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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 (Dahlberg 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 (Mérényi 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-Laguette 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.

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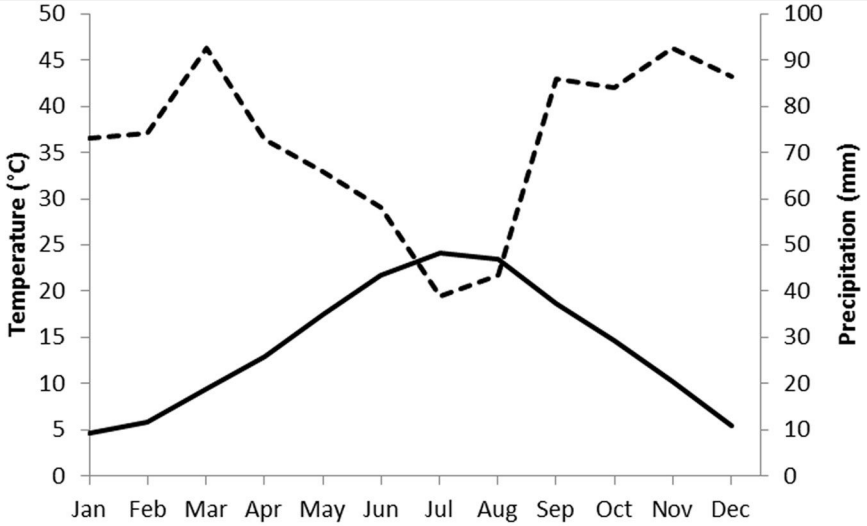

Fig. 1 - Climatic diagram of Bagnouls-Gaussen for the meteorological station of Montelabbate (Pesaro-Urbino, central Italy). Precipitation and temperature data are for the 2000-2016 period. Monthly temperatures (left axis, °C) are indicated with the solid line, monthly precipitations (right axis, mm) are indicated with the dotted line.

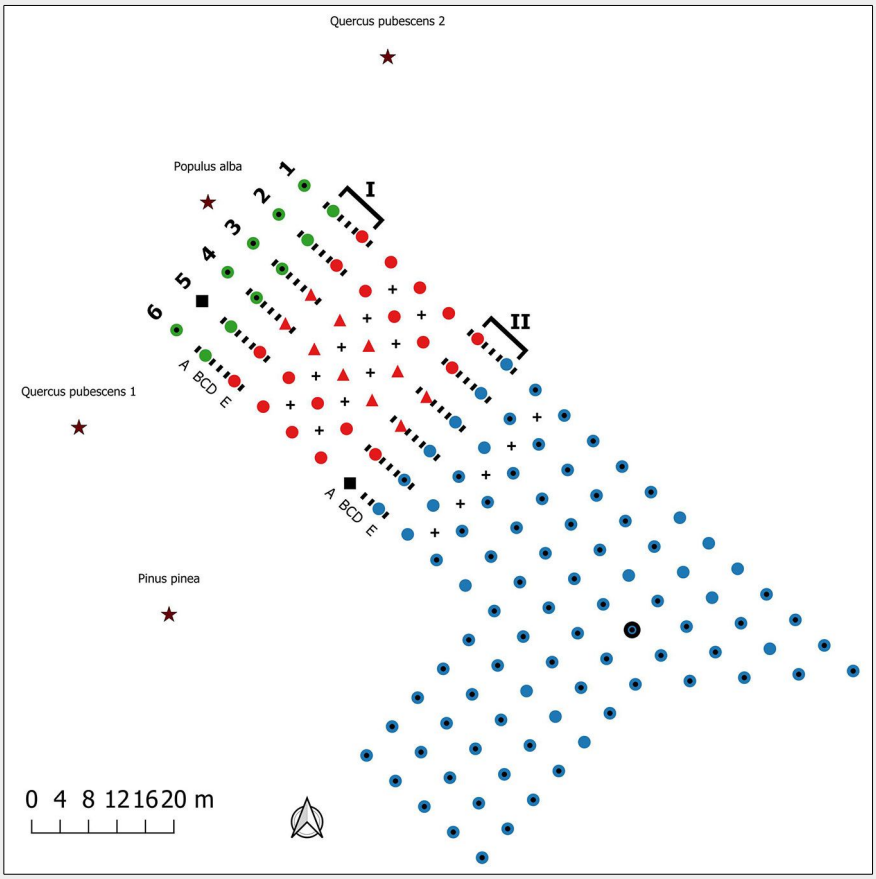

Fig. 2 - Scheme of truffle plantation, transect location and sampling points. Circles indicate *Quercus pubescens* seedlings; triangles *Corylus avellana* seedlings; black squares dead plants. Mycorrhized species: *Tuber brumale* (red), *Tuber aestivum* (green) *Tuber melanosporum* (blue). Dotted circles indicate plants with *brûlé* and the bold circle indicates the only productive plant. Transects are highlighted with a square bracket: (I) aest/brum transect; (II) brum/mela transect. The positions of soil samples collected in each transect are indicated with the "-" symbol. (A-B-C-D-E): soil sample types: A and E were only used for ERM analysis, while B, C and D for both ERM and ECMs analyses. Sampling within plots are indicated with "+" symbol. The black star symbol indicates the adult ectomycorrhizal trees surrounding the plantation: *Quercus pubescens* 1, *Quercus pubescens* 2, *Populus alba* and *Pinus pinea*.

2012, Parladé et al. 2013, Gryndler et al. 2013) the ERM of a *Tuber* species in soil samples.

In the past, a number of plantations were established in Italy by planting groups of seedlings mycorrhized with different black truffles species in the same field (Baciarelli-Falini et al. 2010). This kind of orchard is well suited to study the competitiveness between the different truffle species because they are subjected to the same experimental conditions. In one of these plantations, we aimed to verify the competitiveness of *T. brumale* towards *T. melanosporum* and *T. aestivum* 14 years after planting. Analyses of both truffle ECMs and ERM were carried out in the transects separating *T. brumale* from *T. melanosporum* and *T. aestivum* plots.

Materials and methods

The study site covers an area of 1540 m^2, at an altitude of about 80 m a.s.l., in the municipality of Montelabbate (Pesaro-Urbino, Italy – 43.845488 N; 12.788349 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 ASSAM (*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. brumale* – *T. melanosporum* (II: brum/mela).

The plants 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 shown 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 ERM and ECM 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 dm^3) 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 depth and 10 cm in diameter, ~1.2 dm^3). 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 UncI-UncII (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 ExTaq® DNA polymerase (Takara Bio Inc., Kusatsu, Japan), 1× Buffer solution, 200 µM dNTP, 4 mM $MgCl_2$, 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

Transect I - aest/brum

A total of 5115 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 (32% *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, Fig. S2). 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 col-

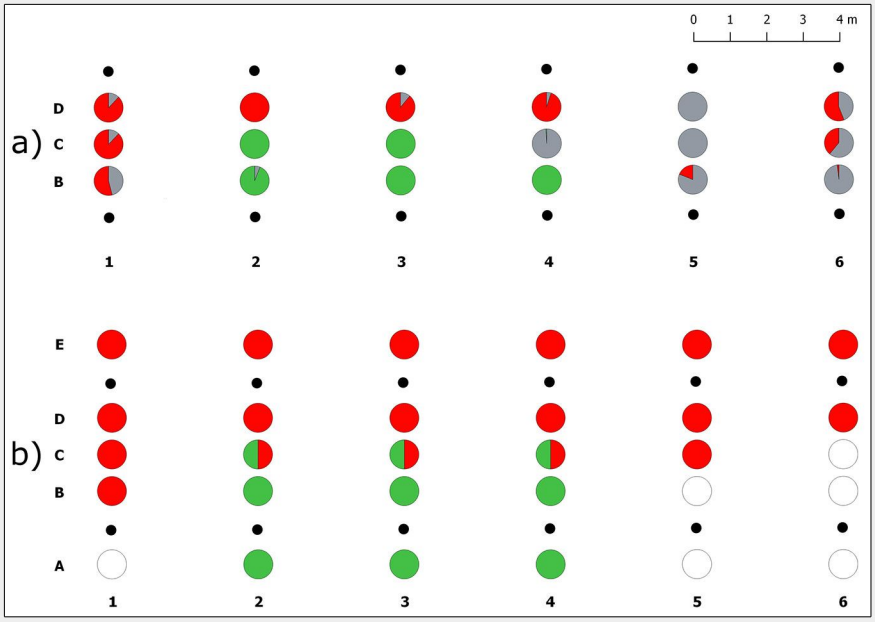

Fig. 3 - Spatial distribution of truffle ERMs and ECMs in the transect I (aest/brum). (a) Percentages of ectomycorrhizal colonization of the inoculated truffle species and native ectomycorrhizal fungi in each sample, from B to C; (b) presence/absence of the truffle ERMs in each sample, from A to E. (red): *Tuber brumale*; (green): *Tuber aestivum*; (gray): ECMs formed by other fungi; (white): no truffle mycelia; (black dots): host plants.

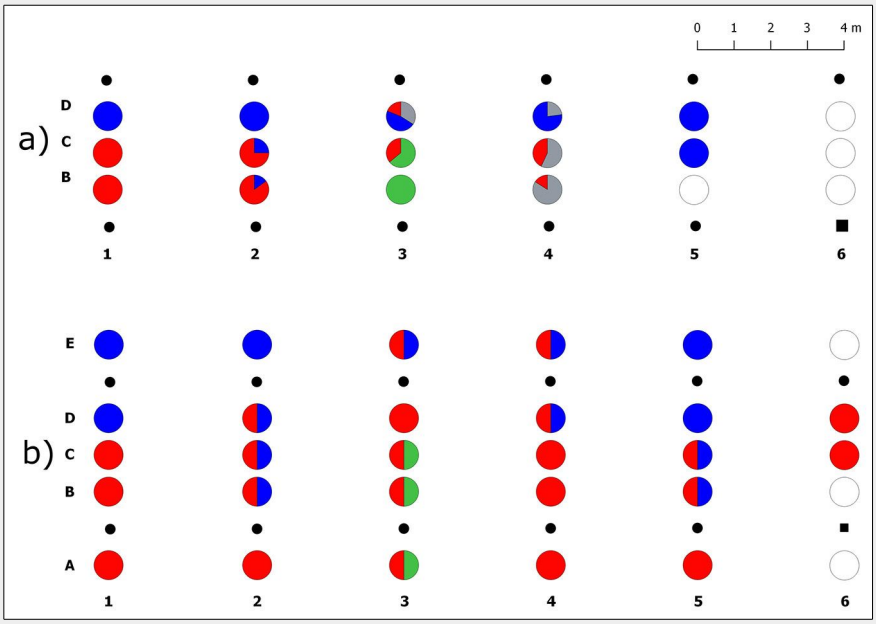

Fig. 4 - Spatial distribution of truffle ERMs and ECMs in the transect II (brum/mela). (a) Percentages of ectomycorrhizal colonization of the inoculated truffle species and native ectomycorrhizal fungi in each sample, from B to C; (b) presence/absence of the truffle ERMs in each sample, from A to E. (red): *Tuber brumale*; (green): *Tuber aestivum*; (blue): *Tuber melanosporum*; (gray): ECMs formed by other fungi; (white): no truffle mycelia; (black dots): host plants; (black square): dead host plant.

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 *truffières* 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 *T. 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 & Dubiau 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-

lium 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, Lefevre & Hall 2001, Riousset 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 pretty low (1.2%) and pH is > 7.5, conditions that could have prevented *T. brumale* from colonizing the *T. melanosporum* plot. Several authors (Jaillard et al. 2016, Callot et al. 2001) claim that a low level of organic matter favours *T. melanosporum* rather than *T. brumale*. Moreover, *T. melanosporum* has a better development in alkaline soils with pH that ranges between 7.5 and 8.3 (Jaillard et al. 2016), while the optimal pH for *T. brumale* is 6.5 (Bratek et al. 2001). The host species may also promote the competitiveness of one or the other *Tuber* species even if, in this case, the results in literature are more ambiguous. Some studies reveal that *T. melanosporum* proved to be more competitive on *Quercus* spp., contrary to *T. brumale* which prefers *C. avellana* (Chevalier & Sourzat 2012, Donnini et al. 2001, Baciarelli-Falini et al. 2010). Another condition that might have also prevented the diffusion of *T. brumale* is the no-tillage condition adopted in the truffle plantation. In fact, soil tillage is considered able to favour the propagation of *Tuber* competing species (Chevalier & Sourzat 2012).

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. borchii* 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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References

Agerer R (1995). Anatomical characteristics of identified ectomycorrhizas: an attempt towards a natural classification. In: "Mycorrhiza: Structure, Function, Molecular Biology and Biotechnology" (Varma AK, Hock B eds). Springer, Berlin, Germany, pp. 685-734.

Baciarelli-Falini L (2005). Prime osservazioni sulla sostituzione delle micorrize di *Tuber* nelle tartufaie coltivate [First observations of replacement of *Tuber* mycorrhizas in cultivated truffle orchards]. In: Proceedings of the "Seminario sullo Stato Attuale della Tartuficoltura Italiana" (Bencivenga M, Donnini D, Gobbini A eds), Spoleto-Norcia (Italy), 21-22 Feb 2004. Associazione "il Tartufo nel 2000", Gruppo Micologico "Valle Spoletana", Norcia, Italy, pp. 75-80. [in Italian]

Baciarelli-Falini L, Bencivenga M, Donnini D, Di Massimo G (2010). Research results in truffle beds of the Umbria region. In: Proceedings of the "3rd International Conference on Truffles" (Donnini D ed). Spoleto (Italy) 25-28 Nov 2008. Comunità Montana dei Monti Martano, Serano e Subasio, Italy, pp. 577-589. [in Italian]

Belfiori B, Riccioni C, Tempesta S, Pasqualetti M, Paolocci F, Rubini A (2012). Comparison of ectomycorrhizal communities in natural and cultivated *Tuber melanosporum* truffle grounds. FEMS Microbiology Ecology 81: 547-561. - doi: 10.1111/j.1574-6941.2012.01379.x

Bencivenga M, Donnini D, Di Massimo G (1992). Analysis of *Tuber melanosporum* mycorrhizae in an 11-year-old truffle-bed. Micologia e Vegetazione Mediterranea 7: 159-171.

Benucci GMN, Raggi L, Albertini E, Grebenc T, Bencivenga M, Falcinelli M, Di Massimo G (2011). Ectomycorrhizal communities in a productive *Tuber aestivum* Vittad. orchard: composition, host influence and species replacement. FEMS Microbiology Ecology 76: 170-184. - doi: 10.1111/j.1574-6941.2010.01039.x

Bever J (2002). Host-specificity of AM fungal population growth rates can generate feedback on plant growth. Plant and Soil 244: 281-290. - doi: 10.1023/A:1020221609080

Bratek Z, Bagi I, Paradi I, Vikor J (2001). Differentiation among truffle species based on habitat preferences. In: Proceedings of the 5th International Conference "Science et Culture de la Truffe" (Courvoisier M, Olivier JM, Chevalier G eds). Aix-en-Provence (France) 4-6 Mar 1999. Federation Française des Trufficulteurs, Paris, France, pp. 193-195.

Callot G, Daignieres Y, Fernandez D, Raymond M, Salducci X (2001). Effect of soil conditions on the fruiting of Perigord truffle (*Tuber melanosporum*). In: Proceedings of the 5th International Conference "Science et Culture de la Truffe" (Courvoisier M, Olivier JM, Chevalier G eds). Aix-en-Provence (France) 4-6 Mar 1999. Federation Française des Trufficulteurs, Paris, France, pp. 257-259.

Chevalier G, Frochot H (1997). La truffe de Bourgogne [The Burgundy truffle]. Pétrarque, Levallois-Perret, France, pp. 257. [in French]

Chevalier G, Sourzat P (2012). Soils and techniques for cultivating *Tuber melanosporum* and *Tuber aestivum* in Europe. In: "Edible Ectomycorrhizal Mushrooms" (Zambonelli A, Bonito GM eds), Soil Biology series, vol. 34, Springer-Verlag, Berlin-Heidelberg, Germany, pp. 163-189. - doi: 10.1007/978-3-642-33823-6_10

Dahlberg A (2001). Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. New Phytologist 150: 555-562. - doi: 10.1046/j.1469-8137.2001.00142.x

De Miguel AM, Agueda B, Sánchez S, Parladé J (2014). Ectomycorrhizal fungus diversity and community structure with natural and cultivated truffle hosts: applying lessons learned to future truffle culture. Mycorrhiza 24: 5-18. - doi: 10.1007/s00572-013-0554-3

Donnini D, Baciarelli-Falini L, Bencivenga M (2001). Analysis of mycorrhization in over 12 year-old cultivated truffle beds of *T. melanosporum* Vittad. under various soil and climate conditions. In: Proceedings of the 5th International Conference "Science et Culture de la Truffe" (Courvoisier M, Olivier JM, Chevalier G eds). Aix-en-Provence (France) 4-6 Mar 1999. Federation Française des Trufficulteurs, Paris, France, pp. 437-440.

Granetti B, Angelini P (1992). Competition between some ectomycorrhizal fungi and *Tuber melanosporum* in a cultivated truffle bed. Micologia e Vegetazione Mediterranea 7: 173-188.

Gryndler M, Trilčová J, Hršelová H, Streiblová E, Gryndlerová H, Jansa J (2013). *Tuber aestivum* Vittad. mycelium quantified: advantages and limitations of a qPCR approach. Mycorrhiza 23: 341-348. - doi: 10.1007/s00572-012-0475-6

Guerin-Laguette A, Cummings N, Hesom-Williams N, Butler R, Wang Y (2013). Mycorrhizas analyses in New Zealand truffières reveal frequent but variable persistence of *Tuber melanosporum* in co-existence with other truffle species. Mycorrhiza 23: 87-98. - doi: 10.1007/s00572-012-0450-2

Hall I, Brown G, Zambonelli A (2007). Taming the truffle. The history, lore, and science of the ultimate mushroom. Timber Press, Portland, OR, USA, pp. 304. [online] URL: http://www.fs.fed.us/pnw/pubs/journals/pnw_2008_trappe001.pdf

Iotti M, Leonardi M, Oddis M, Salerni E, Baraldi E, Zambonelli A (2012). Development and validation of a realtime PCR assay for detection and quantification of *Tuber magnatum* in soil. BMC Microbiology 12: 93-101. - doi: 10.1186/1471-2180-12-93

Iotti M, Piattoni F, Hall IR, Zambonelli A (2016). First evidence for truffle production from

plants inoculated with mycelial pure cultures. Mycorrhiza 26: 793-798. - doi: 10.1007/s00572-016-0703-6
Iotti M, Zambonelli A (2006). A quick and precise technique for identifying ectomycorrhizas by PCR. Mycological Research 110: 60-65. - doi: 10.1016/j.mycres.2005.09.010
Jaillard B, Oliach D, Sourzat P, Colinas C (2016). Soil characteristics of *Tuber melanosporum* habitat. In: "True Truffles (*Tuber* spp.) in the world" (Zambonelli A, Iotti M, Murat C eds). Soil Biology series, vol. 47, Springer International Publishing, Cham, Switzerland, pp. 169-190. - doi: 10.1007/978-3-319-31436-5_11
Kennedy P (2010). Ectomycorrhizal fungi and interspecific competition: species interactions, community structure, coexistence mechanisms, and future directions. New Phytologist 187: 895-910. - doi: 10.1111/j.1469-8137.2010.03399.x
Koide R, Xu B, Sharda J, Lekberg Y, Ositguy N (2005). Evidence of species interactions within an ectomycorrhizal fungal community. New Phytologist 165: 305-316. - doi: 10.1111/j.1469-8137.2004.01216.x
Lefevre CK, Hall IR (2001). The status of truffle cultivation: a global perspective. Acta Horticulturae 556: 513-520. - doi: 10.17660/ActaHortic.2001.556.75
Linde CC, Selmes H (2012). Genetic diversity and mating type distribution of *Tuber melanosporum* and their significance to truffle cultivation in artificially planted truffières in Australia. Applied and Environmental Microbiology 78: 6534-6539. - doi: 10.1128/AEM.01558-12
Martin-Santafe M, Perez-Fortea V, Zuriaga P, Barriuso J (2014). Phytosanitary problems detected in truffle cultivation in Spain. Forest System 23: 307-316. - doi: 10.5424/fs/2014232-04900
Mello A, Cantisani A, Vizzini A, Bonfante P (2002). Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environmental Microbiology 4: 584-594. - doi: 10.1046/j.1462-2920.2002.00343.x
Merényi Z, Varga T, Bratek Z (2016). *Tuber brumale*: a controversial *Tuber* species. In: "True Truffles (*Tuber* spp.) in the world" (Zambonelli A, Iotti M, Murat C eds). Soil Biology series, vol. 47, Springer International Publishing, Cham, Switzerland, pp. 49-68. - doi: 10.1007/978-3-319-31436-5_4
Nara K (2006). Ectomycorrhizal networks and seedling establishment during early primary succession. New Phytologist 169: 169-178. - doi: 10.1111/j.1469-8137.2005.01545.x
Olivier JPM, Savignac JC, Sourzat P (2012). Truffe et trufficulture [Truffles and trufficulture]. FANLAC, Périgueux, France, pp. 400. [in French]
Parladé J, De la Varga H, De Miguel AM, Sáez R, Pera J (2013). Quantification of extraradical mycelium of *Tuber melanosporum* in soils from truffle orchards in northern Spain. Mycorrhiza 23: 99-106. - doi: 10.1007/s00572-012-0454-y
Peay KG, Bruns TD, Kennedy PG, Bergemann SE, Garbelotto M (2007). A strong species-area relationship for eukaryotic soil microbes: island size matters for ectomycorrhizal fungi. Ecology Letters 10: 470-480. - doi: 10.1111/j.1461-0248.2007.01035.x
Ricard JM (2003). La truffe. Guide technique de trufficulture [The truffle: technical guide for truffle cultivation]. Centre Technique Interprofessionnel des Fruits et Légumes, Paris, France, pp. 268. [in French]
Riousset LG, Chevalier G, Bardet MC (2001). Truffles d'Europe et de Chine [Truffles of Europe and China]. INRA, Paris, France, pp. 155. [in French]
Rubini A, Paolocci F, Granetti B, Arcioni S (1998). Single step molecular characterization of morphologically similar black truffle species. FEMS Microbiology Letters 164: 7-12. - doi: 10.1111/j.1574-6968.1998.tb13060.x
Smith SE, Read DJ (2008). Mycorrhizal symbiosis (3rd edn). Academic Press, New York, USA, pp. 800.
Sourzat P (2005). Pressure of contamination mycorrhizal fungi against black truffle in spontaneous and cultivated truffières in France. In: Abstracts of the "4th International Workshop on Edible Mycorrhizal Mushroom" (Honrubia M, Morte A, Torrente P eds), Murcia (Spain) 28 Nov - 2 Dec 2005. Universidad de Murcia, Spain, pp. 116.
Sourzat P (2011). Black truffle cultivation and competing fungi. In: Proceedings of the "7th International Conference on Mushroom Biology and Mushroom Products (Savoie JM, Foulongne-Oriol M, Largeteau M, Barroso G eds), Arcachon (France) 4-7 Oct 2011. INRA, Bordeaux, France, pp. 516-528. [online] URL: http://www.cabdirect.org/cabdirect/abstract/20123168959
Sourzat P, Dubiau JM (2001). Contamination by *Tuber brumale* in truffle plantations: observations, experimentations and strategies of cultivation protection; the example of a truffle plantation at Miers in the Lot department, France. In: Proceedings of the 5th International Conference "Science et Culture de la Truffe" (Courvoisier M, Olivier JM, Chevalier G eds), Aix-en-Provence (France) 4-6 Mar 1999. Federation Française des Trufficulteurs, Paris, France, pp. 466-468.
Turgeman T, Sitrit Y, Danai O, Luzzati Y, Bustan A, Roth-Bejerano N, Kagan-Zur V, Masaphy S (2012). Introduced *Tuber aestivum* replacing introduced *Tuber melanosporum*: a case study. Agroforestry System 84: 337-343. - doi: 10.1007/s10457-011-9478-0
Zambonelli A, Iotti M, Zinoni F, Dallavalle E, Hall R (2005). Effect of mulching on *Tuber uncinatum* ectomycorrhizas in an experimental truffière. New Zealand Journal of Crop and Horticultural Science 33: 65-73. - doi: 10.1080/01140671.2005.9514332
Zambonelli A, Leonardi P, Iotti M, Hall I (2017). Ecological and genetic advances in the cultivation of *Tuber* spp. Revista Fitotecnia Mexicana 40: 371-377.
Zampieri E, Murat C, Cagnasso M, Bonfante P, Mello A (2010). Soil analysis reveals the presence of an extended mycelial network in a *Tuber magnatum* truffle ground. FEMS Microbiology Ecology 71: 43-49. - doi: 10.1111/j.1574-6941.2009.00783.x

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).

Tab. S1 - Ectomycorrhizas (ECM) and extraradical truffle mycelium (ERM) in the *T. aestivum*/*T. brumale* transect.

Tab. S2 - Ectomycorrhizas (ECM) and extraradical truffle mycelium (ERM) in the *T. melanosporum*/*T. brumale* transect.

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