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SHORT NOTE

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Two genetically related strains of *Tuber borchii* produce *Tilia* mycorrhizas with different morphological traits

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Abstract Two genetically related strains of *Tuber borchii* Vittad. (1BO and 43BO) produce mycorrhizas with *Tilia platyphyllos* Scop. with a different degree of efficiency. The aim of this work was to characterize the morphology of the fungal symbiotic structures in order to examine potential relationships between the anatomical traits of the mycorrhiza, the mycorrhizal capacities of the fungal strains and their effect on the host plants. Some morphological features of mantle hyphae (small size, intense staining, vacuolization, abundance of mitochondria) led to a mantle with morphological features that were isolate-specific. There were unexpected differences, at least under our experimental conditions: 1BO strain mantle cells were larger, less reactive to staining, more highly vacuolated and poorer in mitochondria than those of 43BO. These features were found throughout the mantle in 1BO, while the inner mantle hyphae of 43BO were significantly smaller and more intensely stained than the outer cells. In the 43BO strain there was a positive relation between these features and higher infectivity (evaluated as percentage of mycorrhizal tips) as well as a slightly more effective stimulation of plant growth. These observations suggest that genetically related truffle strains produce mycorrhizas with different morphologies, which may be related to a more efficient response of the host plant to inoculation.

Keywords Ectomycorrhiza · *Tuber borchii* · Image analysis · Cell viability · Mantle anatomy

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Introduction

Mycorrhizas have long been held to be an essential feature of the biology and ecology of most terrestrial plants, since they influence plant growth, water and nutrient absorption, and susceptibility to root diseases. A current review of these topics has been provided by Smith and Read (1997). In the past 10 years, a wealth of experimental investigations and the development of new technologies has led to substantial advances in knowledge of mycorrhizal function, in cellular and molecular biology (Harrison 1999; Martin 2001) as well as ecological contexts (Horton and Bruns 2001; Van der Heijden et al.1998).

Much less is known about the effects of ectomycorrhizal fungal symbionts on root structure and eventually root function. While correlations between leaf structural-physiological traits and plant growth strategies have been widely investigated (Eissenstat 2000), investigations of possible correlations between root structure and the potential growth rate and height of the whole plant are very limited. Wahl and Ryser (2000) investigated a range of perennial temperate grasses, identified useful parameters such as tissue density and vessel diameter and found relationships that, by analogy, raise questions for mycorrhizal associations. One of the most interesting findings was a strong negative correlation between tissue density of the axile roots and potential growth rate. This was mirrored by some anatomical traits that fit well (e.g. lignified cells) with a survival strategy shown by plants in low-resource environments (Wahl and Ryser 2000).

This raised the question of whether ectomycorrhizal roots can be investigated in a similar context, first looking for relationships between anatomical traits and plant potential growth. In natural conditions, the roots of ectomycorrhizal plants have an anatomy that is altered significantly by the presence of the fungal mantle (Peterson and Bonfante 1994). On this basis, we focused on truffle mycorrhizas since they may be obtained in in vitro conditions and also because of previous observations showing that two genetically related strains of *Tuber*

borchii Vittad. (1BO and 43BO) produced mycorrhizas with linden with different degrees of efficiency; the latter strain produced a significantly higher number of mycorrhizal tips (Giomaro et al. 2000). Changes in the anatomy and cytology of the mantle of *Tuber borchii* on linden roots were investigated in order to identify morphological traits, some of which were quantified using image analysis. Symbiotic efficiency in terms of effects on plant growth parameters was also evaluated.

Materials and methods

Fungal cultures

Tuber borchii strains (1BO, deposited in the American Type Culture Collection as no. 96540, and 43BO, deposited in the Herbarium of Centro di Micologia, Bologna as no. 1201-43) were isolated from fruitbodies growing on *Pinus pinea* L. roots (Zambonelli et al. 1995). Using molecular techniques, the strains have been shown unambiguously to belong to the same taxon (*Tuber borchii*) (Rossi et al. 1999). Mycelia were cultivated on potato dextrose agar (PDA) for 20 days and mycelial plugs were transferred to 60 ml MMN liquid culture medium (Marx 1969). The cultures were grown in darkness at 23±2°C for 1 month.

Mycorrhizal synthesis

In vitro mycorrhizas were obtained as described by Sisti et al. (1998). Micropropagated plantlets of linden (Chalupa 1984) were transplanted into culture tubes (4 cm diameter × 30 cm height) filled with 80 ml of a peat moss:vermiculite (1:30 v/v) mix (Molina 1979) and 15 ml of MS/2 (Murashige and Skoog 1962), pH 6.4, supplemented with 10 g l^{-1} glucose. Plantlets were inoculated by adding plugs of *Tuber borchii* mycelium, produced as described above, and cultivated at 25±1°C with a 16-h photoperiod under cool white fluorescent lamps (3500 lux).

Twenty, 30 and 90 days after inoculation, tubes containing *Tilia platyphyllos* were scanned under the stereomicroscope and rootlets were sampled. According to the criteria of Giomaro et al. (2000), three groups were identified for each strain: 1) non-mycorrhizal roots, mostly found during the first sampling; 2) roots with hyphae running at the root surface in the first and second samplings, and 3) fully developed mycorrhizas 90 days after inoculation. Samples from each group were selected and prepared for morphological observation.

Microscopy

Mycorrhizal and non-mycorrhizal rootlets from 20, 30 and 90 days were prepared for light (LM) and transmission electron microscopy (EM) according to Balestrini et al. (1996). The root segments were fixed in 2.5% (v/v) glutaraldehyde, postfixed in 1% (w/v) osmium tetroxide, washed three times with water, dehydrated in an ethanol series of 30, 50, 70, 90, 100% (v/v) (15 min each step) at room temperature and then embedded in LR White resin (Polysciences, Warrington, Pa., USA). Semi-thin sections (1 μm) were stained with 1% (w/v) toluidine blue for morphological observations, while thin sections (0.05 μm) were stained using the PATAg method to visualize polysaccharides (Roland and Vian 1991) or contrasted with uranyl acetate and lead citrate. In order to better differentiate the fungal wall, some sections were treated with an antibody against β1–3 glucans according to Bonfante et al. (1998). Sections were observed using a CM 10 Electron Microscope (Philips) at the Laboratorio di Microscopie Avanzate (www.bioveg.unito.it). Hyphae taken at the colony margin from the two fungal strains grown on PDA were processed following the same procedures.

Image analysis

Transverse semi-thin and thin sections of ectomycorrhizas were subjected to morphometric image analysis using Scion Image 4.0.2 beta image analysis software. Ninety-day-old, fully developed mycorrhizas were used. The mycorrhizas of both strains exhibited the shapes and colors described in Giomaro et al. (2000). Twenty sections taken from mycorrhizas on different plants were analyzed for each isolate; at least 50 hyphal cross-sections were considered for each semi-thin section. Analysis was limited to the mantle. All the images were converted into 8-bit grayscale images (0 white; 255 black). The following parameters were measured for each mantle cell: distance between mantle cell and surface of epidermal cells, perimeter, area, major axis and average intensity of staining. Twenty thin sections for each isolate were subjected to a similar analysis and the following parameters were considered: thickness of hyphal wall, host wall area/cell area ratio, vacuole area/cell area ratio and mitochondrial area/cell area ratio. Some parameters are shown as area ratio since images at high magnification cannot include whole cells.

Plant growth parameters

After 4 months, the remaining 60 tubes were gently emptied and vermiculite particles manually removed from the root systems under a stereomicroscope. The shoot heights and numbers of nodes were measured. Leaves, stem and root systems were split and weighed separately (wet weight). Dry weights of leaves, stems and root systems were measured after storage at 70°C overnight.

Statistical analysis

In order to analyze biometric values of the mantle cells of semi-thin and thin sections of the ectomycorrhiza, the data set was analyzed using the Kolmogorov-Smirnov test to verify Gaussian distribution, the F max-test to determine the heterogeneity of the variance and the t-Student test to determine the significance of the difference among means (P <0.05, 2-tailed). Ratio of wall area/cell area, vacuole area/cell area, mitochondrial area/cell area were arcsine square-root transformed to minimize heteroscedasticity.

In order to examine differences in the morphometric features in the outer and inner layers of the mantle, the correlation coefficients (r Pearson) were calculated between the distance of mantle cells/external cortical cells and the area, perimeter, major axis and average intensity of staining; significant correlations (slope significantly different from 0; P <0.05) were shown with a regression line.

One-way ANOVA was performed to analyze plant growth parameters related to control plants and plants colonized by 1BO and 43BO. The Kolmogorov-Smirnov test was used in order to verify the normal distribution of data. The mean values were compared using the Tukey-Kramer test at P <0.05 (Sokal and Rohlf 1995).

Results

Anatomy and cytology of linden mycorrhizal roots

A cross-section of a non-mycorrhizal linden rootlet sampled 20 days after inoculation showed a single layer of epidermal cells, frequent hair roots, and a three-layered cortex surrounding the stele (not shown).

Actively growing hyphae of the fungus, regardless of the strain used, developed along and around roots and established contact with the epidermal cells, (Fig. 1A, Fig. 2A–C). Root hairs are a preferential adhesion point

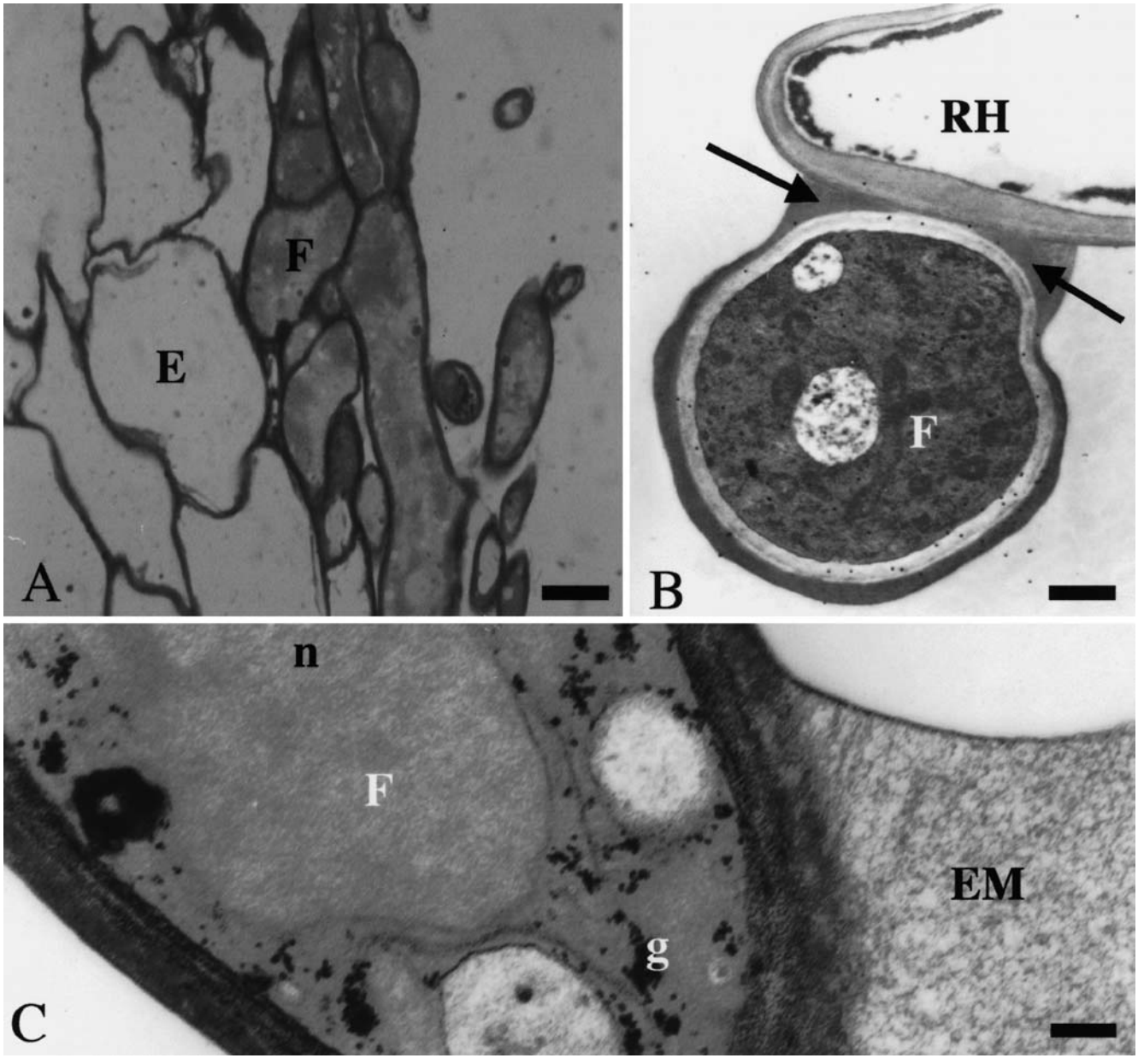

Fig. 1A–C The development at 30 days of *Tuber borchii* 43BO strain in the presence of *Tilia platyphyllos* roots. **A** Light microscopy, showing that actively growing hyphae developing along the root surface establish contacts with the epidermal cells (*E* epidermal cell, *F* hypha); *bar* 7 μm. **B** Electron microscopy, showing contact between a root hair and a hypha. Abundant extracellular material is present at the point of contact (*arrows*). The section is labelled with an antibody against β1–3 glucans (*RH* root hair, *F* hypha); *bar* 0.5 μm. **C** At higher magnification the extracellular material shows a fibrillar structure and reacts to the silver PATAg reaction (*EM* extracellular material, *F* hypha, *n* nucleus, *g* glycogen); *bar* 0.3 μm

(Fig. 2B). Abundant extracellular material contacts hyphae with the root hair surface (Fig. 1B). At the ultrastructural level, this has a fibrillar structure and reacts to the PATAg reaction, suggesting a polysaccharide nature of the fibrils (Fig. 1C). As is typical of ectomycorrhizal development (Bonfante et al.1998), the fungus first developed between the epidermal cells into the first layers of the cortex (Fig. 2C), where it produced the Hartig net (Fig. 2D), and lastly developed the mantle on the root surface.

Fully developed mycorrhizas of the two *Tuber borchii* strains showed anatomical differences at the mantle level. The strain 43BO produced a mantle consisting of 10–15 layers of tightly packed hyphae (Fig. 3A). At higher magnification, the innermost layers showed smaller, strongly stained hyphae, while the outer layers consisted of swollen hyphae (Fig. 3B).

The 1BO strain produced a thinner mantle, with no more than 8–10 hyphal layers (Fig. 4A). These hyphae were large and often less reactive to toluidine blue

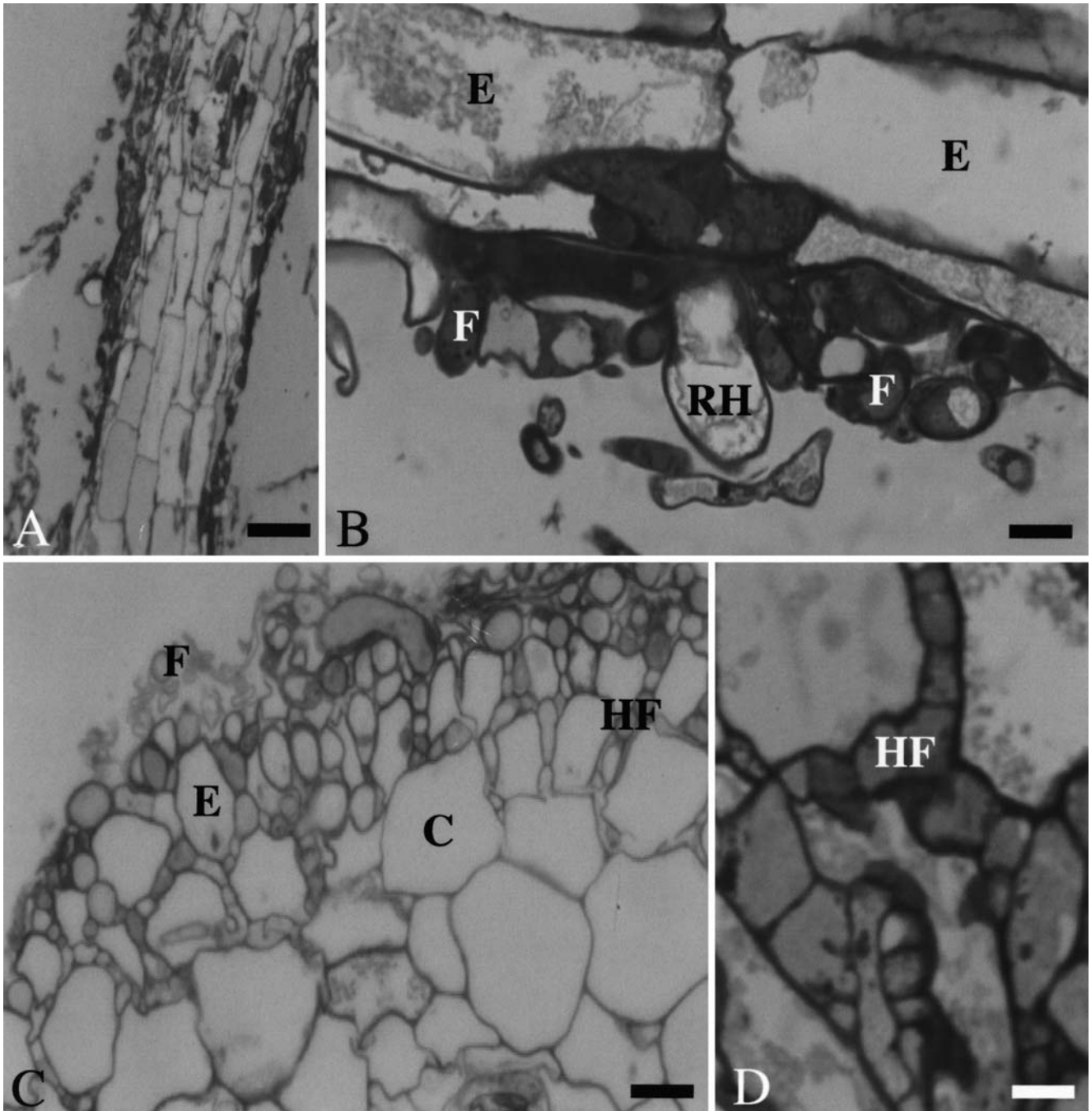

Fig. 2A–D Development at 30 days of *Tuber borchii* 1BO strain in the presence of *Tilia platyphyllos* roots seen under light microscope. **A** Longitudinal section of a colonized root: the fungus loosely surrounds the root; *bar* 55 μm. **B** At higher magnification, hyphae closely surround a root (*E* epidermal cell, *F* hypha, *RH* root hair); *bar* 8.8 μm. **C** In fully established mycorrhiza, the Hartig net is well developed around the epidermal and outer layer of the cortex (*F* fungus, *E* epidermal cell, *C* cortical cell, *HF* Hartig net); *bar* 15 μm. **D** Detail of the Hartig net showing intercellular hyphae (*HF* Hartig net hyphae); *bar* 5 μm

staining than those of strain 43BO (Fig. 4B). Electron microscope observations confirmed the presence of cytological differences. 43BO hyphae showed a regular profile, were rich in organelles (mitochondria, ribosomes) and glycogen particles and were embedded in an abundant extracellular material (Fig. 5A). Conversely, 1BO hyphae consistently showed huge vacuoles, which were sometimes filled with granular material (Fig. 5B). An antibody against β1–3 glucans revealed the presence of these cell wall components in the electron transparent layer of both the strains, in contrast to the loose extracellular material, which did not contain this glucan type (Fig. 5A, B). Similar features were observed in hyphae of 43BO and

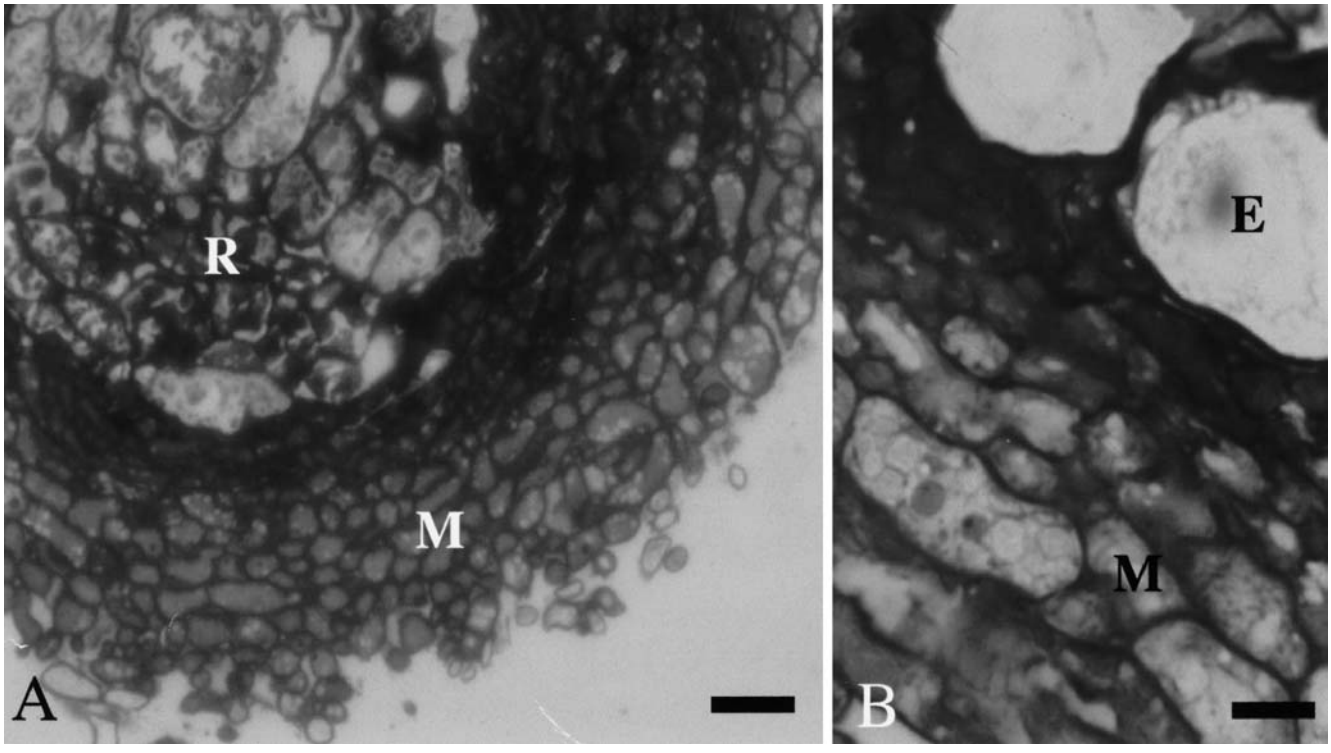

Fig. 3A, B Morphological features of 90-day-old mantle developed by *Tuber borchii* 43BO strain seen under light microscope. **A** The mantle consists of 10–15 layers of tightly packed hyphae on ectomycorrhizal roots (*M* mantle, *R* root); *bar* 20 μm. **B** At higher magnification the innermost layers show hyphae that are smaller in size and strongly stained with toluidine blue. The outer layers consist of living but more vacuolated hyphae (*M* mantle, *E* epidermis); *bar* 7 μm

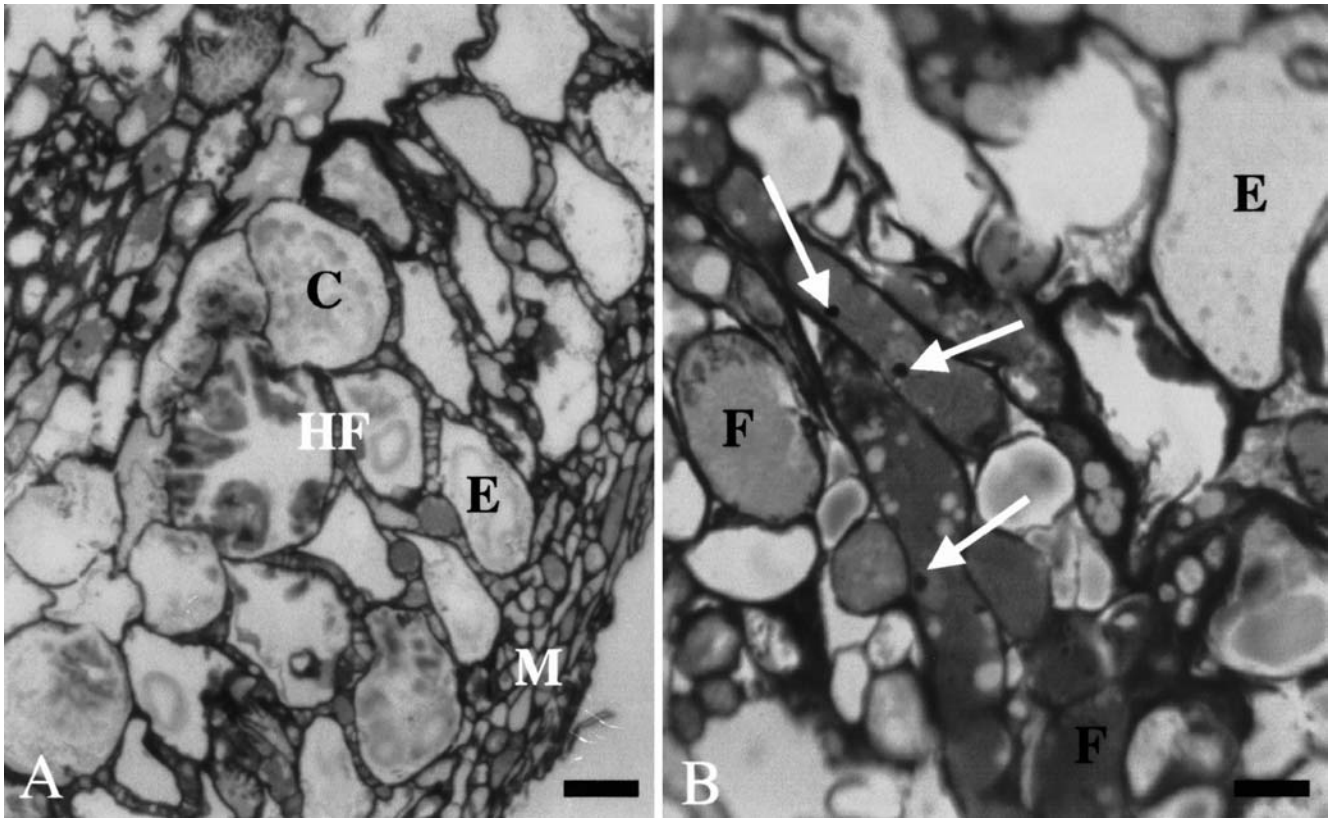

Fig. 4A, B Morphological features of 90-day-old mantle developed by *Tuber borchii* 1BO strain seen under light microscopy. **A** The mantle consists of about 8–10 hyphal layers on ectomycorrhiza (*C* cortical cell, *HF* Hartig net, *E* epidermal cell, *M* mantle); *bar* 17 μm. **B** Details of the mantle. Hyphal size is irregular, some hyphae are poorly reactive to toluidine blue staining, while others show a dense cytoplasmic content. *Arrows* indicate nuclei (*E* epidermal cell, *F* hypha) *bar* 6 μm

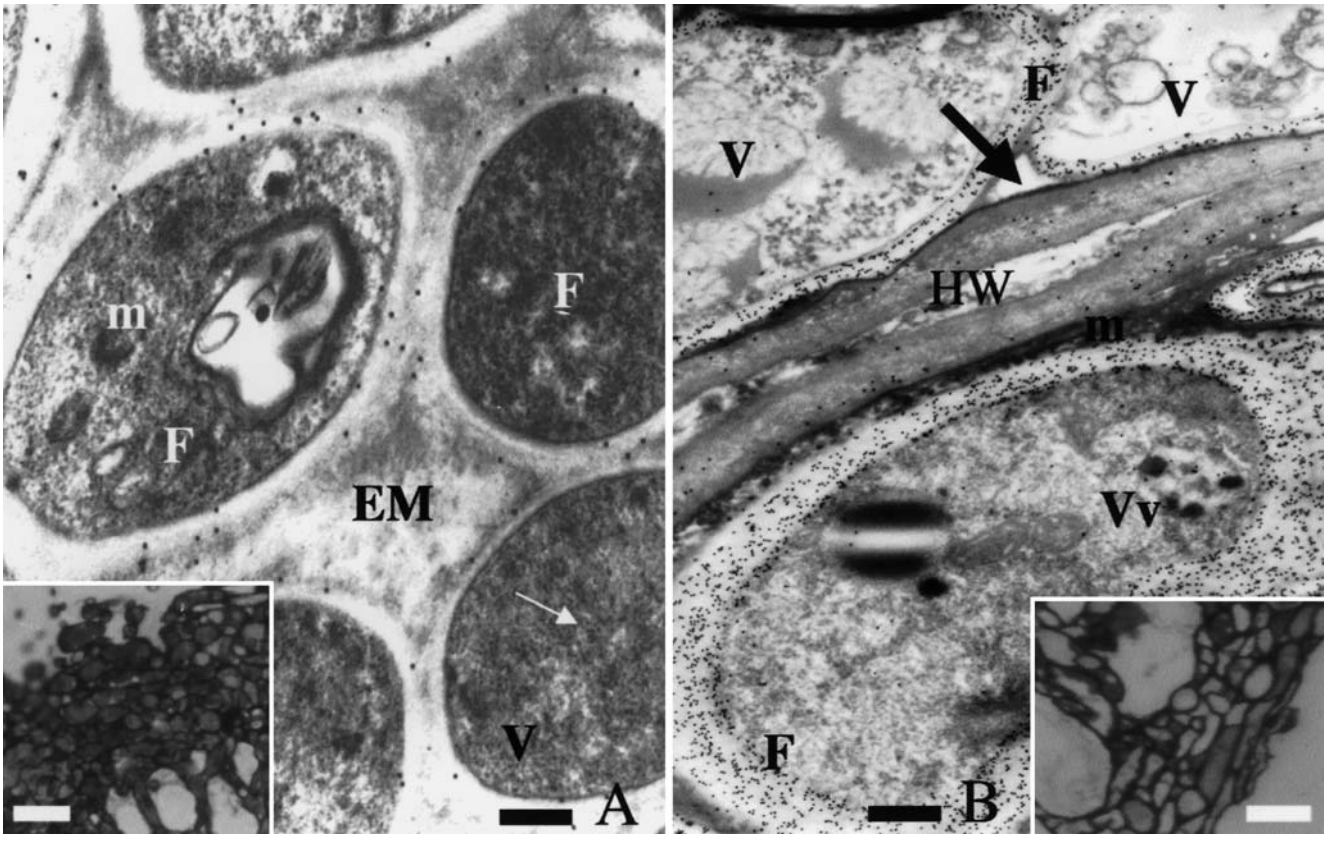

Fig. 5A, B Ultrastructural features of 90-day-old mantles of *Tuber borchii* 1BO and 43BO strains in fully developed mycorrhizas, as shown in the inset seen under light microscope. **A** 43BO hyphae show a regular profile, are rich in organelles (mitochondria, ribosomes, indicated by a *white arrow*) and are surrounded by abundant extracellular material. The sample is labelled with an antibody against β1–3 glucans, which weakly labels the fungal wall (*F* hypha, *EM* extracellular material, *m* mitochondria); *bar* 0.3 μm, *bar* in the inset 19 μm. **B** 1BO hyphae consistently show huge vacuoles, containing granular material (*V*) as well as small electron-dense globules (*Vv*). Interhyphal spaces (*arrow*) are present. The sample is labeled with an antibody against β1–3 glucans, which strongly labels the fungal wall (*F* hypha); *bar* 0.5 μm, *bar* in the inset 19 μm

1BO sampled from growing in in vitro cultures in the absence of the plant (not shown).

Image analysis

In order to understand whether the observed anatomical and cytological differences between the two strains involve the symbiotic potentials of the truffle strains as well as the response of the mycorrhizal host, a morphometric analysis was performed on semi-thin (LM) and thin sections (EM) obtained from the fully developed mycorrhizas.

The results based on the parameters selected to analyze the mantle hyphae are shown in Table 1. The inner mantle fungal cells of the 1BO strain were significantly larger and less reactive to toluidine blue than those of the 43BO strain, which had hyphae cross-sections of about 2/3 of the area of the 1BO hyphae.

EM sections did not reveal significant differences in the fungal wall thickness or the wall area/cell area ratio. Vacuoles occupied a more significant part of the cell of the 1BO isolate, which also had fewer mitochondria, than 43BO. The parameters selected for LM sections were applied to each isolate to examine whether there was a change in size and reactivity to staining along the mantle layers with distance from the outer root cells. The 1BO strain did not show any significant relationship; smaller

Table 1 Morphometric parameters analyzed in the mantle cells of *Tuber borchii* 1BO and 43BO strains in ectomycorrhizal association with linden roots. The data shown are means ±standard deviations. Values with a different letter in the same row are significantly different (*t*-test, $P < 0.05$) (*A* Area of cells, *2p* perimeter of cells, *M axis* major axis of cells, *dye* intensity of staining of cells: 0 white, 255 black)

Morphometric parameter	1BO	43BO
A (μm^2)	25.8±11.7a	17.0±11.5b
2p (μm)	18.9±7.5a	15.9±8.2b
M axis (μm)	7.9±4.1a	5.8±3.6b
Dye	120.9±42.5a	139.6±37.1b
Thickness of hyphal wall (nm)	101.8±17.7	90.3±19.4
Wall area/cell area ratio	0.167±0.103	0.218±0.111
Vacuole area/cell area ratio	0.312±0.163a	0.142±0.113b
Mitochondria area/cell area ratio	0.024±0.008a	0.035±0.012b

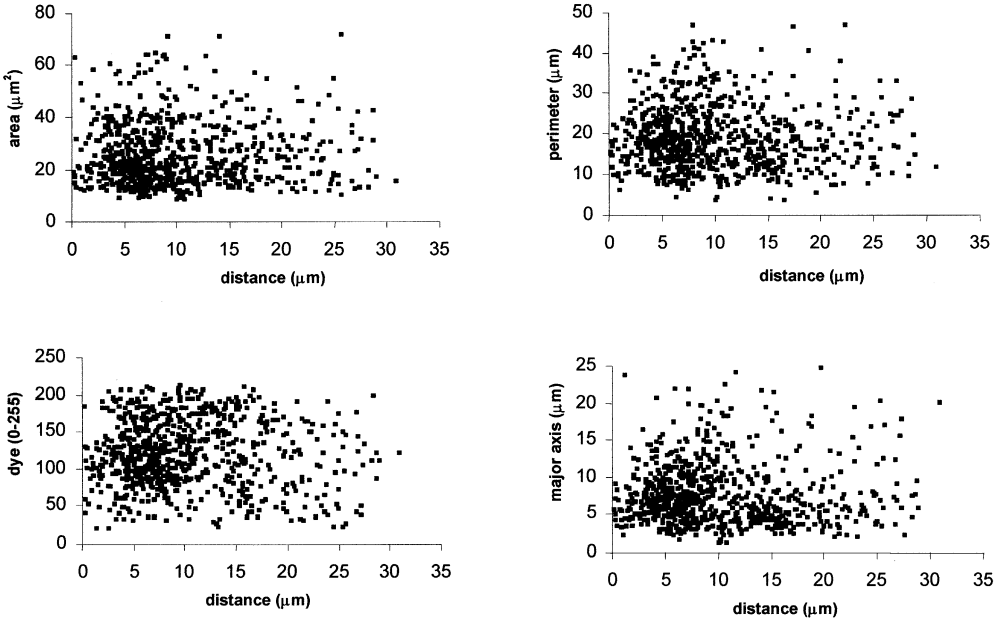

Fig. 6 1BO isolate: relationships between the distance of mantle cells from epidermal cells and area, perimeter, staining (0 white; 255 black), major axis of mantle cells

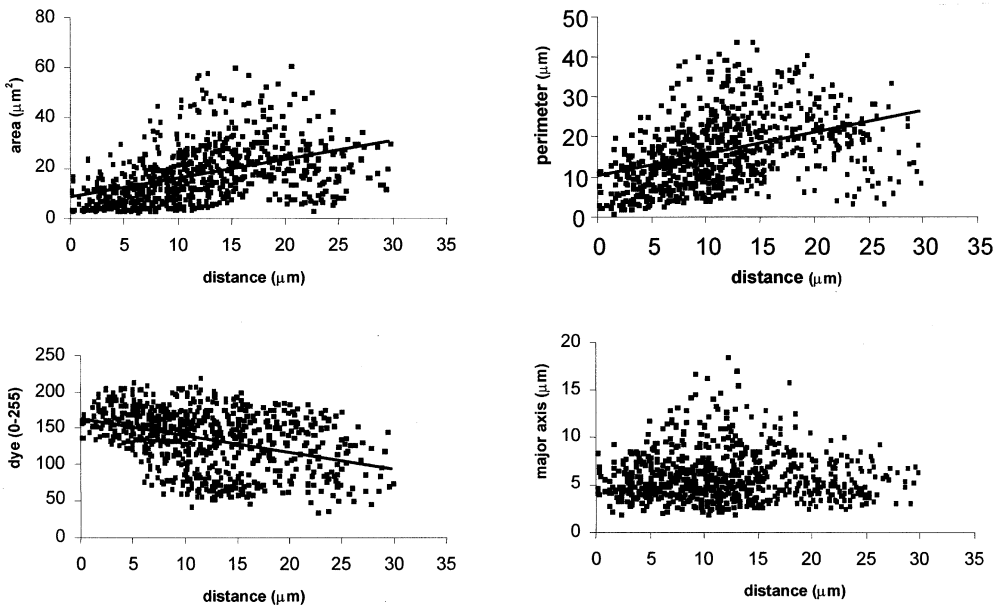

Fig. 7 43BO isolate: relationships between distance of mantle cells from epidermal and area, perimeter, staining (0 white; 255 black), major axis of mantle cells. Significant correlations (P <0,05) are shown with a regression line

and/or more densely stained cells were distributed erratically (Fig. 6). Conversely, 43BO showed a significant positive correlation (P <0.05) between cell size (area and perimeter) and distance from the root surface. This parameter was mirrored by a significant negative correlation (P <0.05) between reactivity to toluidine blue and root surface. This is due to the inner mantle hyphae being smaller and more intensively stained than the outer hyphae (Fig. 7), confirming the morphological results.

Plant growth

Plant growth parameters after 4 months are given in Table 2. While the weight of leaves did not vary significantly between inoculated and control plants, a positive mycorrhizal effect was evident when all the other parameters were considered. Moreover, 43BO was more consistently associated with the largest effect in terms of development of root system and wet stem weight than 1BO. Exceptions were found in height, dry stem weight and node number, whose values were significantly higher than those of non inoculated plants, but not 1BO mycorrhizal plants.

Table 2 Growth parameters measured in non-inoculated and in 1BO- and 43BO-inoculated seedlings of linden. Values are means ±standard deviations for 20 seedlings in each treatment. Values with a different letter in the same row are significantly different according to the Tukey-Kramer test ($P < 0.05$)

Growth parameter	Non inoculated	1 BO	43 BO
Leaves			
Wet weight (mg)	199.3±41.2	255.9±90.7	249.3±82.1
Dry weight (mg)	46.9±15.7	51.6±12.4	52.4±12.5
Stems			
Wet weight (mg)	161.5±38.6a	202.9±50.8b	260.8±63.7c
Dry weight (mg)	49.7±14.7a	56.8±11.1ab	63.9±18.1b
Root system			
Wet weight (mg)	138.0±68.7a	279.1±53.1b	364.9±102.4c
Dry weight (mg)	20.7±10.6a	39.4±13.3b	54.9±14.6c
Height (mm)	47.9±7.7a	59.5±9.4b	66.5±9.5b
Node number	7.6±2.5a	11.8±3.1b	12.3±2.9b

Discussion

A combination of morphological and morphometric analyses of the fungal mantle of linden mycorrhizal roots, together with the assessment of plant growth parameters, demonstrate that two genetically related truffle strains (1BO and 43BO) lead to mycorrhizas with distinct morphological features and different effects on vegetative plant development, given the single set of climatic and environmental conditions in the in vitro experiments performed. It is well known that fungal isolates of the same species can have different symbiotic capacities, depending on environmental factors like nutrients, temperature, and water (Cairney 1999).

A number of approaches have been used to relate fungal behavior to effects on the host plant, e.g. counting of mycorrhizal tips (Boukcim and Mousain 2001), one of the most commonly used, or the assessment of fungal aggressiveness, defined as the capacity of a symbiotic fungus to produce a defense reaction in its host. For example host chitinase activity changed following inoculation with different *Pisolithus* strains (Albrecht et al. 1994), or was located in the epidermal root cells following contact with a *Suillus collinitus* strain, which showed a low symbiotic potential (Bonfante et al.1998). Similar results have been obtained with the truffle strains investigated here. Giomaro et al. (2000) analyzed the symbiotic behavior of five *Tuber borchii* strains and concluded that strain 43BO led to higher number of mycorrhizal tips than 1BO.

The morphometric analysis in the present work has allowed us to identify parameters of the positive response stimulation of linden to 43BO that correlate well with the symbiotic capacities of this fungal strain. Transverse sections of 43BO mycorrhiza show narrow hyphae and a mantle that can be described as having high tissue density, according to the terminology of Whal and Ryser (2000). In addition to the reduced diameter, these hyphae are strongly reactive to staining, regardless of the technique used, are poorly vacuolated and have a thin wall. All these features are typical of metabolically active hyphae (Gow and Gadd 1995). In contrast, the larger 1BO hyphae are less reactive, due to the higher vacuole/cytoplasm ratio. Since the mycorrhizas were sampled at the same time, the observed morphological features suggest that 1BO hyphae have a different differentiation process.

Observations of the two strains (1BO and 43BO) during their growth on culture plates suggest that they are cytologically different. Since they developed in the presence of micropropagated plants under highly controlled conditions (Sisti et al. 1998), we raise the hypothesis that differences in mantle anatomy and in hyphal cytology are isolate specific, mirroring the isolate genotype. Rossi et al. (1999) demonstrated very low genetic variability in the two isolates used here, but the few polymorphisms found were sufficient to elaborate strain-specific markers. 43BO and 1BO strains can grow in liquid medium at a low oxygen tension, while in vitro mycorrhizas with *Tilia* have never been obtained in such conditions (D. Sisti and G. Giomaro, unpublished results), suggesting that active aerobic respiration is necessary in the fungi to provide energy for symbiosis establishment. On the basis of our observations, we suggest that the first contact with the plant surface (or with the root hairs, as the EM pictures show) activates the respiratory activity of the fungus as it makes the transition towards the symbiotic phase. A low-molecular-weight molecule isolated from host plant exudates has been demonstrated to activate branching in arbuscular mycorrhizal fungi (Buee et al. 2000). The same molecule activates genes involved in the respiratory chain and increases fungal respiratory activity (Becard et al., unpublished results). We hypothesize that similar mechanisms are active in ectomycorrhizal fungi, where respiration provides energy to sustain morphogenetic events (i.e. mantle, Hartig net development).

In conclusion, in the experimental conditions tested, a relationship was found between some morphological traits of two *Tuber borchii* isolates and differences in their effects on host plant growth. Some of the morphological traits observed may be a consequence of different molecular/biochemical responses following inoculation onto the roots of the host plant. It will be interesting to test whether the morphological traits and symbiotic efficiencies of the two truffle strains are stable in natural conditions and on a range of plant hosts.

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