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ORIGINAL PAPER

Mycorrhization of *Quercus robur* L., *Quercus cerris* L. and *Corylus avellana* L. seedlings with *Tuber macrosporum* Vittad.

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Abstract *Tuber macrosporum* Vittad. is not a common truffle species, but with remarkable organoleptic qualities and much economic interest. After the addition of truffle spore slurry, 30 seedlings of *Quercus robur* L., *Quercus cerris* L. and *Corylus avellana* L. were grown inside a greenhouse for 11 months before evaluation of the mycorrhizal level. Two different potting mixes were used: a natural soil-based potting mix for *Q. robur*, *Q. cerris* and *C. avellana* and a peat-based potting mix for *Q. robur*. *Quercus robur* planted in soil potting mix was the most receptive towards the truffle spore inoculum, with a level of formation of *T. macrosporum* ectomycorrhizas (ECMs) of approximately 14 %, ranging from a minimum of ~4 % to a maximum of ~44 % in different seedlings. No *T. macrosporum* ECMs developed on *Q. cerris* (soil potting mix) or on *Q. robur* (peat potting mix), whereas a low percentage of ECMs was detected on only three *C. avellana* (soil potting mix) seedlings. The fungus *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička was also detected as a contaminant on almost half the truffle-inoculated seedlings. A new detailed description of the morphological and anatomical characteristics of *T. macrosporum* ECMs and their DNA-based verification with species-specific markers were also reported.

Keywords *Quercus robur* · *Corylus avellana* · *Tuber macrosporum* · Truffle-plant · Mycorrhization

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Introduction

The genus *Tuber* P. Micheli ex. F. H. Wigg., which is in the order Pezizales (Ascomycota) according to the Index Fungorum (http://www.indexfungorum.org/), is an ectomycorrhizal taxon that is associated with ecologically important tree and shrub species that are typical of Mediterranean vegetation. It includes the highly esteemed edible mushrooms that are known as truffles, which have unique organoleptic qualities and a flourishing market (De Román et al. 2006; Smith and Read 2008, Varese et al. 2011).

Nine truffle species can be harvested and marketed legally according to Italian rules (Legge n. 752, 16 Dicembre 1985), although not all of them have the same importance on national and international markets. This is probably because truffle collectors are more interested in the market prices of certain species than their organoleptic properties. For some species (e.g., *Tuber macrosporum* Vittad., *Tuber brumale* Vittad.), there is no real market and they are often sold mixed with other, more appreciated species, such as the black truffle (*Tuber melanosporum* Vittad.). However, *T. macrosporum* is an intriguing species because (1) it has excellent organoleptic characteristics and its aroma resembles that of the esteemed white truffle (*Tuber magnatum* Pico) (Iotti et al. 2002), (2) it is widely distributed across Europe and (3) some Italian nurseries have succeeded in

producing plants that are infected with *T. macrosporum* spores and some experimental man-made orchards have already been established (Vezzola 2005).

Although *T. macrosporum* is reported to be a rare species in France and Great Britain, it appears to be common in Serbia, Hungary and Romania and is collected frequently in Italy and Slovenia. Recent reports have also confirmed the presence of *T. macrosporum* in Germany, the Czech Republic, Switzerland, Ukraine, Croatia, Slovakia, Serbia and Montenegro (Marjanović et al. 2010; Gógán et al. 2011). In addition, *T. macrosporum* has been collected recently in Turkey (Benucci et al. 2011a).

Recent phylogenetic studies on the *Tuber* genus, which were based on the internal transcribed spacer (ITS) region and large subunit of the nuclear ribosomal DNA (nrDNA), have shown that the Macrosporum clade is one of the ancestral lineages of the genus (Jeandroz et al. 2008; Bonito et al. 2010). The Macrosporum group has also been discovered in Japan and probably represents a complex of species (Kinoshita et al. 2011).

Tuber macrosporum is found in forests, from 50 to 950 m above sea level, and preferentially forms associations with angiosperm symbionts, especially with *Corylus avellana* L., *Quercus robur* L., *Quercus cerris* L., *Ostrya carpinifolia* Scop., *Carpinus betulus* L., *Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Populus* spp. and *Salix* spp. (Vezzola 2005). Granetti et al. (2005) have reported this species in symbiosis with *Pinus nigra* Arnold and *Fagus sylvatica* L. *Tuber macrosporum* prefers fresh, wet, occasionally flooded, thick, clay-rich soils with variable levels of calcium carbonate, often in lowlands or near rivers (Vezzola 2005; Marjanović et al. 2010; Gógán et al. 2011). The period during which the fruiting body ripens is not clearly defined, and truffle collectors harvest *T. macrosporum* from early summer to late autumn.

The first mycorrhization of *T. macrosporum* with hornbeam seedlings was established and distinctive traits described and published by Giovannetti and Fontana (1980–1981). Some subsequent works have expanded the topic: Oak and hazel seedlings have been inoculated with *T. macrosporum* spore slurry, and the obtained ectomycorrhizas (ECMs) have been photographed and described (Zambonelli et al. 1993; Granetti 1995; Vezzola 2005). However, some descriptions are controversial and do not really focus on informative morphological traits that could provide a basis for correct species identification. Moreover, no molecular confirmation of ECMs that belong to *T. macrosporum* has been reported so far.

The study described herein focused on the mycorrhization of seedlings of *Q. robur* L., *Q. cerris* and *C. avellana* L. through the addition of a *T. macrosporum* spore inoculum. Two different potting mixes were tested, and mycorrhized seedlings were evaluated with regard to the level of truffle infection. We describe the morphological and anatomical characteristics of *T. macrosporum* ECMs, in addition to a DNA-based verification to enable molecular identification.

Material and methods

Seedling germination and truffle spore slurry inoculations

Acorns from *Q. robur* and *Q. cerris* and seeds from *C. avellana* were surface-sterilised in a 5 % sodium hypochlorite solution for 20 min and soaked in distilled water for 24 h. They were then set in a mix of 50 % sterile perlite and 50 % sterile vermiculite and placed in a nursery until germination. Oak and hazel trees were selected for the inoculation trials because they are considered to be the most important host symbionts in natural *T. macrosporum* orchards (Granetti et al 2005; Vezzola 2005).

Fresh ascomata of *T. macrosporum* (collected in autumn 2009) were washed in tap water with a brush and air-dried. Each truffle was examined macro- and microscopically and selected for suitability for inoculation on the basis of the presence of mature spores. Rotten truffles or those that contained insect larvae were discarded. Selected truffles were placed in a plastic bag and frozen at −20 °C until use in the mycorrhization procedures.

In spring 2010, the tap roots of seedlings at the two-leaf stage (two leaves in addition to the cotyledons) were cut, and plantlets were transplanted into 9×9×13 cm pots that contained the potting mixes listed in Table 1. Immediately before transplantation, the truffle spore slurry, which was prepared by blending frozen truffles for 5 min, was added to seedlings in the proportion of 2 g/plant. Thirty seedlings each of *Q. robur*, *Q. cerris* and *C. avellana* were transplanted into pots that contained a sterile soil potting mix (Pmix1), and a further 30 seedlings of *Q. robur* were transplanted into pots that contained a peat potting mix (Pmix2) to give a total of 120 seedlings. The sterile soil potting mix was used because natural soil potting mixes are commonly

Table 1 Content and ratios of potting mixes used for seedling inoculation and cultivation in accordance with Benucci et al. (2011a) with slight modification

	Soil potting mix (Pmix1)	Peat potting mix (Pmix2)
Natural sterilised soil[a] (%)	50	–
Peat (Agrochimica ©) (%)	–	50
Vermiculite (Asfaltex ©) (%)	25	25
Perlite (Saint-Gobain ©) (%)	25	25
$CaCO_3$ (g/L)	–	20
NPK (Osmocote®)[b] (g/L)	2	2
pH_{H2O}[c]	7.91	7.86

[a] For soil physicochemical characteristics, refer to Benucci et al. (2011a)

[b] Slow-release fertilizer with nitrogen, phosphorus and potassium

[c] Measured at the time of evaluation of the mycorrhization level

used with excellent results by Italian commercial nurseries that produce *Tuber*-inoculated plants (Granetti et al. 2005), whereas the peat-based potting mix was used because it is more standardised and all components are easily available on the market.

After inoculation, seedlings were grown (side by side, in blocks of 15 each) in an unheated greenhouse until levels of mycorrhization were checked. The plants were irrigated twice weekly during June and August and once weekly during all other months of their cultivation in the greenhouse (Benucci et al. 2011a).

Level of *T. macrosporum* infection and ECM morphology

In spring 2011, 15 seedlings of each of *Q. robur*, *Q. cerris* and *C. avellana* cultivated in Pmix1 and 15 seedlings of *Q. robur* cultivated in Pmix2 were sampled randomly for analysis, and shoot length, stem diameter and length of the root system were measured. We analysed only 15 out of 30 seedlings per treatment because (1) we wanted a completely randomized design, which could be achieved by sampling; (2) 15 seedlings were a sufficient number on which to base the morphological, molecular and statistical analyses; (3) it was a valid compromise between the accuracy of the data obtained and the time taken and (4) analysis of 50 % of the plants in a batch was higher than that required by local government rules (Regione dell'Umbria) for the evaluation of the mycorrhization level in relation to the marketing of truffle-inoculated plants produced by commercial nurseries (Regolamento Regionale 16 luglio 2007 n. 8). The root system of each seedling was washed gently and placed in a Petri dish (Avis et al. 2003). Ectomycorrhizas were identified using a stereomicroscope (Leica Leitz Wild MZ8) and a light microscope (Leica Leitz DMRB). Photographs were taken with a Leica DFC320 digital camera and analysed with LAS software version 3.3.1 (Leica Microsystems Ltd., Switzerland). Cell sizes were measured along the major (cell length) and minor (cell diameter) cell axes. Types of mantle cell were identified on the basis of cell shape (i.e. angular, epidermoid) and labelled as mantle type L and type M, respectively, following the online key DEEMY (http://www.deemy.de) and Agerer (1987–2008, 1991) as guide references.

To determine the level of infection, each ECM was considered as a single individual (Bruns 1995; Dickie et al. 2002). *Tuber macrosporum* ECMs, ECMs of other species and non-ectomycorrhizal tips were subjected to visual evaluation (VE), in which the percentage of ECMs present in the entire root system was estimated visually (Benucci et al. 2011a), and to a relative abundance valuation (RAV), as described by Bencivenga et al. (1995a). The RAV method is currently the standard quality test for truffle-infected plants produced by commercial nurseries in some Italian regions. The root system of each seedling was divided into proximal and distal parts: four root portions from each part were selected, and 50 root tips in each portion were counted to give a total of 400 tips. The abundance of ECMs and of non-ectomycorrhizal root tips was calculated by dividing the number of tips counted for each group by the total number of tips counted for each seedling (400). The number of *T. macrosporum* ECMs with mycelia was also counted and calculated by averaging the number of ECMs with mycelia (i.e. vegetative mycelium, emanating hyphae and/or cystidia) that were counted in 100 tips from each of the 15 *Q. robur* seedlings cultivated in Pmix1.

Molecular identification of *T. macrosporum* ECMs

Genomic DNA was extracted with the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) from single ECMs at various developmental stages (a range of ECMs were represented on the basis of colour, mantel thickness and cystidial development) and from a pool of 10 ECMs with or without peritrophic mycelia that originated from different seedlings. The selected marker from nrDNA was amplified using ITS species-specific primer pairs for *T. macrosporum* (Benucci et al. 2011b).

Polymerase chain reactions (PCRs) were carried out in accordance with Benucci et al. (2011b), except that only 10 pmol each of the Tmacr For and Tmacr Rev-specific primers was used. The amplifications were performed with the following cycling parameters: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 45 s. Twelve microlitres of each PCR product was run on 1.6 % (*w*/*v*) agarose gels and stained with ethidium bromide.

Statistical analysis

Both the seedling measurements and data for the mycorrhization level were checked for a normal distribution with the Shapiro–Wilk and Anderson–Darling tests (α=0.05). Significant differences between the dimensions of *Q. robur* seedlings cultivated in the two potting mixes, the levels of mycorrhization obtained with the different evaluation approaches and types of ectomycorrhizal mantle were detected by analysis of variance (ANOVA). Differences between means were addressed with Tukey's test (p<0.05).

Results

Seedling dimensions and mycorrhizal level

No significant differences were detected in relation to the shoot length and length of the root system of *Q. robur* in the

different potting mixes (Pmix1 vs. Pmix2). However, the stem diameter of the *Q. robur* seedlings was higher on average in peat potting mix (Pmix2) than in Pmix1 (ANOVA, $p<0.05$) (Table 2).

Tuber macrosporum ECMs were formed on both *Q. robur* and *C. avellana* seedlings planted in Pmix1, but the oak seedlings produced the most *T. macrosporum* ECMs. *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička was detected as a co-occurring contaminant fungus in the majority of the seedlings. *Quercus cerris* seedlings planted in Pmix1 and *Q. robur* seedlings planted in Pmix2 did not form any ECMs. *Tuber macrosporum* was detected on 10 out of 15 *Q. robur* seedlings with a relative abundance that ranged from ~4 to ~44 % (~14 % with RAV and ~20 % with VE on average, respectively), whereas *S. brunnea* was detected in all *Q. robur* seedlings with a relative abundance that ranged from ~11 to ~51 % (~25 % with RAV and ~27 % with VE on average, respectively) (Table 3). *Tuber macrosporum* ECMs were detected in only three out of 15 *C. avellana* seedlings, but with a high relative abundance (~35, ~24 and ~44 % with the RAV method).

Morphoanatomical description of *T. macrosporum* ECMs

Significant differences were detected in relation to the sizes of mantle cells among the different parts of the *T. macrosporum* ECMs along the major and minor axes (ANOVA, $p<0.05$) (Table 4). In particular, cells at the base were significantly bigger than cells at the tip and in the middle part of the ECM mantle, in which two different cell shapes were detected (type L and type M). Type L and type M cells in the middle part were also significantly different from each other on the basis of the measurement along the minor axis.

Tuber macrosporum ECMs on *Q. robur* and *C. avellana* were simple or ramified in a monopodial-pinnate or monopodial-pyramidal pattern (Fig. 1a–c). Simple ectomycorrhizal tips were almost straight, cylindrical or club-shaped with rounded ends. The colour of the ECMs varied considerably: the youngest were light yellow with pale grey shades, whereas the oldest were dark ochre or brownish, sometimes with a yellowish apex that was growing (Fig. 1d, g, h).

Cystidia were sinuous, septate, with very thick walls and branched at various angles (more often with sharp angles). The colour of the cystidia varied from light yellow (sometimes with a greyish shade) to strong ochre. They tended to merge, creating anastomoses that formed an abundant web of mycelium around the ECM that was typically orange in colour (Figs. 1e, f and 2f, g). It is worth noting that we frequently found adult well-formed ECMs without peritrophic mycelia (Fig. 1d–h); the percentage of *T. macrosporum* ECMs with mycelia was only 8.7 % Table 3.

The outer mantle surface was either covered densely by mycelium (cottony) or was smooth to loosely grainy; in both cases, it was composed of angular/epidermoid cells that formed an uneven, regular puzzle-like pattern, which differed among the apex, middle part and base of the mantle (Fig. 2a–e). The mantle was pseudoparenchymatous and built up from four to six cell layers, and the Hartig net penetrated into the first two to three cell layers of the root parenchyma (Fig. 2h).

The middle part of the ectomycorrhizal root tip was the most variable part of the mantle: cells varied in both shape and size (Table 4). Mantle cells ranged from ~12.3×4.7 μm (sinuous, ellipsoid) at the ectomycorrhizal tip to ~15.2×4.7 μm for type M cells in the middle part and from isodiametric, almost rectangular (~15.8×8.3 μm) for type L cells in the middle portion to elongated (~20.7×6.0 μm) in the basal part of the ECMs.

Molecular identification of *T. macrosporum* ECMs

Genomic DNA from single ECMs at different developmental stages and from a combined sample of 10 ECMs, with or without typical cystidia, was subjected to PCR amplification with *T. macrosporum*-specific primers for an ITS nrDNA marker. All samples gave the expected amplicon of ~230 bp, which matched perfectly the amplicon size obtained when DNA from *T. macrosporum* ascocarps was amplified with the same primers (Benucci et al. 2011b). No bands were detected in negative controls (no DNA added) (Fig. 3).

Discussion

Formation of *T. macrosporum* ECMs with *Fagales* hosts has been reported in the literature (Giovannetti and Fontana

Table 2 Values for seedling measurements [mean±standard error (SE), $n=15$]

Plant species	Mix type	Stem length (cm)	Stem diameter (mm)	Root system length (cm)
Q. robur	Soil (Pmix1)	62.3±3.21	7.8±0.18a	15.5±0.64
Q. cerris	Soil (Pmix1)	45.1±2.80	6.8±0.19	13.7±0.49
C. avellana	Soil (Pmix1)	60.2±4.22	7.7±0.24	14.0±0.58
Q. robur	Peat (Pmix2)	80.0±5.58	8.9±1.27b	15.3±0.77

Values with different letters were significantly different at $p<0.05$ and degrees of freedom (df)=1, 28

Table 3 Values (mean±SE, $n=15$) for *T. macrosporum* ectomycorrhizal levels (RA and VE) in *Q. robur* seedlings cultivated in Pmix1 and number of ECMs with mycelia (mean±SE, $n=100$)

	Tuber macrosporum	*Sphaerosporella brunnea*
Mycorrhizal counts (RA)	0.142±0.04	0.254±0.03
Mycorrhizal estimation (VE)	0.202±0.05	0.277±0.03
Number of ECMs with mycelium	0.087±0.008	–

1980–1981; Zambonelli et al. 1993; Granetti et al. 2005). However, some points must be noted. (1) Some reports lack detailed descriptions accompanied by extensive photographic documentation of the taxonomic elements of macroscopic and microscopic morphology that are useful for correct species identification. (2) Some descriptions are controversial. (3) No reports combine the morphological description of ECMs with molecular verification. (4) There are no reports on the receptivity of different host species in different potting mixes towards spore inocula of *T. macrosporum*.

The mycorrhization experiments reported herein demonstrated that *T. macrosporum* ECMs were formed on *Q. robur* and *C. avellana* seedlings after spore slurry inoculation. Although the level of mycorrhizal infection was low on average and *S. brunnea* was detected as a fungal contaminant, a high percentage of truffle ECMs were detected in some seedlings.

Concerning the morphology and anatomy of truffle ECMs, our findings were in accordance with previous reports published by Giovannetti and Fontana (1980–1981) and Zambonelli et al. (1993). We observed that the fungal cells that formed the ectomycorrhizal mantle of *T. macrosporum* varied in shape and size from the apex to the base of the ECM. The middle part of the ECM mantle showed two different mantle types: a typical puzzle-like cell pattern (type M) and an almost irregular rectangular cell pattern (type L); the two types of cell showed significant differences with regard to length along the major and minor axes. Significant differences in relation to the sizes of mantle cells in other *Tuber* ECMs have already been described by Giomaro et al. (2000), who reported that the form of mantle cells in *Tuber borchii*×*Pinus pinea* L. varies depending on the host plant and the fungal strain used in the in vitro mycorrhization experiments. Moreover, in a comparative study of the ECMs formed by *T. brumale* Vittad. on *Tilia americana* L. and *Quercus pubescens* Willd., Giomaro et al. (2002) have reported that several biometric characteristics of the mantle (e.g., mantle cell perimeter and area) differ according to the host plant. To the best of our knowledge, no reports have been published that show significant differences in the size and shape of mantle cells within the same ECM. Our results highlight the possibility that the mantle cell pattern could be formed by the presence of cells of different shapes in different parts of the mantle, namely at the tip, middle part (type L and type M) and base of a single ECM.

We did not observe needle-shaped cystidia, which had been proposed to be specific to *T. macrosporum* (Granetti 1995) in any of our samples. According to the earlier reports, these cystidia are simple, thin, hyaline or light yellow in colour, with one or two septa, and resemble those described for *T. borchii* (Giomaro et al. 2000). In contrast, the *T. macrosporum* cystidia that we observed were long, intensely branched, pluri-septate, had thickened walls, a yellowish colour that tended to orange and were rich in anastomoses. These cystidia could be distinguished from those of *T. melanosporum*, which have perpendicularly oriented branches, a yellow-ochre colour, thinner walls and do not form anastomoses (Granetti et al. 2005).

However, it is worth noting that emanating elements, such as mycelia and cystidia, were present in only a small portion of the ECMs in the entire root system. This finding is important because *T. macrosporum* cystidia are a fundamental taxonomic key to correct morphological identification, and if lacking, the ECMs of *T. macrosporum* could be confused with those of other truffles (e.g. *T. brumale* and *T. borchii*), whose mantle cell pattern is similar (Zambonelli et al. 1993; Giomaro et al. 2002). It is known that morphological traits, such as mycelia and cystidia, are variable under changing environmental and climatic conditions (Rubini et al. 1998; Zambonelli et al. 1993). For these reasons, the morphoanatomical taxonomic characteristics of *T. macrosporum* ECMs are not sufficient to distinguish ECMs from

Table 4 Morphological and anatomical characteristics of *T. macrosporum* ECMs with *Q. robur* seedlings (values are mean±SE, $n=100$)

Tuber macrosporum		
Length of ECMs (mm)	1.934±0.050	
Diameter of ECMs (mm)	0.329±0.007	
Thickness of cystidia (μm)	3.100±0.058	
	Major axis	Minor axis
Mantle cell, tip (μm)	12.33±0.381c	4.74±0.193c
Mantle cell, middle part, type M (μm)	15.23±0.442b	4.73±0.196c
Mantle cell, middle part, type L (μm)	15.78±0.394b	8.28±0.336b
Mantle cell, base (μm)	20.71±0.625a	6.02±0.197a

Values with different letters were significantly different at $p<0.05$ and $df=3, 398$

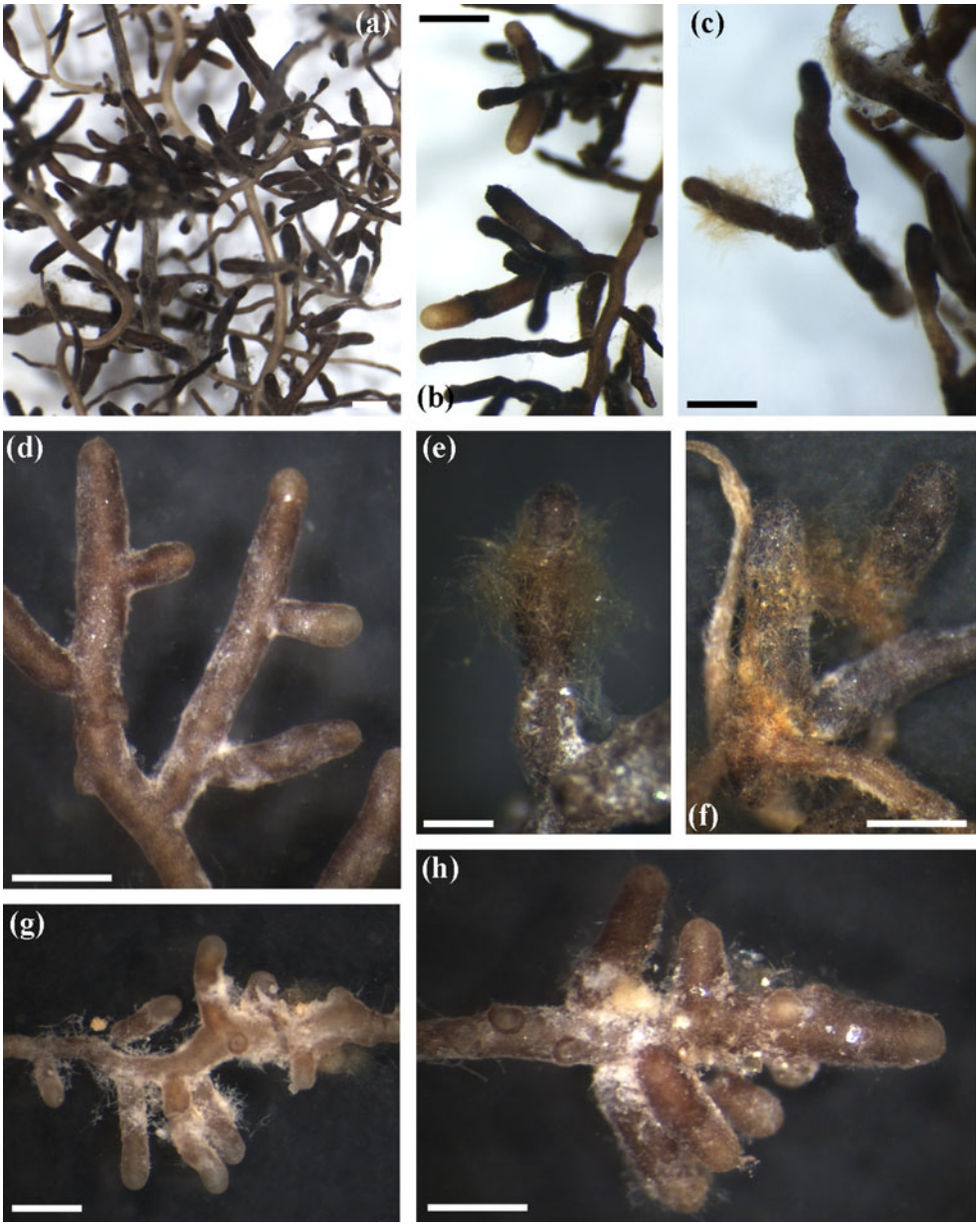

Fig. 1 Morphological characteristics of *T. macrosporum* ECMs. **a** Mycorrhized roots (*bar*=700 μm); **b** young, light-ochre tips (*bar*=700 μm); **c** adult ECMs with abundant specific mycelia (*bar*=500 μm); **d** ECMs without mycelia (*bar*=700 μm); **e** simple ECM with abundant ochre mycelium (*bar*=700 μm); **f** some ECMs with mycelia (*bar*=500 μm); **g** young ECMs with light-ochre mycelia (*bar*=700 μm); **h** growing ECMs with mycelia and yellowish apexes (*bar*=500 μm)

other species of *Tuber*, and molecular PCR-based tools must also be used to ensure unambiguous identification.

Quercus robur, as one of the most important hosts for *T. macrosporum* in its natural habitat (Vezzola 2005), was the most receptive of the tree species tested towards the fungal inocula. It showed the highest level of mycorrhization among different treatments and might be the best species for the establishment of future plantations. We should also mention that several *Q. robur* seedlings were contaminated abundantly with *S. brunnea*, which is a common contaminant fungus in nurseries that produce *Tuber*-inoculated plants, and it is often favoured by the high humidity inside of the greenhouse (Bencivenga et al. 1995b; Donnini and Bencivenga 1995). Poor results were also obtained with *C. avellana*, for which roots were often contaminated with *S. brunnea*, and with *Q. cerris* cultivated in soil-based potting mix and *Q. robur* cultivated in peat-based potting mix. In the latter two cases, no ECMs of *T. macrosporum* or other fungal contaminants were formed, even though all seedlings were grown side by side under the same nursery conditions.

As shown in Table 1, the pH of the soil potting mix (7.91), as measured at the time of evaluation of the mycorrhization level, was almost the same as that of the peat potting mix (7.86). Thus, a difference in pH between the two types of potting mix could not account for the absence of *T. macrosporum* or presence of *S. brunnea* ECMs in seedlings grown in the soil and peat potting mixes. It is likely that the moisture content of the potting mixes, together with other abiotic (e.g., humic substances) or biotic (e.g., bacteria or yeasts) agents, have a key role in spore germination and the

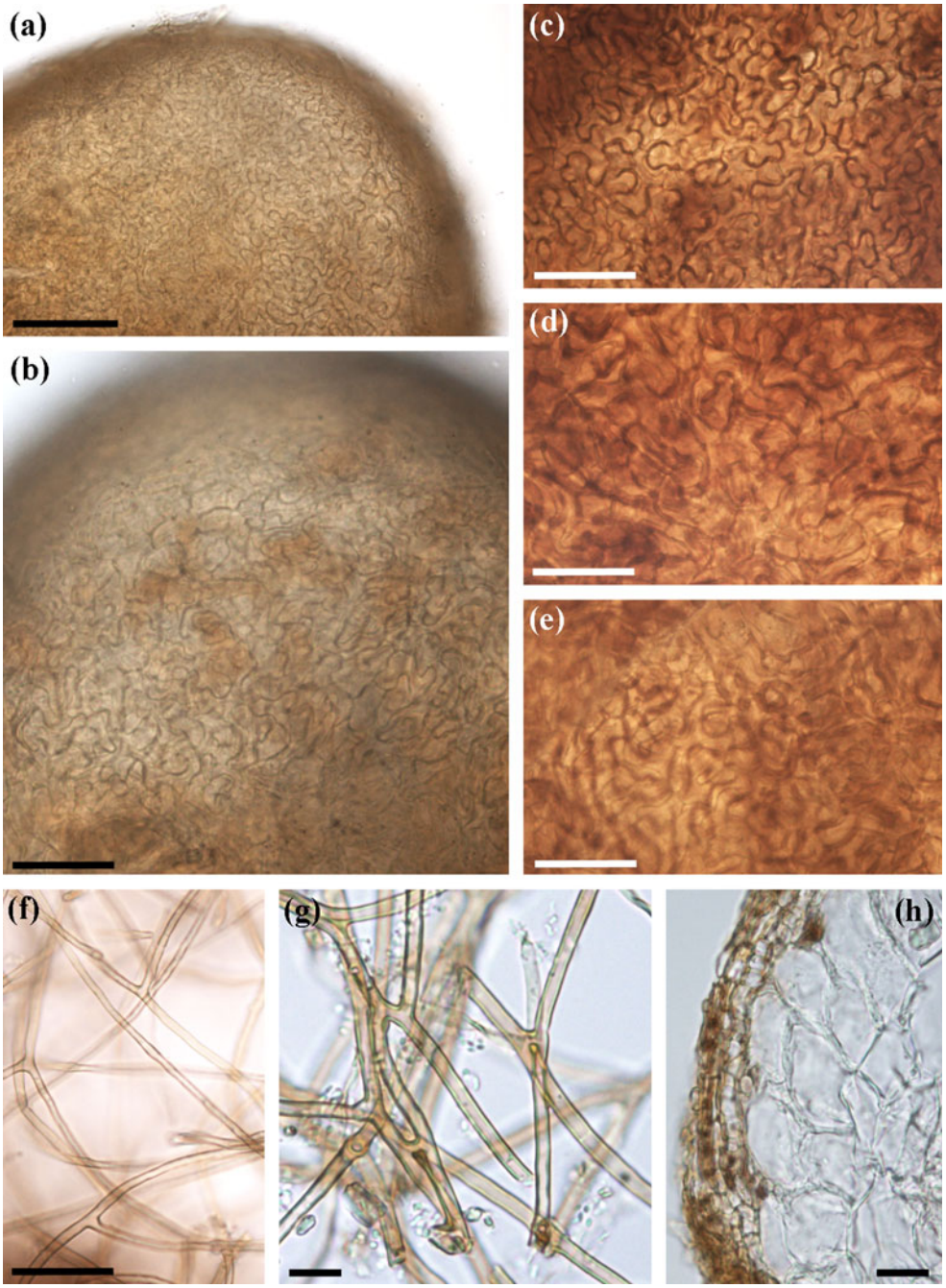

Fig. 2 Morphological and anatomical traits of *T. macrosporum* ECMs. **a** Young, light-ochre mycorrhizal tip (*bar*=20 μm); **b** outer mantle cell pattern of an ectomycorrhizal tip (*bar*=30 μm); **c** outer mantle cell pattern in the middle part of an ECM, cell type M (*bar*=30 μm); **d** outer mantle pattern in the middle part of an ECM, cell type L (*bar*=30 μm); **e** outer mantle cell pattern at the base of an ECM (*bar*=30 μm); **f** differently ramified ochre mycelium (*bar*=30 μm); **g** mycelial anastomosis (*bar*=10 μm); **h** cross section of the mantle and Hartig net (*bar*=10 μm)

subsequent formation of ECMs by truffles and other fungal contaminants (Angelini et al. 1998; Vaughan-Martini et al. 2001; Sbrana et al. 2002). Further inoculation trials are needed to understand why no truffle ECMs formed with *Q. cerris* cultivated in the soil potting mix, given that this is one of the most important host trees in *T. macrosporum* natural truffle grounds.

In regard to new cultivation possibilities for alternative truffle species that have excellent organoleptic properties and might be advantageous to the truffle market, the evidence that *T. macrosporum* seedlings

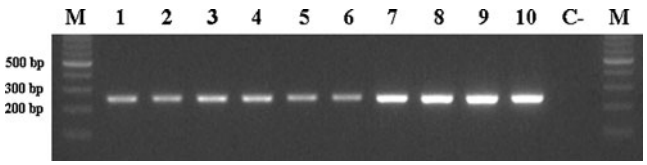

Fig. 3 Amplicons obtained with the Tmacr For/Tmacr Rev-specific primers for *T. macrosporum*, visualized in a 1.6 % (*w*/*v*) agarose gel with ethidium bromide staining. *M* GeneRuler marker (Fermentas); *C−* negative control (no DNA added to the PCR); *lanes 1–6* amplicons obtained from single ECMs at different developmental stages; *lanes 7* and *8* amplicons obtained from a pool of 10 ECMs that comprised young and old ECMs, with or without peritrophic mycelia; *lanes 9* and *10* amplicons obtained from *T. macrosporum* ascocarps

can be obtained by spore slurry inoculation might boost its cultivation. Although some experimental orchards with *T. macrosporum* seedlings have been created (Vezzola 2005), the production of truffle-infected seedling using this species is still in its infancy and further trials are needed to standardise and optimise the quality of the process in a routine nursery setting.

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