Is *Tuber brumale* a threat to *T. melanosporum* and *T. aestivum* plantations?

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True truffles in the genus *Tuber* are the most valuable ectomycorrhizal fungi and their cultivation has become widespread around the world. Competition with other ectomycorrhizal fungi and especially with undesired *Tuber* species, like *T. brumale*, can threaten the success of a truffle plantation. In this work, the competitiveness of *T. brumale* towards *T. melanosporum* and *T. aestivum* was assessed in a 14 year-old plantation carried out planting seedlings inoculated with these three truffle species in adjacent plots. Analyses of both truffle ectomycorrhizas and extra-radical mycelium were carried out in the transects separating the *T. brumale* plot from *T. melanosporum* and *T. aestivum* plots. The results confirm the competitiveness of *T. brumale* against *T. aestivum* and *T. melanosporum* due to its major ability to colonize the soil around its ectomycorrhizas. However, its competitiveness is limited to the transect areas and it was never found inside *T. melanosporum* plot. These results remark that, in presence of optimal conditions for *T. melanosporum* and *T. aestivum*, the greatest risk of contamination with *T. brumale* is due to wrong greenhouse activity.

**Keywords:** Competition, Black Truffles, Extra-Radical Mycelium, Ectomycorrhizas, Species-Specific Primers

**Introduction**

Ectomycorrhizas (ECMs) are symbiotic associations between fine roots of woody plants and soil fungi. They represent the most frequent root symbioses in boreal, temperate and subtropical forests and woodlands (Smith & Read 2008). Different species of ectomycorrhizal fungi may live together and share the same environment, establishing competition for nutrients/water in the soil and carbon on the host roots (Kennedy 2010). Competition among these fungi can be highlighted through the analysis of their communities (Koide et al. 2005, Peay et al. 2007). Although the first studies on below-ground ectomycorrhizal fungal communities date back to the mid-1990s (Dahlgberg 2001), the mechanisms determining competitive outcomes are not yet entirely understood. The full understanding of these mechanisms is crucial because they affect the survival and spreading of ectomycorrhizal fungi, and different plant-fungus pairings can result in notable modifications in performance for both symbionts (Bever 2002, Nara 2006). Competition between ectomycorrhizal fungi becomes of practical relevance when a commercially valuable fungus is introduced in the field through inoculated seedlings obtained in greenhouse. ECMs of the introduced fungal species can be replaced by other native ectomycorrhizal fungi on the host roots (Hall et al. 2007) and threaten the success of the plantation.

True truffles in the genus *Tuber* are the most valuable ectomycorrhizal fungi and their cultivation has become widespread around the world (Zambonelli et al. 2017). The most cultivated Tuber species are the black truffles *Tuber melanosporum* Vittad., *T. aestivum* Vittad. and, to a lesser extent, *T. brumale* Vittad. These species often share the same natural sites and compete for space on the host roots (Hall et al. 2007, Chevalier & Sourzat 2012). Due to the lower value of the ascomata and the high competitiveness, *T. brumale* has often been considered as a contaminant, able to replace the ECMs of *T. melanosporum* in greenhouse or in truffle plantations (Meény et al. 2016). Most of the studies on ectomycorrhizal communities of *T. melanosporum* plantations carried out in Italy, France and Spain report the presence of *T. brumale* (De Miguel et al. 2014). It was also found able to compete with, and in some cases to replace, *T. melanosporum* in New Zealand and Australia where true truffles are not endemic and *T. brumale* was probably introduced with the inoculum (Guerin-Lagouette et al. 2013, Linde & Selmes 2012). ECMs of *T. brumale* were also identified in *T. aestivum* plantations (Zambonelli et al. 2005, Benucci et al. 2011) but competition between these two black truffles has been poorly investigated. Rather, *Tuber aestivum* was found replacing *T. melanosporum* in several black truffle plantations around the world (Bencivenga et al. 1992, Granetti & Angelini 1992, Turgeman et al. 2012, De Miguel et al. 2014).

Most of the studies focusing on the competition between black truffles only considered the distribution of their ECMs, while the extra-radical mycelium (ERM) has been little considered, as it is a relatively new target of investigation. Recent studies have shown that the use of species-specific primers on DNA extracted from soil is a sensible and reliable method to identify (Zampieri et al. 2010) or quantify (Iotti et al. 2007) the extra-radical mycelia of black truffles.
Materials and methods

The study site covers an area of 1540 m², at an altitude of about 80 m a.s.l., in the municipality of Montelabbate (Pesaro-Urbino, Italy – 43.845488 N; 12.78349 E). The truffle orchard under investigation was established in 2002, using plants mycorrhized by spores. The field had been used for arable crops for at least 30 years before planting. The sandy-clay-loam soil (sand 49%, clay 28%, silt 23%) is calcareous (total carbonate 22%) and has a pH of 7.75. The soil organic matter is 1.2%. The climate of this area is characterized by a short summer drought period (Fig. 1); March and November are the wettest months while July (24.1 °C) and January (4.7 °C) are the hottest and coldest months, respectively. The region is suited to T. melanosporum, T. brumale and T. aestivum, which can also naturally occur in the same forest stands.

A total of 130 seedlings (120 Quercus pubescens Willd. and 10 Corylus avellana L.) mycorrhized with T. melanosporum, T. brumale or T. aestivum were planted 4 × 4 m apart as shown in Fig. 2. The seedlings were certified by the regional authority AS-SAM (Agenzia Servizi Settore Agroalimentare Marche, Osimo AN, Italy) which ensured a minimum mycorrhization of 30% with the target truffle species. At the time of the study, the plantation was grass-covered and the planted trees were 3 to 4 m high with a canopy cover of approximately 50% (Fig. S1 in Supplementary material). Most of the plants mycorrhized with T. melanosporum (79%) and T. aestivum (64%) showed the characteristic brûlé (a vegetation-devoid area around the host plant), while the T. brumale plants did not show it. The plantation was surrounded by mature tree species, some of them ectomycorrhizal such as Q. pubescens, Populus alba L. and Pinus pinea L. The only cultural practices carried out on the truffle orchard after planting were pruning, grass-mowing and irrigation. No tillage was performed. Irrigation was provided during summer by a drip system for the first 3 years after planting and, subsequently, by a sprinkler system every two weeks.
At the time of the study, only one ascoma of T. melanosporum was collected (February 2016) under a plant mycorrhized with the same truffle species (Fig. 2).

**Soil sampling**

The soil sampling was carried out in late spring 2016 within two transects between the plots of plants mycorrhized with different Tuber species (Fig. 2): T. aestivum – T. brumale (I: aest/brum) and T. melanosporum (II: brum/mela). The plots on the margins of the two transects (23 plants in total because one plant died) were selected for soil sampling. Only 3 out of 23 plants on the margin of the two transects showed a brûlé (two in the aest/brum transect and one in the brum/mela transect). Five sample types (A to E) were collected along each tree row as showed in Fig. 2. Samples were 1 m (B and D) and 2 m (C) far from the trunks into the transect areas, while samples A and E were 1 m far from the trunks into the respective truffle plots. Samples B to D were used for both ECM and ERM analyses while samples A and E were used only for ERM analysis. The 58 soil cores for ERM analysis (5 cores × 6 rows × 2 transects, excluding 2 cores not collected close to the dead plant) were extracted through disposable PVC tubes (30 cm depth and 20 mm in diameter, ~0.1 dm³) to avoid cross contamination. The 35 soil cores for ECM analysis (3 cores × 6 rows × 2 transects, excluding 1 core not collected close to the dead plant) were extracted through a steel soil corer (15 cm × 15 cm × 20 cm, ~0.3 dm³). Five and ten soil samples were also collected within T. melanosporum and T. brumale plots, respectively, to assess the presence/absence of truffle ECMs and ERMs. These samples were taken as described above, following the scheme reported in Fig. 2.

**Analysis of truffle ectomycorrhizas**

The roots were removed from the soil cores by a 2 mm mesh sieve, washed in sterile water and then examined under a stereomicroscope (~20). The ECMs were assigned to T. aestivum, T. brumale, T. melanosporum or other fungal species on the basis of their morphology (Agerer 1995). When morphotyping was not able to distinguish T. brumale and T. melanosporum ECMs (e.g., lacking in cystidia), molecular identification was carried out by applying a direct PCR approach (Iotti & Zambonelli 2006) using the species-specific primers designed by Rubini et al. (1998).

The degree of infection was measured by counting the number of living ECMs of each morphotype in all the root samples and expressing the results as a percentage on the total number of ECMs examined.

**Analysis of truffle extra-radical mycelia**

Soil cores collected for ERM analysis were extracted from the PVC tubes and transferred to 15 ml tubes, taking care to avoid cross contamination. Any root fragments were removed under a stereomicroscope. The soil was stored at -80 °C and lyophilized for three days using a Virtis Benchtop 2K™ freeze dryer (SP Industries, Warminster, PA, USA). After drying, soils were ground in a mortar and stored at -20 °C pending further DNA analyses.

The DNA was isolated from the soil samples using the protocol developed by Iotti et al. (2012), adapted for 0.5 g of soil. Yield and quality of isolated soil DNAs were evaluated by a Nanodrop® ND-1000 (Thermo Fisher Scientific, Waltham, MS, USA).

The presence/absence of the ERM of the target truffle species was verified by using species-specific primers. The primer pair Uncl-Uncl (Mello et al. 2002) was used to detect T. aestivum while the presence of T. brumale and T. melanosporum was verified by a multiplex PCR approach using ITSB and ITSML as forward primers and ITS4LNG as the sole reverse primer (Rubini et al. 1998). PCRs targeting T. brumale and T. melanosporum ERMs were repeated separately with the primer pair combinations ITSB-ITS4LNG and ITSML-ITS4LNG, respectively, to allow the amplification of the low amount of target DNAs that remained undetected by multiplex PCRs.

PCRs were conducted using a T-gradient Thermal Cycler (Biometra, Göttingen, Germany) in a 30 µL mixture volume containing 300 nM each primer, 50 ng DNA, 1 U Ex-Taq® DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 1× Buffer solution, 200 µM dNTP, 4 mM MgCl₂, 10 µg of Bovine Serum Albumine. The cycling parameters were as follows: 3 min of initial denaturation step at 95 °C, followed by 23 cycles of 30 s at 94 °C, 30 s at 63 °C, 45 s at 72 °C and a final extension step at 72 °C for 7 min.

**Results**

**Transsect I - aest/brum**

A total of 515 ECMs were counted in the 18 soil samples collected in the aest/brum transect. More than half the ECMs (60%) belonged to the target Tuber species (52% T. brumale and 28% T. aestivum) whereas other fungi formed only 40% of ECMs (Tab. S1 in Supplementary material). ECMs of T. aestivum and T. brumale were never found mixed in the same soil core (Fig. 3a). Tuber brumale ECMs were abundant in samples D (~70%) although they were also found in samples B, collected 1 m far from the T. aestivum plants (Fig. 3a, S1). ECMs of T. aestivum were found in only 3 out of 6 plant rows, where it successfully expanded its colonization only as far as the samples collected along the transect midline (samples C, Fig. S2). ECMs of T. melanosporum were never detected in this transect. ECMs formed by other fungi were absent in 5 out of 18 samples where T. aestivum (4 samples) and T. brumale (1 sample) dominated. ERM of the target truffle species was detected in 24 out of the 30 analysed soil samples (Fig. 3b). Tuber brumale and T. aestivum were exclusively found in 15 and 6 samples, respectively, and occurred together in only three samples collected along the transect midline (samples C). In contrast to T. brumale, T. aestivum ERM was never detected in soil samples collected along the transect midline (samples C).

**Fig. 3** - Spatial distribution of truffle ERMs and ECMs in the transect I (aest/brum). (a) Percentages of ectomycorrhizal colonization of the inoculated truffle species and native ectomycorrhizal fungi in each sample, from B to C; (b) presence/absence of soil truffle ERMs in each sample, from A to E. (red): Tuber brumale; (green): Tuber aestivum; (gray): ECMs formed by other fungi; (white): no truffle mycelia; (black dots): host plants.
lected 1 m far from the T. brumale plants (types D and E) and it was completely absent in the whole sample sets (types A to E) of half plant rows. Soil samples collected within the two brûlés in this transect did not have ECMs or ERM of T. brumale.

**Transect II - brum/mela**

A total of 4310 ECMs were counted in 14 soil samples collected in the brum/mela transect. No roots were found in three soil samples, all collected close to the T. brumale dead plant in row 6. ECMs of the target Tuber species amounted to about 62%, whereas the remaining 38% was formed by other ectomycorrhizal fungi (Tab. S2 in Supplementary material). Tuber brumale dominated the community in this transect with 35% of ECMs, while T. melanosporum reached 27% (Fig. 4a). ECMs of both species showed the same frequency but they co-occurred in only three soil cores. Tuber melanosporum was mainly found in samples collected 1 m far from the trees inoculated with this species (type D), whereas T. brumale was mainly found in samples B (1 m far from its plants) and C (transect midline – Fig. S2 in Supplementary material). ECMs of T. aestivum (26% in total) were also found in two soil cores collected close to a T. brumale inoculated seedling in row 3. One of these samples (type C) showed ECMs of both T. aestivum and T. brumale.

ERM of the target truffle species was detected in all the 28 analysed soil samples (Fig. 4b). Tuber brumale DNA was amplified in ~90% of samples including those collected 1 m far from the T. melanosporum plants (types D and E). However, five of these samples had a little amount of T. brumale mycelium with respect to that of T. melanosporum since its DNA was not amplified by multiplex PCR. T. melanosporum ERM was found in 13 soil cores, ~61% of which occurred together with T. brumale. The presence of T. aestivum DNA was detected in three soil cores, in row 3, where its ECMs were also found. Soil samples collected within the brûlé in this transect did not have ECMs or ERM of T. brumale.

No truffle ECMs and ERMs different from the inoculated species were detected in the soil samples collected within T. melanosporum and T. brumale plots.

**Discussion**

*Tuber brumale* has often been considered as a common contaminant in commercial *tuberiès* throughout Europe (Merényi et al. 2016). The competitive interactions between *T. brumale* and the other valuable black truffles were usually studied by targeting ECMs (Benucci et al. 2011) and, to a lesser extent, soil mycelium (Belfiori et al. 2012). Here, for the first time, we evaluated the spatial distribution of *T. brumale* against both *T. aestivum* and *T. melanosporum* in the same experimental site and cultural conditions, by targeting either their ECMS or ERM.

In general, the ERMs of the three target species were more diffuse than their respective ECMS, a trend particularly evident for *T. brumale*. Moreover, different truffle ERMs co-occurred in a number of soil cores much higher than the respective ECMS.

Fourteen years after the establishment of the investigated orchard, the competition for space among the three black truffle species seems to be almost confined to the transect areas. *Tuber aestivum* and *T. melanosporum* ECMs and ERM were only partially replaced in the transects and *T. brumale* does not appear to colonize the other truffle plots. This consideration is also supported by the distribution of the brûlés in the truffle plantation. These characteristic areas devoid of vegetation are much more evident in *T. aestivum* and *T. melanosporum* than *T. brumale* growth sites (Olivier et al. 2012). In our plantation, brûlés were completely absent within the brumale plot and rare within the transect areas, whereas they were visible around almost all the plants within *T. aestivum* and *T. melanosporum* plots. Further confirmation of this hypothesis was obtained by the analyses of ECMS and ERM sampled within the *T. melanosporum* plot.

ECMs formed by other fungi were less abundant than those of the introduced truffles. The highest diversity and abundance of these fungi were found in the aest/brum transect, where three mature ectomycorrhizal trees at the edge of *T. aestivum* plot might have facilitated the colonization of truffle plants. This method of colonization by native ectomycorrhizal fungi has been discussed by several authors (Sourzat & Dublau 2001, Chevalier & Sourzat 2012), who consider native host trees as one of the first issues to solve when a new truffle plantation has to be established.

Competition between *T. brumale* and *T. aestivum* has been only poorly investigated. ECMs of *T. brumale* have been found in *T. aestivum* plants (Baciarelli-Falini 2005, Zambonelli et al. 2005, Benucci et al. 2011) but specific studies supporting the possibility that these species can significantly replace each other in cultural or natural conditions have never been carried out. In our study site, ECMs and ERM of *T. brumale* showed a more even distribution than those of *T. aestivum* that, conversely, dominated a single soil patch in the centre of the aest/brum transect. The lack of *T. aestivum* ECMS and ERM in half the plant rows could be due to the gradual soil and root colonization of *T. brumale* or, more likely, of native ectomycorrhizal fungal species. *Tuber aestivum* ECMS and ERM were also found in three soil samples in the brum/mela transect. In this case too, *T. aestivum* dominated a soil patch (smaller than the previous one) where only *T. brumale* co-occurred both as ECMS and ERM. These truffle species seem to adopt two opposite ecological strategies. As a matter of fact, *T. aestivum* appears to be less efficient in soil exploration than *T. brumale* but its presence strongly reduces the ectomycorrhizal fungal diversity, as also reported by other authors (Sourzat 2011, Belfiori et al. 2012). The presence of *T. aestivum* in the brum/mela transect may be explained as the consequence of a cross contamination during nursery mycorrhization (Iotti et al. 2012, Linde & Selmes 2012) rather than a myce-
lum or spore movement from the aest/brum transect.

Tuber brumale has been ranked by several authors as a damaging species for truffle plantations (Martin-Santafe et al. 2014), able to displace *T. melanosporum* and reduce its productivity (Chevalier & Frochot 1997, Callot et al. 2001, Lefever & Hall 2001, Riouset et al. 2001, Ricard 2003, Sourzat 2005, Olivier et al. 2012). It is considered so threatening that cultural practices in *T. melanosporum* plantations in France are mostly devoted to reducing its presence in the soil (Sourzat & Dubiau 2001, Olivier et al. 2012).

In this study, *T. brumale* was never found invading *T. melanosporum*, although in some plant rows its ECMs expanded up to the sampling points closest to *T. melanosporum* plants. *Tuber brumale* ERM was found in two cores collected in the sampling points beyond the *T. melanosporum* plants, mixed together with *T. melanosporum* ERM. At the same time, however, *T. melanosporum* is still well represented both as ECMs and ERM inside the transect.

Competition between black truffles mostly depends on a number of edaphic factors, such as organic matter content and soil pH. In the truffle plantation under investigation, the organic matter percentage is still well represented (*T. melanosporum*), although in *T. brumale* transect.

**Conclusions**

In this study site, *T. brumale* was not able to spread out into the *T. aestivum* and *T. melanosporum* plots and the competition seemed to be confined to the transect areas. When the selection of the plantation site is appropriate for *T. aestivum* and *T. melanosporum*, the main issue is the risk of nursery contamination, the primary cause of subsequent contaminations in the field (Linde & Selmes 2012). It is therefore crucial to carefully monitor the quality of both the spore inoculum and the *Tuber* mycorrhized plants before the establishment of the plantation. The use of mycelial inoculated plants, which recently proved to be successful with *T. brachii* cultivation (Iotti et al. 2016), could also be a valid solution because mycelium has several advantages over spore inoculum, such as fewer contamination risks and higher percentages of root colonization.

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Supplementary Material

Fig. S1 - View of the truffle plantation at the time of sampling. Detail of the first two plant lines in the T. melanosporum plot (a). T. melanosporum plants with the characteristic brûlé (b).

Fig. S2 - Mean percentage of truffle ECMs at 1, 2 and 3 m from the trunk of plants mycorrhized by T. brumale (A and B), T. aestivum (C) and T. melanosporum (D) in the transects I (A and C) and II (B and D)