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Anatomical and morphological characterization of mycorrhizas of five strains of *Tuber borchii* Vittad

Abstract *Tilia platyphyllos* Scop. plantlets were inoculated in vitro with five *Tuber borchii* Vittad. strains (1BO, 17BO, 43BO, 71BO and 10RA) to test their intraspecific variability. The ability of the strains to form mycorrhizas varied, with the mean degree of ectomycorrhizal infection ranging from 50.6% (for 1BO) to 82.1% (for 10RA). The anatomical/morphological characteristics of the resulting mycorrhizas were determined. Although the morphological features of the mycorrhizas and the characteristics of the cystidia were similar for all strains tested, differences were found in the anatomical features of the mantle. The form of the mantle cells was examined in the surface and inner layers (6 and 12 μm deep, respectively) by both conventional and confocal microscopy. These cells were polygonal in 1BO, and 71BO, epidermoid in 43BO and intermediate in 17BO and 10RA. The structure of the mantle also varied and thus provided little information with which to identify *T. borchii* mycorrhizas.

Keywords *Tuber borchii* · Ectomycorrhiza · *Tilia platyphyllos* · Mantle features

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Introduction

Diversity of ectomycorrhizal fungi is high in natural forests (Bruns 1995) and the total number of soil fungi involved in mycorrhizal symbiosis was recently estimated by Molina et al. (1992) in more than 5000 species. Anatomical and morphological characteristics of ectomycorrhizas are often used to study biodiversity in ectomycorrhizal fungi (Agerer 1987–1998). However, the recent development of molecular techniques now allows ectomycorrhizal fungi to be identified even in the presence of conflicting, ambiguous or missing morphological characters (Lanfranco et al. 1998).

The arrangement and organization of the fungal structures in mycorrhizas are species specific and well conserved (Agerer 1991). Variation in mycorrhizal morphology seems to depend on the host plant rather than on the strains of a single taxonomic fungal species (Jacobson and Miller 1992). However, it has been observed under controlled conditions that different fungal strains can affect plant growth in different ways and can behave differently during ectomycorrhiza formation (Tonkin et al. 1989; Thoen et al. 1990). In contrast, nothing is known about the influence of different *Tuber* strains on the formation and structure of the mycorrhiza. This is due above all to the difficulty of isolating these fungi in pure culture (Fontana 1968; Mischiati and Fontana 1993).

Truffles (*Tuber* spp.) are ectomycorrhizal fungi of particular economic importance in that many of them have valuable ascocarps. In fact, seedlings infected with edible *Tuber* species are grown by commercial nurseries in Europe on a large scale for truffle cultivation. Up to now, verification of the ectomycorrhizal infection of these plants has been performed using only morphological methods (Fischer and Colinas 1996; Govi et al. 1997; Gandeboeuf et al. 1998) but PCR-based identification methods have recently been set up (Henrion et al. 1994; Amicucci et al. 1996, 1998; Bertini et al. 1998; Rubini et al. 1998; Mello et al. 1999). The anatomical and morphological features of *Tuber* ectomycorrhizas

with various host plants have also been described by several authors (Zambonelli et al. 1993, 1995; Granetti 1995; Rauscher et al. 1995). In particular, the anatomical structure of the mantle and the features of the cystidia (size, ramification type, etc.) have been shown to be the most important characteristics with which to distinguish the ectomycorrhizas formed by different *Tuber* species.

The aim of this present work was to compare the anatomical/morphological features of the mycorrhizas of different strains of *Tuber borchii* Vittad. on *Tilia platyphyllos* Scop. using a standardized clonal model to synthesize ectomycorrhizas in vitro (Sisti et al. 1998).

Materials and methods

Origin of the *T. borchii* strains examined

Five different isolates of *T. borchii* were obtained from fresh fruit bodies harvested from different areas of north-central Italy in different years (Table 1). Dried voucher specimens are preserved in the herbarium of the Centro di Micologia of Bologna. The fungal cultures were isolated on PDA (potato dextrose agar, Difco) and subcultured on the same medium in agar slants every 6 months. Four of the strains (1BO, 17BO, 43BO, 10RA) had been previously identified using PCR (Rossi et al. 1999). The DNA extracted from the mycelium culture of strain 71BO was analysed by species-specific multiplex PCR according to Amicucci et al. (1998) and by RFLP analysis using *Afa*I, according to Bertini et al. (1998).

Mycorrhizal synthesis in vitro

Mycelia were grown for 30 days on MMN liquid medium (Molina 1979) before being used for the inoculation of micropropagated shoots of *Tilia platyphyllos*. Ectomycorrhizal synthesis was performed in tubes filled with peat moss-vermiculite embedded in liquid media, as described by Sisti et al. (1998). In order to limit somaclonal variation, the plantlets were obtained from microshoots of sprouts from a single *T. platyphyllos* plant. The synthesis experiments were conducted in a growth chamber with 16-h daylight at 22 ± 1 °C. The experiments were performed on 10 tubes of each strain and repeated three times.

Colonization studies

The test tubes were checked weekly under a stereomicroscope (Leica Wild) to monitor mycelium growth and ectomycorrhizal development, measuring the distance between the point of inoculation and the maximal depth reached by the hyphae. After 4 months, all plantlets were removed from the tubes and the percent mycorrhization determined using the method of Rocchi et al. (1999). The anatomical/morphological characteristics of the mycorrhizas were then determined.

Morphological characterization of ectomycorrhizas

The morphological features of the mycorrhizas were examined under a dissecting microscope with incident light and a black background. As suggested by Agerer (1987–1998), only fresh material was used. The colour was recorded using the colour identification chart of the Royal Botanical Garden, Edinburgh (1969) as reference. The mycorrhizas were then stored in FAA (formaldehyde: 70% ethanol: acetic acid, 5:90:5) at 5 °C. Anatomical characterization of the mantle and cystidia was performed using both a light transmission microscope (LM) (Dialux 20 EB Leitz) with high magnification ($\times 1000$ in immersion oil) and video-confocal microscopy (VCM), using a prototype system developed at the Institute of Biophysics, CNR, Pisa (Benedetti et al. 1994, 1995).

For VCM, mycorrhizal tips were bleached by heating in 10% w/v KOH and adding a few drops of H_2O_2 for 20–30 s. The mycorrhizal tips were then dyed with fluorescein isothiocyanate (FITC) (Schelkle et al. 1996) or observed in the UV range without fluorescent dyes. With VCM it is possible to excite autofluorescence in the UV range and to obtain confocal images in the visible and near-infrared spectral ranges. Slices of the tissue were taken at 800-nm intervals using a $\times 40$ oil immersion lens. The mantle structure was described using the glossary of terms published by Agerer (1987–1998). The dimensions of the cells were determined using the NIH Image software (Scion Corporation). Ectomycorrhizal tips fixed in FAA were dehydrated in an aqueous series of ethanol (70, 80, 95, 100%), clarified in xylol, embedded in paraffin wax (52–54 °C) and cut with a rotary microtome (8–10 μm thickness). Serial sections were stained with cotton blue, mounted in lactic acid and observed under LM ($\times 400$).

Index of lobation

In order to quantify the differences in shape observed in mantle cells of the different strains, a mathematical formula was proposed as an index of lobation.

$$i = \frac{1}{n\sqrt{\pi}} \sum_{i=1}^{n} \frac{p_i}{\sqrt{A_i}}$$

where:
i = index of lobation
p = semiperimeter of the cell
A = area of the cell
n = number of cells measured

This formula describes the relationship between the measured perimeter of a cell and the circumference of a circle having the same area. In the case of the mantle cells of *Tuber* spp. (in which cell shape ranged from polygonal to epidermoid) (Zambonelli et al. 1993), the value was higher in more lobate cells and can thus be used as an indicator of lobation.

Table 1 Characteristics of the *Tuber borchii* strains used. Herbarium numbers are of dried specimens in the Mycology Center, University of Bologna, Italy

Strain	Collection locality	Herbarium number	Host plant	Isolation year
1BO (ATCC n. 96540)	Cervia (RA)	762	*Pinus pinea* L.	1987
17BO	Marina di Ravenna (RA)	1183-17	*Pinus pinea* L.	1996
43BO	Marina di Ravenna (RA)	1201-43	*Pinus pinea* L.	1996
71BO	Sant'Angelo in Vado (PS)	1274	*Quercus pubescens* Willd.	1997
10RA	Marina di Ravenna (RA)	1187	*Pinus pinea* L.	1996

Results

Strain 71BO analysed by species-specific multiple PCR and by RFLP was confirmed as *T. borchii* (data not shown).

The kinetics of mycelial growth in the test tubes were similar in the five strains. After a lag phase of 8–10 days, all strains showed an exponential phase of about 6 weeks. After 10 weeks, the mycelia had colonized the entire substrate (Fig. 1).

The hyphal pattern of the five strains differed: the hyphae of 43BO, 10RA, and 17 BO grew uniformly and loosely throughout the substrate (Fig. 2), while the hyphae of 1BO and 71BO were thicker around the root tips (Fig. 3). Three months after inoculation, the first ectomycorrhizas were observed in the upper portion of the tubes for all strains. These ectomycorrhizas were free of external mycelia with the exception of those formed by 1BO, which still surrounded the tips. Four months after inoculation, the mean degree of ectomycorrhizal infection ranged from 50.6% for 1BO to 82.1% for 10RA (Table 2).

The mycorrhizas of all strains were either unramified or showed monopodial pinnate ramification. The percentage of unramified ECM was only 11.6% for 10RA, but otherwise ranged from 45.7% (1BO) to 55.2% (17BO). The number of ECM tips in the mycorrhizal system was also highest for 10RA (mean 8.7) and ranged from 3.7 (71BO) to 6.1 (43BO). The colour of the proximal part of the mycorrhizas was fulvous in 1BO, 17 BO, 43 BO, 10 RA, while 71 BO was darker (hazel-coloured) (Fig. 4). The growing tips of all strains were often lighter coloured (whitish-yellow).

The lengths and diameters of both simple and ramified mycorrhizas were similar with the exception of 1BO, for which the values were slightly lower.

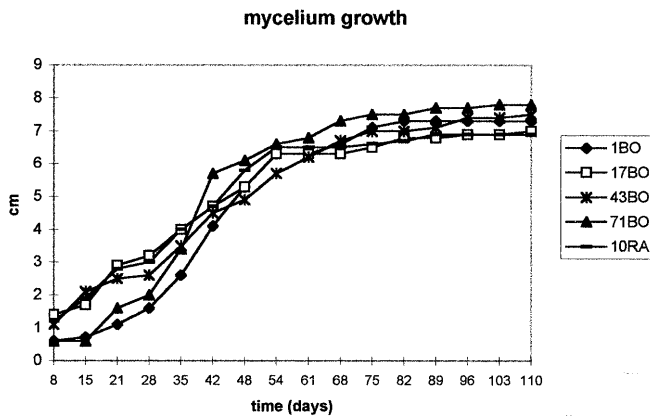The mean percentage of root tips with cystidia was basically very similar for 17BO, 43BO, 71BO and

Fig. 2 Intensive hyphal proliferation in close contact with the root surface;1BO isolate after 40 days; *bar* 300 μm
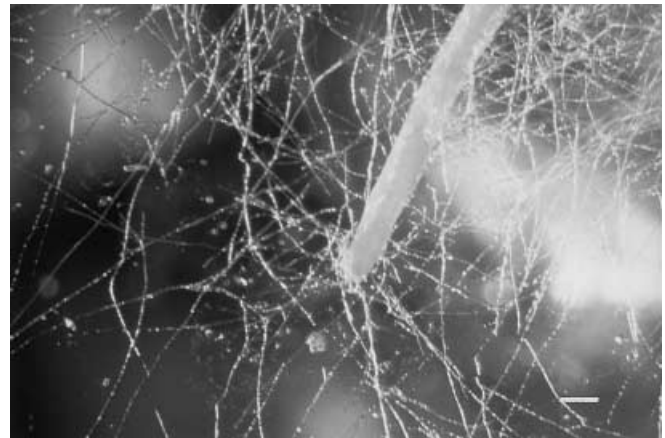
Fig. 3 Non-intensive hyphal proliferation in the root surface; 10RA isolate after 40 days; *bar* 300 μm

10RA but significantly lower for 1BO. The lengths of cystidia were similar in all strains and the number of septa varied from 0 to 2. Only 43BO presented cystidia with three septa. The anatomical structure of the mantle of the mycorrhizas formed by the five strains was observed at different depths below the surface (6 and 12 μm) using both LM (Figs. 5, 6) and VCM (Figs. 7, 8, 9 and 10). The mantle was pseudoparenchymatic and composed of more or less lobate cells, depending on the strain. The mantle cells were polygonal, with rounded edges in mycorrhizas of 1BO and 71BO, with lobate cells in 17BO and 10RA and with marked lobes of epidermoid form in 43 BO. However, all anatomical measurements of the mantle (main and lesser axes, area, perimeter) were similar in all strains.

The calculated indices of lobation are shown in Table 3. As foreseen, these values were greatest for 43BO, and lowest for 1BO and 71 BO. No significant differences were found in the biometric characteristics of the mycorrhizas.

Fig. 1 Mycelial growth of different strains of *Tuber borchii* in vitro. Data are the means of 30 independent measurements; the standard deviation (not shown), ranged from 6.1% to 18.3% of the mean value
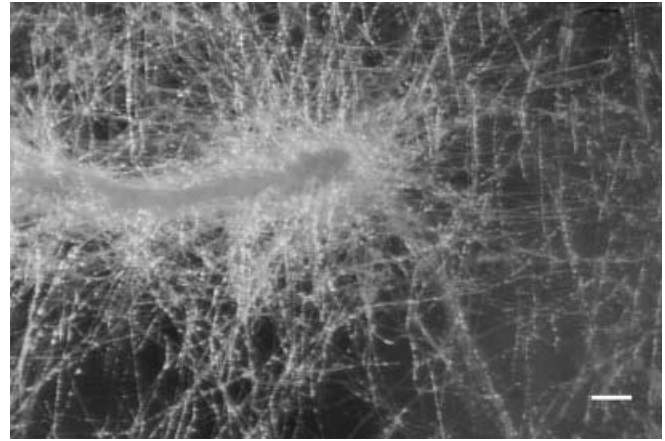
Table 2 Characteristics of ectomycorrhizas (ECM) formed by the different strains of *T. borchii*. The colour code numbers refer to the colour identification chart published by the Royal Botanical Garden (1969).
The data shown are the means of at least 30 independent measurements ± SD

| Strain	1BO	17BO	43BO	71BO	10RA
ECM infection (%)	50.6±25.5	81.7±13.4	77.5±16.8	62.5±21.1	82.1±11.3
Morphological features of the mycorrhizas					
Colour	Fulvous (12)	Fulvous (12)	Fulvous (12)	Hazel (27)	Fulvous (12)
ECM tips (%)	45.7±10.2	55.2±15.8	49.1±13.5	54.5±20.4	11.6±6.4
Length of ECM tips (mm)	0.85±0.50	1.20±0.57	0.97±0.20	0.95±0.65	1.07±0.38
Diameter of ECM tips (mm)	0.18±0.03	0.26±0.04	0.25±0.03	0.26±0.04	0.26±0.03
ECM tips with cystidia (%)	3.1±2.8	38.2±10.8	52.6±18.7	50.3±15.4	48.2±9.7
Ratio ECM tips: ECM ramified system	3.8±1.9	4.5±3.0	6.1±4.9	3.7±2.3	8.7±6.1
Length of ECM system (mm)	1.9±1.2	2.3±1.2	3.1±2.3	1.9±1.1	2.0±0.8
Diameter of ECM system (mm)	0.23±0.03	0.27±0.02	0.27±0.03	0.28±0.02	0.29±0.02
Anatomical features of mantle and cystidia					
Surface view of mantle (6 μm deep)					
Hyphal arrangement	Pseudoparenchymatous	Pseudoparenchymatous	Pseudoparenchymatous	Pseudoparenchymatous	Pseudoparenchymatous
Shape of hyphal cells	Roundish	Epidermoid	Epidermoid	Roundish	Epidermoid
Cell diameter (μm)	14.0±5.9×6.9±3.0	17.5±6.1×8.7±3.0	16.9±6.6×7.6±3.7	16.6±4.5×7.5±3.3	15.2±5.5×7.2±2.8
Cell area (μm^2)	127.1±104.5	130.4±95.3	128.5±92.2	115.6±67.2	102.9±55.1
Perimeter (μm)	46.1±18.6	46.9±18.8	48.7±20.0	43.1±13.2	43.8±13.7
Plan view of mantle inner layers (12 μm deep)					
Hyphal arrangement	Pseudoparenchymatous	Pseudoparenchymatous	Pseudoparenchymatous	Pseudoparenchymatous	Pseudoparenchymatous
Shape of hyphal cells	Roundish	Epidermoid	Epidermoid	Roundish	Epidermoid
Cell diameter (μm)	16.0±7.2×7.9±2.6	17.9±5.2×8.1±2.9	17.8±9.3×7.4±3.2	17.4±5.9×8.6±4	15.7±5.5×8.2±2.7
Cell area (μm^2)	113.0±77.4	128.0±74.5	144.6±105.1	133.6±73.6	130.5±64.5
Perimeter (μm)	45.1±17.2	46.4±14.0	52.1±18.2	51.5±17.5	49.1±13.7
Cystidia					
No. of septa	0.81±0.69	0.78±0.58	1.73±0.59	1.09±0.55	0.91±0.61
Length (μm)	98.2±11.5	108.7±11.4	113.8±24.6	90.7±26.5	97.8±17.8
Transversal sections					
Mantle thickness (μm)	18.8±2.9	24.1±4.1	20.3±3.7	23.4±4.0	19.8±3.1
Cell dimensions (rad × tang) (μm)	1.7±0.9×6.2±3.6	3.0±0.8×6.2±2.9	3.4±1.5×7.1±4.1	3.4±1.2×5.5±3.1	2.8±0.8×6.3±2.3
Depth of Hartig net (μm)	38.7±5.1	20.65±3.2	30.8±10.4	36.1±10.1	31.6±8.1
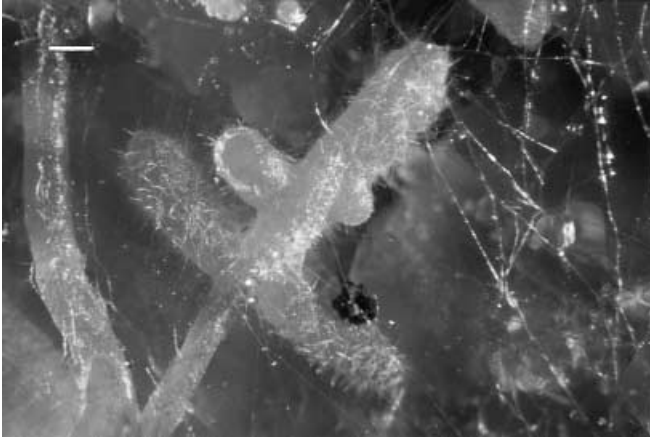
Fig. 4 In vitro monopodial ectomycorrhizal system of isolate 71 BO after 3 months of culture; *bar* 200 μm
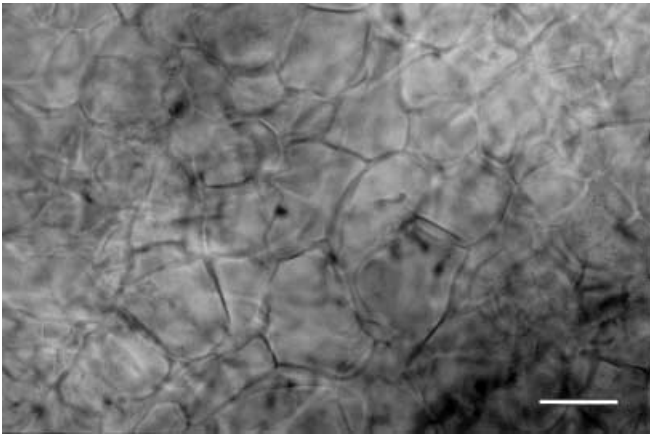

Fig. 5 Mycorrhizal mantle of isolate 1BO with a light transmission microscope (LM); *bar* 10 μm
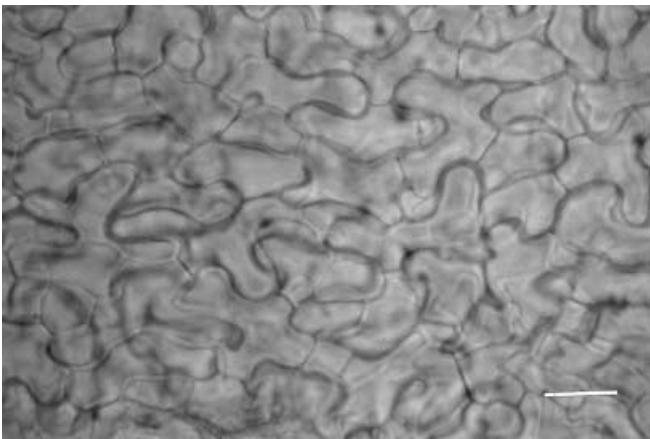

Fig. 6 Mycorrhizal mantle of isolate 43BO with LM; *bar* 10 μm

Discussion

All five strains tested were able to form ectomycorrhizas in vitro on *Tilia platyphyllos*, although with different mycorrhizal competence. The lowest percentage of mycorrhizal infection was shown by 1BO, which had been in axenic culture for more than 10 years. Several reports have suggested that the ability of some fungal taxa to form ECM strains decreases with time in culture (Marx 1981; Thompson et al. 1993; Cairney 1999). Accordingly, the partial loss of ability to form mycorrhizas observed for strain 1BO may reflect physiological variation due to prolonged axenic culture. In previous work, Saltarelli et al. (1999) studied the intraspecific variability in vitro among four of these five *T. borchii* strains (1BO, 17BO, 43BO, 10RA) and found differences in the electrophoretic profiles of their polypeptide and mRNA populations, especially for 1BO. In liquid medium containing glucose, 1BO also presented the lowest level of hexokinase activity, which may represent the glucose sensor in symbiotic fungi (Saltarelli et al. 1999). These results support the hypothesis that partial loss of mycorrhiza-forming ability and physiological changes in 1BO are the result of modification of genes involved in growth during storage in axenic culture (Di Battista et al. 1996).

Intraspecific variability in physiological characteristics of ectomycorrhizal fungi has already been demonstrated by several authors (e.g. Cairney 1999). Our results show the occurrence also of anatomical and morphological differences among strains of the same species. The morphological features and cystidia characteristics of ECM from these five different *T. borchii* strains developed in vitro were similar to those described by other authors (Fontana et al. 1992; Zambonelli et al. 1993, 1995; Rauscher et al. 1996), in particular those given by Granetti et al. (1995) using the some host plant (*T. platyphyllos*). In contrast to the many morphological characteristics shared by mycorrhizas of the tested strains, the pattern of the mantle cells showed intraspecific variability. VCM was a useful tool to identify these differences in that it allowed optical non-disruptive sectioning and examination of the mantle layers as with Normaski's contrast microscope but with the additional advantage of being able to select the depth of the views. The usefulness of confocal microscopy in studying fungal cytology is well known (Kwon et al. 1993) but was only recently applied to the characterization of mycorrhizas (Schelkle et al. 1996; Melville et al. 1998; Comandini et al. 1998). Using the proposed index of lobation, it was also possible to quantify the differences in cell shape in the mantle. Thus, this index could be a further tool to characterize the fungal mantle.

Our work has shown that the form of mantle cells in *T. borchii*, which were found by Zambonelli et al. (1993, 1995) to vary depending on the host plant, also vary with the fungal strain used in in vitro mycorrhiza-
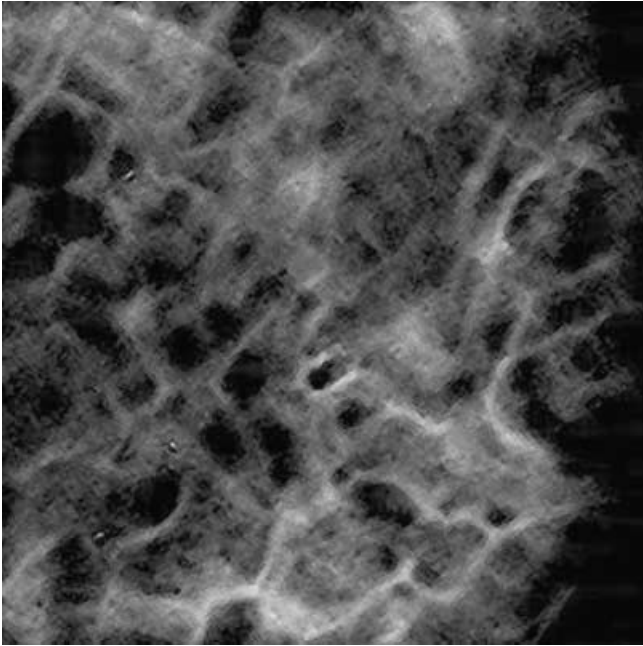
Fig. 7 Video-confocal microscopy of the different layers of the mantle: external view (6 μm deep) of the mantle of 1BO; *bar* 10 μm
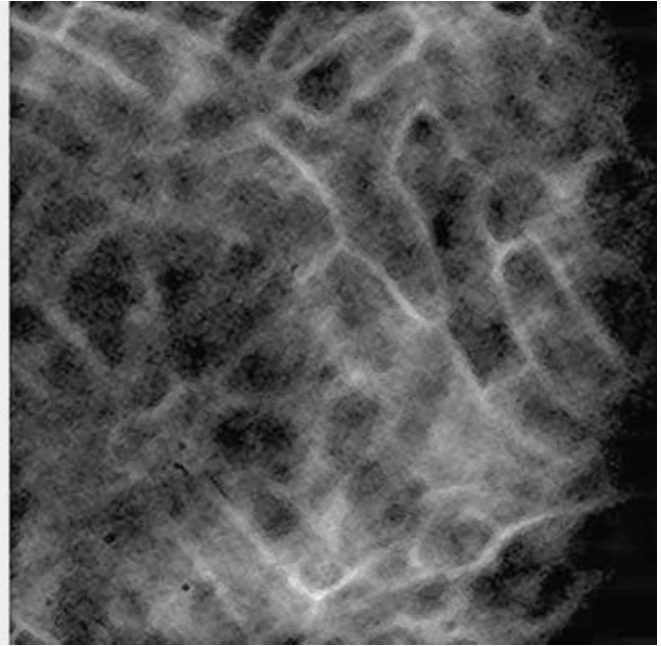

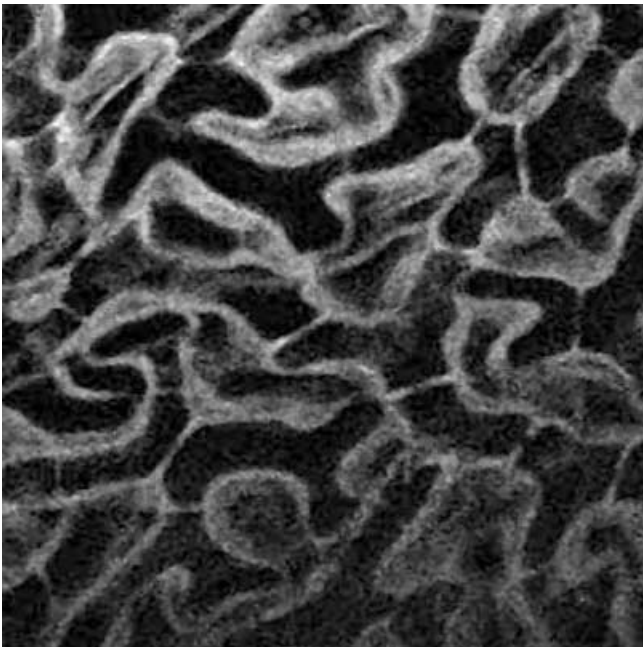**Fig. 8** Internal view (12 μm deep) of the mantle of 1BO; *bar* 10 μm

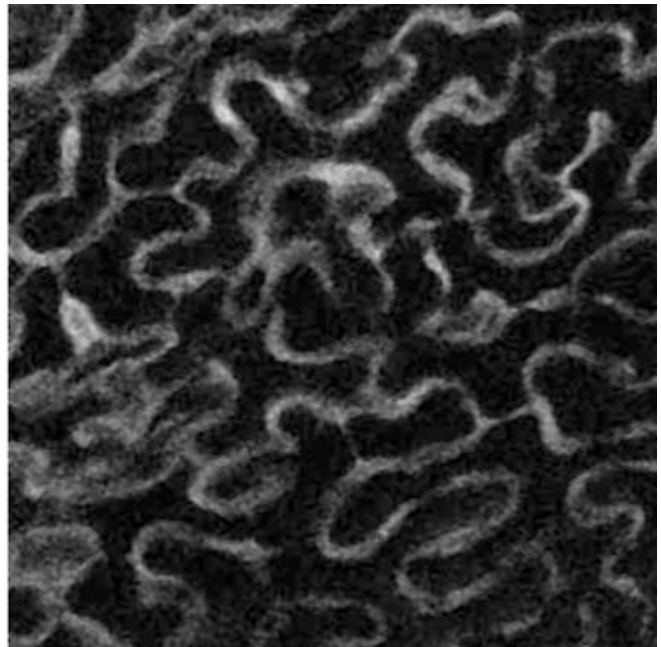**Fig. 9** External view (6 μm deep) of the mantle of 43BO; *bar* 10 μm

Fig. 10 Internal view (12 μm deep) of the mantle of 43BO; *bar* 10 μm

Table 3 Index of lobation in the mantle of ectomycorrhizas formed by different *T. borchii* strains

Index of lobation	1BO	17BO	43BO	71BO	10RA
External layer	1.21±0.12	1.27±0.18	1.34±0.24	1.17±0.12	1.25±0.13
Internal layer	1.19±0.10	1.25±0.15	1.32±0.24	1.16±0.11	1.23±0.14

tion experiments. These differences were influenced not only by the intraspecific variability of the strains but also by the origin of the plant host and the age of the strains. Such intraspecific variability makes it more difficult to use morphological methods to identify mycorrhizas of the white truffles of the genus *Tuber* (*T. magnatum* Pico, *T. borchii*, *T. maculatum* Vittad. and *T. puberulum* Berk & Br.), which have different commercial value. The ectomycorrhizas of these truffles have similar awl-shaped hyaline cystidia (Blaschke 1987; Fontana et al. 1990, 1992; Zambonelli et al. 1993, 1995; Granetti 1995; Granetti et al. 1995) and this characteristic is not as distinctive as in other *Tuber* species (*T. melanosporum* Vittad. and *T. brumale* Vittad.), where length, shape and colour are species specific (Palenzona 1969). Our results also show that the pattern of the mantle cannot be used to distinguish mycorrhizas formed by *T. borchii* from mycorrhizas of other white truffles. In fact, the cell shape of the *T. borchii* strains examined varied from slightly lobed, as already described for *T. borchii* (Fontana et al. 1992; Rauscher et al. 1996), to the epidermoid shape typical of *T. maculatum* (Zambonelli et al. 1999), *T. magnatum* (Fontana et al. 1990; Zambonelli et al. 1993; Granetti 1995) and *T. puberulum* (Blaschke 1987). These results underline the need to support morphological studies with molecular identification techniques to ensure correct certification of commercially available seedlings infected with white truffles.

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