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ORIGINAL PAPER

Self-anastomosing ability and vegetative incompatibility of *Tuber borchii* isolates

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Abstract In this work, different mycelial isolates of the ectomycorrhizal fungus *Tuber borchii* were analysed for their ability to form self-anastomoses, which were monitored by time-lapse live-cell imaging, providing a description of the anastomosis process. Self-fusions were evidenced in three out of five isolates, with frequencies ranging between 50 and 88% of hyphal contacts. Time-lapse video microscopy evidenced that during pre-contact events, hyphae were capable of growth re-orientation functional to hyphal contact: the time elapsed between hyphal growth re-direction and complete fusion ranged from 115 to 200 min. After anastomosis, protoplasmic flow occurred between fused hyphae and nuclei could be detected in fusion bridges. Vegetative incompatibility was also assessed by analysing macroscopic and microscopic hyphal interactions between paired *T. borchii* mycelia. Both plate-pairing tests and microscopic analyses showed vegetative compatibility only within the same isolate, whereas different degrees of incompatible responses were observed in inter-isolate pairings. The diversity of *T. borchii* strains revealed by cytomorphological approaches is consistent with their genetic diversity obtained by molecular methods.

Keywords Self-anastomosing ability · Vegetative incompatibility · Live-cell imaging of anastomosis · *Tuber borchii* · Mycorrhizal fungi

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Introduction

In filamentous fungi, hyphal fusion (anastomosis) is a widespread phenomenon, and it may be considered one of the main morphogenetic events of fungal life cycle. By means of anastomoses, fungal hyphae are repaired, and mycelial colonies become interconnected, forming hyphal networks, fundamental for communication and nutrients translocation within fungal individuals (Gregory 1984; Rayner 1996). It is interesting to note that the ability to form hyphal fusions has been reported to affect fungal fitness: for example, *Rhizoctonia solani* non-self-anastomosing isolates (NSAIs) showed lower survival when compared to co-specific self-anastomosing isolates (SAIs; Hyakumachi and Ui 1987; Hyakumachi et al. 1987).

The cellular events leading to hyphal recognition and anastomosis formation have been described by Buller (1933) and furtherly studied in detail by pairing isolates belonging to the same or to different species (Ainsworth and Rayner 1986, 1989; Worrall 1997; Saupé 2000; Glass and Kaneko 2003). Fungal hyphae, which become involved in anastomosis, often show a mutual attraction (autotropism)—also termed 'homing'—whereas negative autotropism commonly occurs among adjacent hyphal branches at the growing edge of a fungal colony (Trinci 1984).

In mycorrhizal fungal species, hyphal anastomoses represent a fundamental cytological event in the formation of interconnected extraradical networks, which spread from colonized roots, uptake soil mineral nutrients and translocate them to the host plants (Brownlee et al. 1991; Giovannetti

et al. 2001; He et al. 2004). Moreover, anastomosis formation is one of the mechanisms allowing the establishment of belowground plant interconnections (Brownlee et al. 1991; Arnebrant et al. 1993; Martins 1993; Amaranthus and Perry 1994; Simard et al. 1997; Giovannetti et al. 2004, 2006; He et al. 2004).

Moreover, the phenomenon of hyphal fusion has been exploited to assess mycelial compatibility and genetic diversity among isolates of pathogenic, saprophytic and mycorrhizal fungi (Ainsworth and Rayner 1986, 1989; Worrall 1997; Saupé 2000). Such cytomorphological experimental method proved to be very sensitive and revealed the existence of different vegetative compatibility (or anastomosis) groups of co-specific fungal isolates (VCGs), consistent with their genetic diversity (Sen 1990; Chillali et al. 1998; Stenlid and Vasiliauskas 1998; Mes et al. 1999).

Studies on the genetic variations of different species and isolates belonging to the ectomycorrhizal symbiotic genus *Tuber* allowed the development of species-specific typing methods and the detection of geographic diversification among species (Lanfranco et al. 1993; Amicucci et al. 1998; Gandeboeuf et al. 1997a, b; Mabru et al. 2001; Mello et al. 2001, 2002; Rubini et al. 2005). Furthermore, the improvement of techniques for obtaining mycelial pure cultures from ascocarps collected in the field revealed differences among strains in growth patterns, carbohydrates assimilation, proteins and messenger ribonucleic acid synthesis (Saltarelli et al. 1999; Vallorani et al. 2000), as well as in genetic features (Paolocci et al. 1997; Henrion et al. 1994; Rossi et al. 1999; Murat et al. 2004; Mello et al. 2005; Rubini et al. 2005).

In this work, we analyse different *T. borchii* mycelial isolates for self-anastomosing ability, monitored hyphal fusions by time-lapse live-cell imaging and provide a description of the anastomosis process. Moreover, the occurrence of vegetative compatibility among paired *T. borchii* mycelial strains is assessed.

Materials and methods

Fungal material Mycelia of *T. borchii* strain 1BO, deposited as ATCC 96540 (kindly provided by Dr. A. Zambonelli, University of Bologna, Italy), and strains 10RA, 17BO, Z43 and B2 (kindly provided by Dr. B. Citterio, University of Urbino, Italy) were maintained on potato dextrose agar (PDA, Difco, MI).

Microscopic assessment of hyphal interactions Mycelial discs (3-mm diameter) were cut from the edge of actively growing *T. borchii* mycelium of all isolates and placed upside down on two opposite sides of 20×20-mm cellophane membranes (GE Healthcare Europe GmbH, Milan, Italy) placed in 55-mm-diameter Petri dishes on PDA (Difco), Hagem–Modess (HM) agar or Modified Melin–Norkrans (MMN) agar, for optimal imaging of hyphae. Five replicate membranes of all parings were prepared. After 10–18 days of incubation at 25°C in the dark, membranes were stained for histochemical localization of succinate dehydrogenase (SDH) activity (Smith and Gianinazzi-Pearson 1990) and transferred to microscope slides to be observed under a Reichert-Jung Polyvar (Reichert, Wien, A)-transmitted light microscope. Hyphal fusions showing protoplasm continuity among fused hyphae were classified as perfect, as the lack of such continuity is the result of incompatible interactions. Frequency of anastomoses was calculated by determining the proportion of hyphal contacts, which led to hyphal fusions, and 95% confidence intervals of the obtained values are given. To detect the occurrence of nuclei, some membranes were stained with either 0.01% (w/v) solution of diamidinophenylindole (DAPI, Sigma, Milan, Italy) or SYTO 13 (prepared following supplier's information, Molecular Probes, Oregon) and observed under the Reichert-Jung Polyvar microscope using epifluorescence, with filter combinations U1 (BP 330–380, LP 418, DS 420) and B1 (BP 450–495, LP 520, DS 510), respectively. Some membranes were also stained with 0.01% (w/v) solution of Calcofluor White (Sigma-Aldrich Srl, Milan, Italy) to visualize hyphal interactions (Sbrana et al. 1995), and observed under epifluorescence with the filter combination U1.

Time-lapse live-cell imaging of fusion events in T. borchii Continuous observations of living *T. borchii* hyphae were carried out by using Petrislide plates (Millipore, Milan, Italy) as imaging chambers. A thin layer (2 mm) of PDA or HM agar was poured on the lid and inoculated in the middle with hyphae from *T. borchii* isolate 10RA colonies. Petrislides were incubated in the dark at 25°C and observed daily under the Polyvar microscope equipped with a 3CCD UP-890 CE video camera (Sony Italia SpA, Milan, Italy). Time-lapse imaging was performed at scan intervals of 1–60 min, for periods up to 15 s. Images and movies were captured and edited using Studio version 8 software, which also allowed the conversion into animated movies in avi. format. At least 20 anastomosing hyphae were monitored, and pre- and post-fusion behaviour and elapsed times were recorded.

Mycelial compatibility assays Isolates were paired in all possible combinations in Petri plates on PDA, HM and MMN agar media, by placing two mycelial plugs (5-mm diameter) on agar, about 3 cm apart. At least three plates were prepared for each pairing and each growth medium, and after 20 days incubation at 25°C in the dark, the pairings were analysed for macroscopic mycelial reactions.

These reactions were assigned to four categories, according to MacNish et al. (1997; see "Results"). Major and minor semi-axes of each fungal colony were measured under a dissecting microscope (Leica, Milan, Italy), by using a transparent grid with lines 1-mm apart, to calculate the areas.

Results

Microscopic assessment of hyphal interactions The use of cellophane membranes allowed us to obtain a loose growth of *T. borchii* mycelium and to detect hyphal fusions, after SDH localization. In intra-isolate pairings, contacting hyphae of strains 10RA, 17BO and Z43 were able to establish self-fusions, whereas hyphae of 1BO and B2 never formed any anastomoses (Table 1). Hyphal anastomoses were often observed in multiple fusion events, which were characterised by the formation of short fusion bridges between parallel, large trunk hyphae (Fig. 1a–d). Self-anastomosis frequencies of 10RA and 17BO ranged between 66.7 and 88.0% of hyphal contacts: Values obtained were 73.6% (95% confidence interval 65.5–80.6%) in 10RA and 76.8% (95% confidence interval 68.9–83.6%) in 17BO, both with average number of fusions of 60±2 per mm of hypha.

Anastomosis frequency recorded in Z43 was 55.7% (95% confidence interval 64.1–68.9%): Both this value and the number of fusions per hyphal length—40±2 fusions/mm hypha—were significantly lower that those recorded in the other isolates ($P=0.004$ and $P<0.01$, respectively).

All hyphal anastomoses observed in the SAIs 10RA, 17BO and Z43 could be classified as 'perfect,' according to the relevant literature, as they were characterised by complete fusion of hyphal walls and protoplasm continuity, detected by formazan salt depositions in hyphal bridges after staining for SDH activity (Fig. 1b–c). Fluorescence microscopy of DAPI- and SYTO 13-stained mycelium of isolate 10RA evidenced nuclei in hyphal bridges formed by anastomosing hyphae (Fig. 1e–f). Neither cellular vacuolization nor lysis, characteristic features of hyphal incompatibility reactions, were detected after self-fusions.

When different isolates were paired (1 BO–10 RA, 1 BO–17 BO, 1 BO–Z 43, 1 BO–B2, 10 RA–17 BO, 10 RA–Z 43, 10 RA–B2, 17 BO–Z 43, 17 BO–B2, Z 43–B2), no perfect anastomoses were detected. Microscopic observations showed that pairings between different SAIs (10 RA–17 BO, 10 RA–Z 43, 17 BO–Z 43) failed to establish hyphal connections, as most contacts showed mycelial inter-mingling without any hyphal interaction, and the low number of fusions recorded (4–8% of hyphal contacts) were always followed by incompatible reactions leading to death of fused cellular compartments (Fig. 1g–h, Table 1).

Hyphal contacts recorded in pairings between NSAIs or between SAI and NSAI showed mycelial inter-mingling without any evidence of hyphal recognition (Fig. 1i, Table 1).

Time-lapse live-cell imaging of fusion events in T. borchii The development of self-anastomoses in mycelial isolate 10 RA was monitored by time-lapse microscopy, recording the growth of several pairs of hyphal tips when their distance was shorter than 10 μm. Attraction (homing) of nearby hyphae or branches was detected at distances of 8–10 μm and consisted in growth re-orientation of hyphal tips, which formed angles of 25–35° with previous growth direction (Fig. 2a–b). In some cases, the receiving hypha developed a lateral tip in response to the approaching hypha nearby, before physical contact. Hyphal behaviour observed in all the monitored hyphal pairs appeared consistent, although analyses of time-lapse pictures showed the occurrence of different contact angles in tip-to-tip or tip-to-side hyphal anastomoses.

After contact (Fig. 2c), hyphal extension ceased, hyphal tips became slightly swollen and cell walls could not be resolved as being separate from each other: This phenomenon was paralleled by increased vacuolization of the contacted hypha (Fig. 2d).

The following step was represented by the lysis of both hyphal walls (Fig. 2e). Although fusion pores could be sometimes visualized, the only unequivocal criterion that could be used to reveal protoplasmic continuity between two fusing hyphae was represented by the cytoplasm and organelle flow through the hyphal bridge. Protoplasmic streaming and organelle flow was detectable through anastomosed hyphae (Fig. 3a–f). The time elapsed between hyphal homing and complete fusion, detected by protoplasmic flow, ranged from 115 to 200 min.

Fusing hyphal walls lysis was often paralleled by septum formation in the vacuolized trait of the contacted hypha, 8–10 μm from the fusion site (Fig. 2e–f).

Table 1 Mycelial interactions between *Tuber borchii* strains recorded on membranes (columns m=microscopic interactions) and on PDA, MMN and HM agar media (columns M=macroscopic interactions)

	1 BO		10 RA		17 BO		Z 43		B 2	
	m	M	m	M	m	M	m	M	m	M
1 BO	I	I	I	T	I	T	I	T	I	B
10 RA			PF	M	IF+I	T	IF+I	T	I	B
17 BO					PF	M	IF+I	T	I	B
Z 43							PF	M	I	B
B 2									I	I

PF Perfect fusion, *IF* incompatible fusion, *M* merging, *I* inter-mingling, *T* tuft, *B* barrage

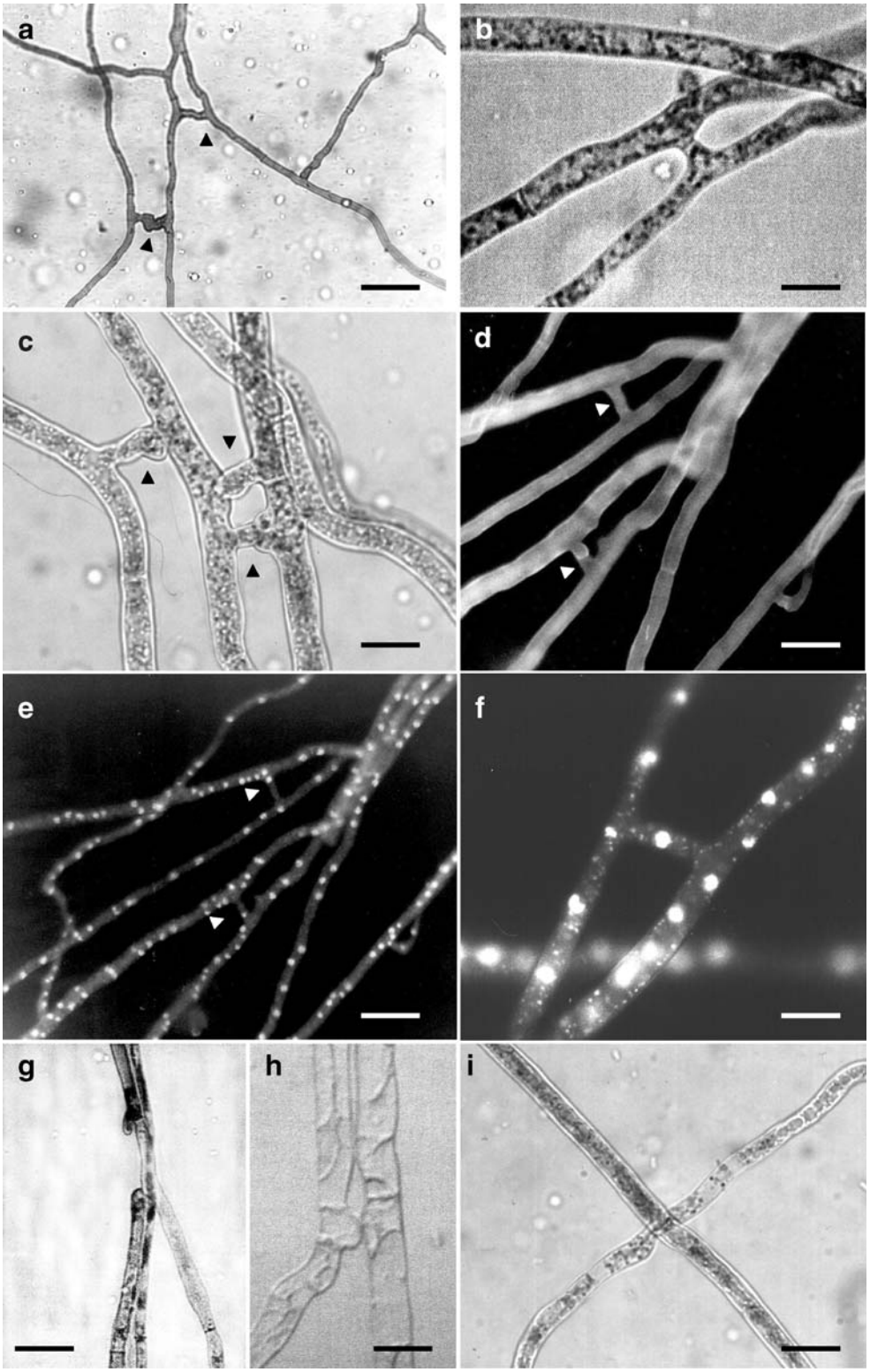

Fig. 1 Visualisation of interactions between hyphae originating either from the same (**a–d**) or from different *Tuber borchii* mycelial isolates (**e–f**). **a** Anastomosis bridges (*arrowheads*) connecting large trunk hyphae of the isolate 10 RA. *Scale bar*=20 μm. **b–c** Localization of succinate dehydrogenase activity in fused hyphae (*arrowheads*) belonging to the isolate Z 43. *Scale bars*=6 μm. **d** Calcofluor staining revealing anastomosed hyphae (*arrowheads*) in 10 RA mycelium. *Scale bar*=8 μm. **e–f** Occurrence of nuclei in the middle of fusion bridges (*arrowheads*) revealed after DAPI (**e**) and SYTO13 (**f**) staining, in 17 BO mycelium. *Scale bars*: **e**=20 μm, **f**=6 μm. **g–h** Incompatible reactions, evidenced by protoplasm-lacking cell compartments, occurring between contacting hyphae of mycelial isolates 10 RA and 17 BO. *Scale bars*=12 and 6 μm, respectively. **i** Intermingling of hyphae belonging to isolates 1 BO and 10 RA, showing no contact interference. *Scale bar*=8 μm

Mycelial compatibility assays The five *T. borchii* isolates paired on PDA, MMN and HM agar media showed different interaction behaviours, which were classified into four different classes: barrage (incompatible interaction characterised by mycelial absence in the interaction zone), tuft (incompatible interaction characterised by aerial hyphae formation in the interaction zone), merging (compatible interaction involving hyphal fusions) or inter-mingling of hyphae without reaction (MacNish et al. 1997). The analysis of the interaction areas revealed four kinds of

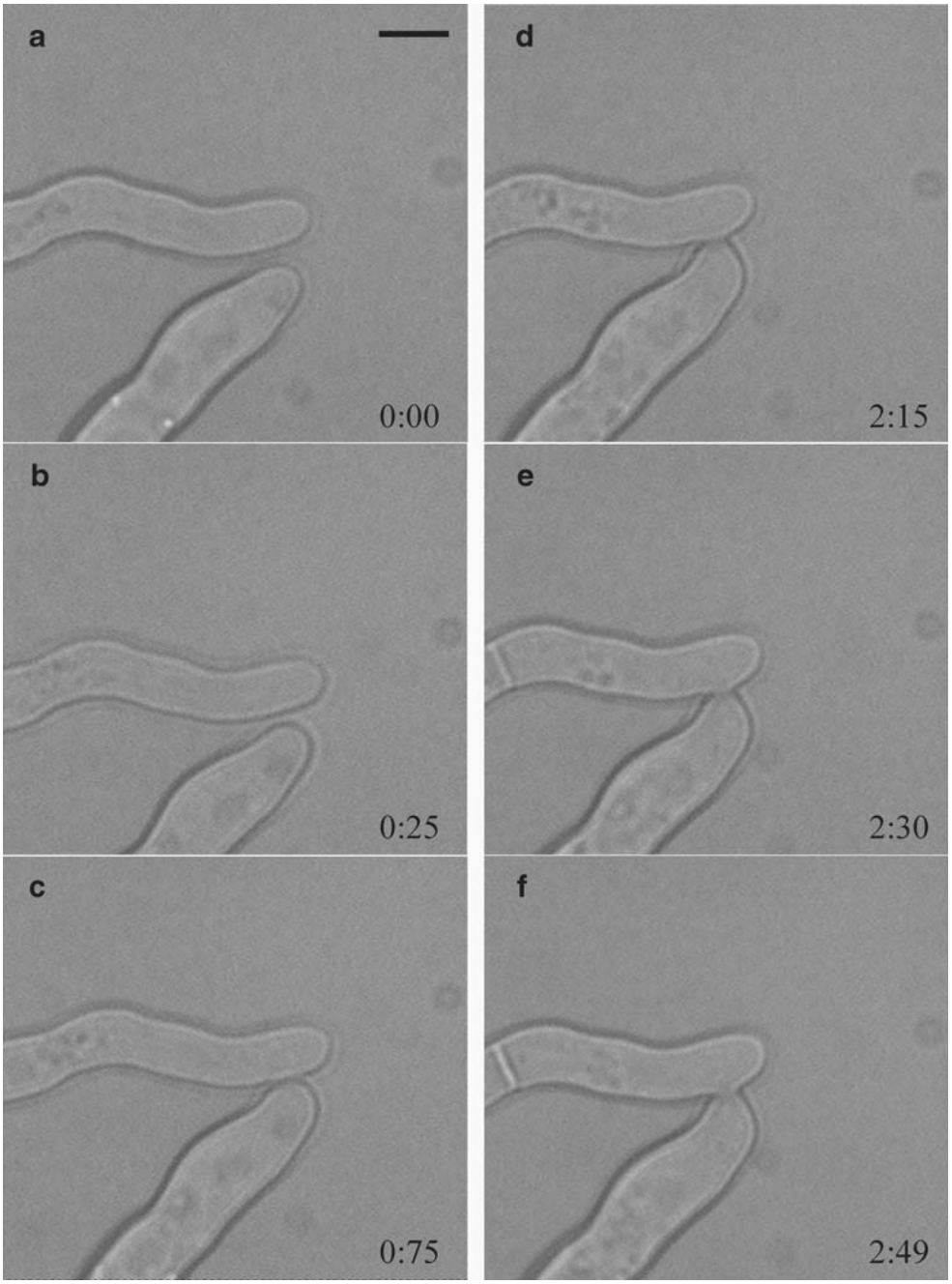

Fig. 2 a–f Time-lapse micrographs of a hyphal fusion in 10 RA living mycelium. *Scale bar*=3 μm in all pictures. After re-orientation, tip 1 extended towards hypha 2 for 7 μm and then changed its direction so that it moved through an angle of −30°, maintaining this pattern until contacting hypha 2, at a distance of about 3 μm from the tip and at an angle of about 60°. Time elapsed is indicated on each picture (a movie sequence from the experiment is available in the electronic supplementary material Movie 1)

hyphal behaviours: colony merging, hyphal inter-mingling, tuft and barrage formation (Figs. 4, 5 and Table 1). Merging, indicating compatible interactions with formation of hyphal anastomoses between mycelia originating from paired colonies, occurred only in self-pairings of 10 RA, 17 BO and Z 43 (Fig. 5a). Self-pairings of 1 BO and B2 isolates showed hyphal intermingling without any macroscopic incompatible response in the interaction zones.

Pairings between different isolates showed tuft responses because of the development of aerial mycelium (Fig. 5b–d) or barrage caused by hyphal growth inhibition with formation of a separation zone (Fig. 5e–f).

It is interesting to note that merging and inter-mingling responses recorded in plates were consistent with results obtained from microscopic assessment, whereas tuft and barrage formation, both suggesting the occurrence of incompatible interactions, were not consistent with microscopic analysis of hyphal interactions (Table 1).

Isolates 10 RA, 17 BO and Z 43 showed a higher competitive ability to colonize agar plates than isolates 1

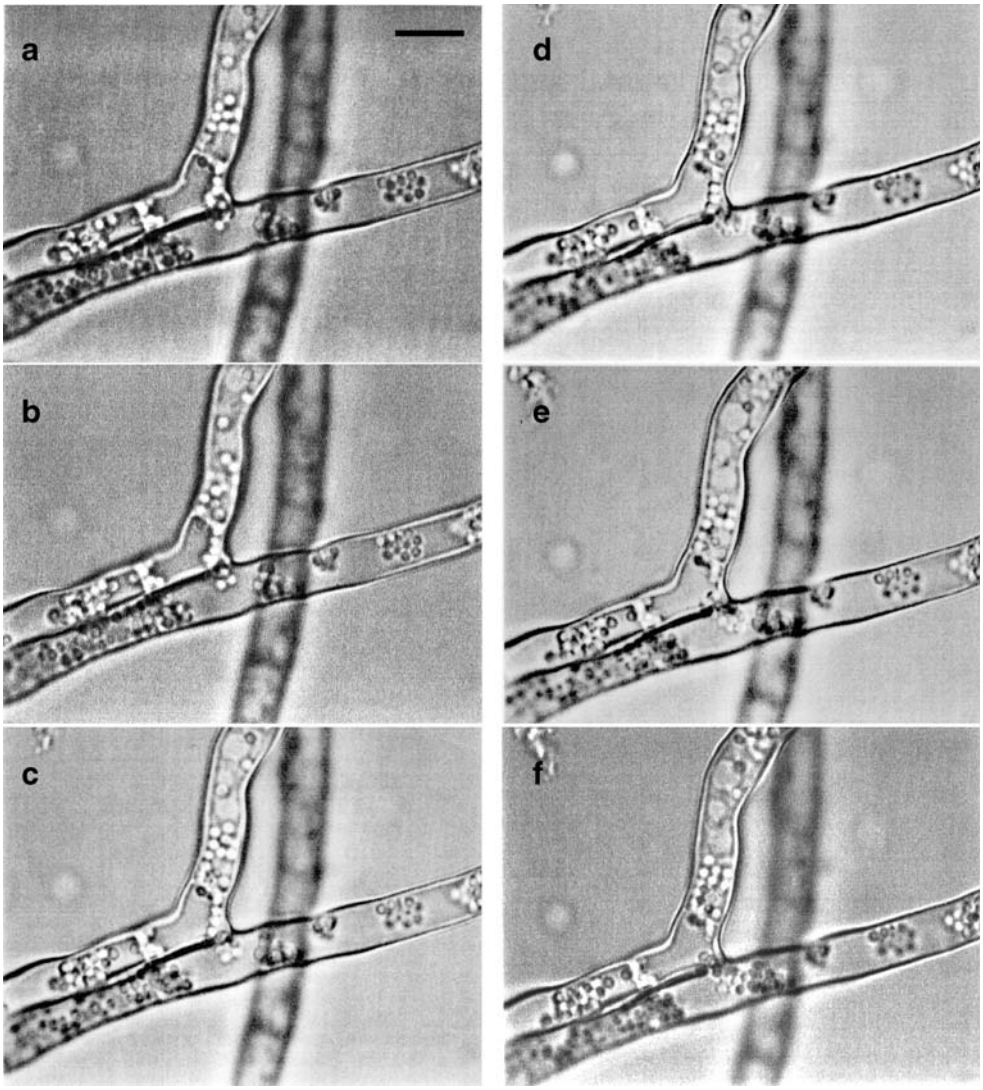

Fig. 3 a–f Time-lapse micrographs of protoplasmic flow through an hyphal fusion formed in 10 RA mycelium. Frames were recorded at 1-min intervals, and *scale bar*=3.8 μm in all pictures (a movie sequence from the experiment is available in the electronic supplementary material Movie 2)

BO and B2 (Fig. 4). The most competitive strain, 10 RA, spread on the agar up to 62% of the Petri dish surface in pairings with 1 BO and up to 60% in pairing with B2.

Discussion

In this work, we showed, by living cell imaging, the occurrence of self-fusions in the ectomycorrhizal fungus *T. borchii*. Moreover, our data show that: (1) SAIs and NSAIs occur within the ectomycorrhizal fungal species *T. borchii*, (2) SAIs are able to produce larger growth than NSAIs in plate pairing tests and (3) no vegetative compatibility occurs between different isolates, either belonging to SAIs or to NSAIs.

In this study, three out of five different isolates of the ectomycorrhizal fungus *T. borchii*—isolates 10RA, 17BO and Z43—were able to develop self-anastomoses, which occurred with frequencies ranging between 68 and 75%, whereas 1BO and B2 did not form any anastomosis on three different growth media. It is interesting to note that other authors, utilizing a woody plant medium, observed some fusions in *T. borchii* isolate 1 BO (Iotti et al. 2002). Because such isolate showed also a low mycorrhizal ability (our unpublished results), the physiological changes involved in its prolonged axenic culture may be at the basis of its differential behaviour. The detection of SAIs and NSAIs within *T. borchii* suggests that different hyphal behaviour may be observed within the same species, as observed for other fungi where genetically distinct mycelia (genets) co-exist and may be considered the population units (Worrall 1997).

Vegetative hyphal fusion in filamentous fungi is a multistep process preceded by signalling, recognition and attraction between approaching hyphae, which are guided to contact by polarized growth (Hickey et al. 2002). Live-cell imaging of growing hyphae of the SAI 10 RA allowed us to monitor the events leading to anastomosis formation:

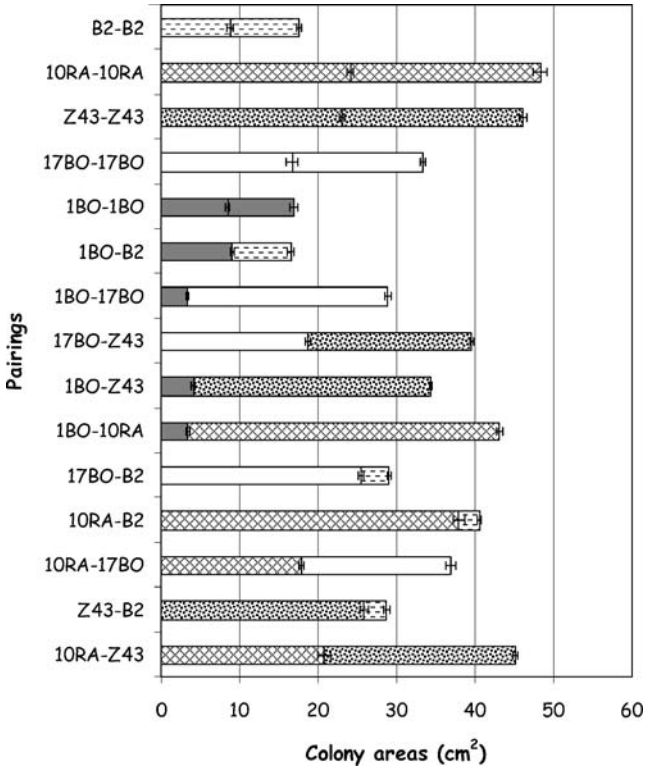

Fig. 4 Mycelial spread of different *Tuber borchii* isolates in self- and non-self-pairings on PDA plates. *Stacked bars* represent average colony areas of each paired mycelium (indicated by different *bar fill patterns*), with standard errors of the means

hyphal homing and contact, cell wall lysis and protoplasmic mingling. According to current terminology, all the fusion processes observed in our work belonged to tip-to-side and to tip-to-tip morphological types (Hickey et al. 2002). The cascade of events detected in *T. borchii* is similar to that described in *Phanerochaete velutina*, *Stereum hirsutum*, *Cryphonectria parasitica* and *Neurospora crassa* (Ainsworth and Rayner 1986, 1989; Newhouse and MacDonald 1991; Hickey et al. 2002). Although, the time elapsed between hyphal homing and complete fusion, detected by protoplasmic flow, ranged from 115 to 200 min, which is higher than that of *Neurospora* (Hickey et al. 2002).

In many studies on saprophytic, pathogenic and symbiotic fungi, macroscopic reactions, detected in plate, have revealed mycelial compatibility/incompatibility among strains, allowing the detection of intra-specific VCGs (Glass and Kuldau 1992; Leslie 1993; Cortesi et al. 1996; Worrall 1997). Some works assessed also microscopic hyphal interactions to verify the occurrence of non-self-fusions (MacNish et al. 1997). In this work, macroscopic reactions (tuft, barrage) in plate pairings showed a high dependence on the growth medium used, confirming previous findings (MacNish et al. 1997).

The discrimination of co-specific isolates on the basis of their macroscopic and microscopic vegetative reactions—i.e. compatibility (hyphal fusions between colonies) or incompatibility (lack of fusions, hyphal rejection and/or cell death)—in our *T. borchii* isolates showed compatible responses only in intra-isolate pairings, whereas all inter-isolate pairings showed vegetative incompatibility, irrespective of their self-anastomosing ability. Microscopic analyses showed the occurrence of incompatible fusions in 4–8% of hyphal contacts between different SAIs. Such interactions were characterised by cell death and compartment isolation, as described in pairings between isolates belonging to different VCGs (Ainsworth and Rayner 1986; Jacobson et al. 1998; Newhouse and MacDonald 1991).

Our results on *T. borchii* strain diversity, obtained by cytomorphological approaches, are in agreement with previous data on genetic and biochemical diversity occurring among *T. borchii* mycelial isolates (Lanfranco et al. 1993; Henrion et al. 1994; Potenza et al. 1994; Guillemaud et al. 1996; Bertini et al. 1998; Saltarelli et al. 1999; Rossi et al.

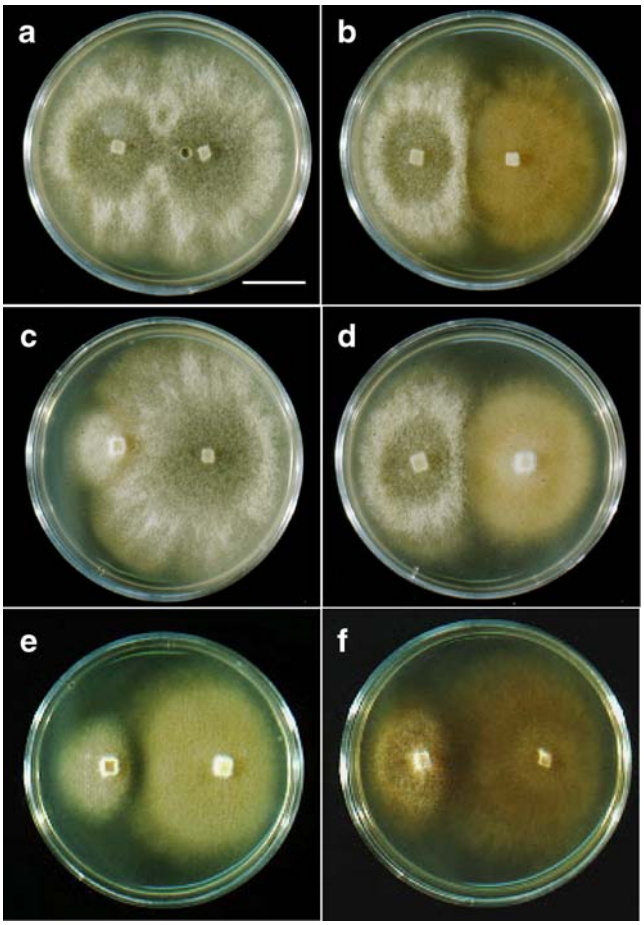

Fig. 5 Pairings of *Tuber borchii* isolates showing different mycelial behaviours in the interaction region after 3 weeks of growth on potato dextrose agar. *Scale bar*=2 cm. **a** Self-pairing of isolate 10 RA showing colony merging. **b–d** Pairings between isolates 10 RA/Z 43, 1 BO/10 RA and 10 RA/17 BO, respectively, showing aerial tuft development. **e–f** Pairings between isolates B 2/17 BO and B 2/Z 43, respectively, showing barrage development

1999), confirming the reliability of vegetative compatibility tests reported by other authors (Sen 1990; Keijer et al. 1996; Mes et al. 1999; Sen et al. 1999).

It is interesting to note that recent data obtained on *Tuber magnatum* suggest that vegetative mycelial isolates originating from ascocarps represent maternal homokaryotic hyphae, which may need a vegetative incompatibility system to maintain their individual characters (Paolocci et al. 2006). Polymorphism in vegetative incompatibility phenotypes is relatively widespread, although both the significance and the factors maintaining incompatibility remain largely unknown (Cortesi et al. 1996).

Our data suggest that self-/non-self-hyphal compatibility may play an important role in truffle life cycle, allowing either hyphal interconnection and protoplasm mingling within the same genet or the maintenance of individual genetic identity.

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