T. pseudoexcavatum* live in calcareous soils (Wang and Li 1991; Riousset et al. 2001; Wang and Hall 2001; Hu and Wang 2005). *Tuber sinense* carpophores have been found in the 0–30 cm layers of calcareous, high pH, and clay soils (Zhang and Wang 1990; Wang and Hall 2001). *T. indicum* var. *yunnanense* Yamanaka carpophores have been found at a depth of 2–12 cm, also in very poor, calcareous, and purple soils, with a pH of 6.5–7.4 (Yamanaka et al. 2000, 2001). *Tuber formosanum* Hu is found only in calcareous soils with a wide range of soil parameters (pH, total nitrogen, carbon, sulfur, and available nutrients), suggesting that *T. formosanum* can adapt to a wide range of soil conditions (Hu et al. 2005).

Riousset et al. (2001) report that *T. indicum* develops at a depth of 30–40 cm under a mulch of pine needles in mountain pinewoods, in soils free from calcium carbonate and with a moderate pH, rich in organic matter and with a high C/N ratio. However, other authors indicate that *T. indicum* inhabits calcareous soils. Fourré et al. (1996) report that *T. indicum* occurs on calcareous plateaus at

Table 3 Analytical results of the two soil samples from *Tuber melanosporum* burns (*C.E.C.* cation exchange capacity, *%V* exchangeable cation saturation percentage; Sand, clay, silt, total carbonate, active carbonate, T.O.C., and N expressed in g kg^{-1}; C.E.C. and exchangeable cations expressed in cmol kg^{-1})

No	Sand	Silt	Clay	pH_{H2O}	Total carbonate	Active carbonate	T.O.C.	N	Ca^{2+}	Mg^{2+}	K^+	Na^+	C.E.C.	%*V*
1	600	130	270	7.92	69.10	56.30	42.60	2.70	23.59	1.20	0.88	0.45	25.94	100
2	660	220	120	7.88	78.80	70.10	35.60	2.70	21.15	0.74	1.10	0.27	23.44	100

2,000–3,000 m. Granetti et al. (2005) indicate that *T. indicum* inhabits calcareous substrates with a pH which varies between 5.5 (due to organic matter) and 8.5.

Our results confirm that *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae develop well in calcareous substrates which are rich in active carbonate, whose properties fulfill the necessary values for the development of *T. melanosporum* according to Callot (1999), Ricard (2003), and García-Montero et al. (2006) (Table 3). Active carbonate is a finely divided fraction of calcareous rock, smaller than 50 μm in size, very chemically active, and constitutes an important reserve of exchangeable Ca^{2+}. The presence of both soil components is very important to *T. melanosporum* due to the action of several factors (García-Montero et al. 2007a, b). These results contrast with the soils described by Riousset et al. (2001) for *T. indicum*; these are devoid of calcium carbonate, have a moderate pH and are rich in organic matter. Therefore, these results and the studies of Wang (2006a) have confirmed that *T. indicum* appears to adapt to a wide range of soil conditions.

Regarding host plants, some authors have reported that *T. pseudoexcavatum* is associated with pines, whereas *T. indicum* is associated with pines and Asian *Quercus* (Cooke and Masse 1892; Zhang and Minter 1988; Riousset et al. 2001; Wang and Hall 2001; Granetti et al. 2005). We have established that *T. pseudoexcavatum* and *T. indicum* showed a high capacity for mycorrhization with *Quercus ilex* subsp. *ballota*. Comadini and Pacioni (1997), Zambonelli et al. (1997) and Di Massimo et al. (1998) also mycorrhized *T. indicum* with *Quercus ilex* L. subsp. *ilex*, *Q. pubescens* Willd., and *Q. cerris* L. These four *Quercus* taxa are the principal host plants of *T. melanosporum* in European Mediterranean areas. Ferrara and Palenzona (2001) also indicate that the spores of *T. indicum* have a high germination capacity, and form young ectomycorrhizae and abundant preotrophic mycelia 3 months after inoculation of spores in *Quercus pubescens* plants. In this regard, not only *T. indicum* but also *T. melanosporum* is able to form mycorrhizae as soon as 2.5 months after inoculation.

We conclude that *T. pseudoexcavatum* and *T. indicum* ectomycorrhizae grow well in calcareous substrates rich in active carbonate inhabited by *T. melanosporum*; they also mycorrhize with the principal *T. melanosporum* host plants. Therefore, both Chinese truffle species have the potential capacity to penetrate numerous *T. melanosporum* plantations and Mediterranean ecosystems. The ectomycorrhizae of these Chinese truffles are morphologically similar to *T. melanosporum* ectomycorrhizae, and it is, therefore, important to develop new protocols, based on morphological and genetic analyzes, for the quality control of commercial plants mycorrhized with *Tuber*.

Acknowledgments We thank Margarita, Luis, Pablo and Miriam for their support and collaboration. We would also like to express our gratitude to Ma. Cruz Gómez-Llano and Faustino Correas of the University Library of E.T.S.I. Montes (UPM) for their help with the bibliography research, J. Orellana, Ruben Valbuena and Francisco Mauro for their image design assistance and to Prudence Brooke-Turner for her linguistic assistance.

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